SOME ASPECTS OF THE TISSUE CULTURE AND MICROPROPAGATION OF *BORONIA MEGASTIGMA* NEES

by

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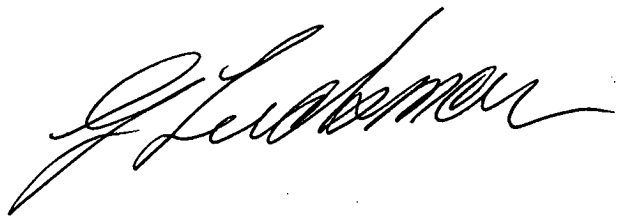
This thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge, it contains no material previously published or written by another person, except where due reference is made in the text of the thesis.



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CONTENTS

ABSTRACT

This study investigates the effects of a number of factors on the growth and development of Boronia megastigma Nees in "in vitro" culture.

A number of non-nutrient factors were found to be influencing the growth and development of cultures. The pH of the medium was found to have an effect on the subsequent growth of the cultures, as did the orientation of explants on the medium and the mode of trimming the explants. Relative Growth was found to be an inappropriate measure of the growth of the cultures.

Nitrogen nutrition was by far the most important factor affecting growth in the nutrient media. The concentration of nitrogen was found to be influencing the amount of growth and the balance between shoot initiation and shoot extension. The cultures grew poorly in the presence of either ammonium or nitrate as a sole source of nitrogen. Changes in the pH of the medium were found to be the cause of the poor growth on media containing ammonium as the sole nitrogen source. Changes in the pH of the medium were also associated with the use of nitrate as the nitrogen source, however correction of the pH change using succinate buffer did not improve growth on this medium.

Adenine hemi-sulphate and vitamins had little influence on the growth and development of the cultures. Sucrose

concentration was found to influence growth, possibly by affecting the osmotic potential of the medium.

The rate of root initiation in culture was also substantially affected by the level of nitrogen in the medium. Development of cultures after root initiation was found to be substantially affected by both the level of nitrogen in the root initiation medium and the level of nitrogen in the proliferation medium, prior to root initiation. Root initiation was also affected by the concentration of sucrose in the medium, while adenine hemi-sulphate had no effect. Survival of plantlets after planting in soil was substantially affected by the length of the time spent on root medium. The effect on plantlet survival of temperature conditions during the root initiation phase was also investigated.

The results obtained from this research should assist in the commercial micropropagation of Boronia megastigma and provide a basis for future research on the in vitro culture of this species.

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I. INTRODUCTION

Boronia megastigma Nees (referred to hereafter as boronia) is a plant, native to Western Australia, which is harvested for the essential oil that can be extracted from its flowers. In its native area the flowers are harvested by hand from wild stands of the plant, making the harvesting and processing of this low yielding oil particularly expensive.

The oil extracted from boronia flowers is highly prized and highly priced (in excess of $3000 per kilogram). It is used primarily in high class perfumes. The major components extracted from the boronia flowers are dodecanol, dodecyl acetate, tetradecyl acetate and beta-ionone (Legget and Menary, 1980).

Boronia has been cultivated in Tasmania for many years for use by florists and as a garden shrub. In the last few years research has been undertaken to establish plantations of boronia, in Tasmania, for the extraction of the oil. In order to make the cultivation of boronia a commercially attractive proposition, a system for mechanical harvest of the flowers has been devised. Mechanical harvesting is made more efficient if the harvester is able to harvest from bushes of uniform height and maturity. It is also important to ensure that the plants have a suitable growth habit, to allow the flowers to be removed from the bush without causing excessive damage to the plant. Careful selection based on yield, oil and harvest characteristics has given a

number of plants that have these harvest characteristics as well as good oil quality and yield. It is necessary to propagate the selected clones to provide large numbers of identical plants suitable for mechanical harvesting. Micropropagation offers the possibility of propagating very large numbers of cloned plants in quite short periods to allow the rapid expansion of the industry. It also offers the opportunity to propagate clones that are not readily propagated through normal vegetative methods.

In the longer term, the techniques now being developed in plant tissue culture offer exciting and important possibilities in the field of plant genetic improvement, germplasm storage and cell suspension culture. In order for these techniques to be successfully applied there needs to be a sound knowledge of the requirements of boronia in tissue culture. As this species has not been reported to have been cultured before there are a wide range of techniques and information that need to be acquired regarding its growth and culture in vitro.

This thesis is intended to provide a starting point, in terms of providing basic information on the in vitro culture of boronia and on its successful micropropagation.

II. LITERATURE REVIEW

The Uses of Plant Tissue Culture and Micropropagation

Plant tissue culture and micropropagation are techniques which have gained a great deal of popularity as methods of obtaining sigificant improvements in the efficiency of horticulture. The term plant tissue culture is described by Krikorian (1982) as referring purely to the aseptic culture of randomly proliferating plant cell masses, often referred to as callus cultures. The term micropropagation usually refers to the use of plant tissue culture techniques to propagate selected plant types, often in very large numbers. However, the term tissue culture is also often used to describe the a wide range of techniques used in the culture of plant cells or parts under aseptic conditions. From this generic term there are a number of subheadings and techniques that have developed. These were grouped under into three areas of importance to horticulture, by Withers and Alderson (1986).
These are :

a) Clonal propagation

b) Plant Health and Germplasm Storage

c) Genetic Improvement

Clonal Propagation

Micropropagation is employed as an alternative to vegetative propagation or seed propagation for a number of quite specific reasons:

a) As a cheap method of large scale clonal propagation of plants in commercial nurseries.

b) As a method of clonal multiplication of plants which cannot be successfully propagated by seed or by normal cuttings.

In both cases one of the main advantages is the very much faster rate at which plants can be produced, in comparison with other macropropagation methods. Micropropagation offers the opportunity to greatly lower the cost of production of cloned plants and at the same time offers a very much faster method of propagation. This allows the production of commercial quantities of new varieties in as little as a few months rather than the several years previously required for slow growing or difficult to root plants. Subsidiary benefits that are obtained from the use of micropropagation are reported to include faster growth and earlier maturation than seed grown plants (Vasil and Vasil, 1980).

Micropropagation is also proving useful for crops which have not previously been considered for clonal production. It has been suggested for use to produce plants for hybrid seed production in Broccoli (Anderson and Carstens, 1977)

and Asparagas (Evans, Sharp and Flick, 1981). It is also being used to propagate some tree species, both for the establishment of seed orchards as well as for the mass propagation of superior individual genotypes for use in forest industries (Haines, 1987).

Micropropagation refers mostly to the techniques of propagation through the use axillary shoot proliferations, followed by a period of root initiation and establishment of the plant in soil. However, it can also refer to a number of other techniques, which result in the production of large numbers of cloned plants. Adventitous shoot production is a closely allied technique for production of shoots prior to root initiation but it needs to be considered separately because of the difficulties that can occur using this technique. In practice, it is often the case that shoots will be produced using a combination of axillary proliferation and adventitious shoot production in the same culture.

The distinction between axillary shoot production and somatic embryogenesis for mass propagation is much more clearly drawn. Somatic embryogenesis is at present still largely an experimental technique, used more in the field of genetic improvement. However, some reports suggest that it has considerable potential for the future, particularly in the area of artificial seed production (Ammirato, 1983). This technique may allow for significant reductions in the labour input required for clonal propagation schemes.

Difficulties Associated With Micropropagation

There is often a tendency to consider the possible benefits of micropropagation without adequate consideration of the difficulties and potential problems that can occur. The example of propagation of large numbers of virus infected orchid plants is well known (Langhans, Horst and Earle, 1977). This lead to the situation where a very large proportion of orchid stocks were derived from a small number of original cultures, most of which were virus contaminated.

Similarly, the large scale propagation of plants gives rise to the possibility of propagating large numbers of plants that are not true to type. This may arise as a result of the chance mutations, as might normally occur as a bud sport on a whole plant, or it may arise as a result of the techniques and chemicals under use. It is now widely recognised that some techniques give rise to a much higher incidence of 'off-type' plants than others (Hussey, 1986). Adventitous shoot production and somatic embryogenesis are apparently much more susceptible to the occurrence of 'off-type' plants. This is mostly because the shoots that arise can do so from a single original cell, which may have undergone some form of mutation. The shoots arising from axilliary proliferations on the other hand will by definition have arisen from the subdivision of a pre-existing meristem. According to Hussey (1980) mutations are likely to be excluded from the meristem because of the

formation of unstable chimeras or because the mutant cell is unable to grow as rapidly as its neighbouring cells due to incompatability. However the existence of bud-sports is an indication that such mutations do occur in natural populations and can be expected to arise in micropropagated plants. In one comparison it was found that banana cultures produced about 3 % of off-type plants when grown purely from axillary shoots cultures. When grown from a culture in which there had been a period of callus growth the incidence of off-type plants rose to 22 % (Drew and Smith, 1987). It might be expected that the amount of variation occurring in a particular culture would depend on the genetic and chimeral stability of that plant species as well as the culture conditions under which it is grown.

Plant Health and Germplasm Storage

Tissue culture has uses in two areas associated with plant health, namely the production of disease free plants and the transportation of plant material through quarantine. In this context, disease free plants refers almost exclusively to freeing plants from infections of viruses. This is a technique which has now been applied to more than 57 species and many more varieties and cultivars (Barlass, 1987). Plant tissue cultures offer a ready means of eliminating viruses from plants through the culture of apical meristems, which are known to be free of some viruses or may have a low titre of virus particles. This is, in

many cases, a simple technique for the elimination of virus infections, provided that a sufficiently small section of the meristem is used. The efficiency of virus elimination can be greatly enhanced by the use of heat treatment and chemical suppressants to lower the titre of virus particles in the plant before excision of the meristem. Exclusion of viruses has also be achieved by the use of antiviral compounds, such as ribavirin, incorporated in the culture medium (Long and Cassells, 1986).

The use of sterile tissue cultures has greatly facilitated the free movement of plants through quarantine and so allows the rapid interchange of genetic resources around the world. The standards applied to plant material transported in vitro is usually the same as those required for in vivo plants (Anon 1987). However, the possibilities for importation of soil borne diseases, insects and some classes of bacterial and fungal pathogens are greatly reduced. Of course freedom from viruses and fungal pathogens not readily detected in tissue culture, must still be certified by other means.

Germplasm Storage

The concept of using plant tissue cultures as a method of conserving valuable genotypes was apparently first suggested in the mid 1970s (Withers, 1986). It is generally considered that the use of cryopreservation will not prove to be a viable replacement for seed storage in all cases but

it has potential for the storage of vegetatively propagated material. This is especially true for virus free stocks and for plants with recalitrant seeds, due either to their short life or low rate of germination.

Early workers in this field suggested that the long term culture of material under conditions that gave slow, stable growth of meristems would be the most practical application but the use of long term storage in liquid nitrogen is now the most common approach (Withers, 1986). It is now being suggested that the huge numbers of variants that are being produced through somaclonal variation may need to be stored for future reference, particularly in species where the techniques may not have been developed to make full use of variants selected in cell culture (Nitzsche, 1983). Cell lines so stored may be kept for later inclusion of the genes into cultures that have retained regenerative competance, or for use in combination with other genes.

Genetic Improvement

It is perhaps ironic that some of the techniques that are now being used to preserve existing germplasm resources are also those being used to create new varieties of plants and hybrids. The plant tissue culture techniques that are of most importance in the area of genetic improvement are those of protoplast culture and the selection of somaclonal variation.

Protoplast Technology

The use of protoplast technology in plant improvement offers new possibilities to supplement conventional breeding programs. Techniques of parasexual hybrization can allow exchange of germplasm between sexually immature superior plants and the transfer of genes between unrelated species, sterile or imcompatible parent plants. This combination of new genetic material can occur at both the nuclear and cytoplasmic levels (Glimelius, 1988). The techniques for isolating protoplasts, inducing fusion of different cell lines and re-establishing hybrid plants, are now quite well described for a wide range of species. Protoplast fusion is not the only means by which genetic modification of protoplasts can occur. The use of Agrobacterium tumifaciens, Ti plasmid has been found to be a successful method of gene transfer in dicotyledons (Davey et al 1986), while the transfer of DNA fragments has been attempted using bacterial spheroplasts (Harding and Cocking, 1986) and liposomes (Mathews, 1983). The direct injection of DNA into protoplasts is a technique that appears to have potential in many species (Gunn and Day, 1986). More recently, lasers have been used to make temporary holes in cell membranes to allow the inclusion of DNA into both cells and chloroplasts within cells (Weber et al, 1988). The opportunities these techniques offer for providing new genotypes and previously un-achievable gene combinations are a very important. As yet, the number of species that the techniques have been

applied to is very limited, many of the techniques being restricted to Nicotiana species and other less commercially significant Solanacea. The greatest potential for the techniques lie in being able to apply them to important food crops, an area of technology that is much less advanced.

Somaclonal Variation

Establishment of suspension cultures from either callus or protoplasts give the possibility of selecting for new characteristics in plants arising either through natural variation or through the agency of induced variation in somatic cell cultures. This offers the opportunity for selection using characteristics that do not lend themselves well to selection through normal breeding techniques. Examples where somatic variation has already been used to provide unique plant characteristics are well documented. The technique has been of particular use in sugar cane, where mutations have been isolated for resistance to a number of diseases, such as Fiji virus disease, downy mildew, eyespot disease and culmicolous smut, as well as for increased sucrose content (Evans and Sharp, 1988). In some cases, the somaclones selected have proved to be unstable but other clones have been found to be stable over several years. Sugarcane is considered to be particularly amenable to selection of somaclonal variants because it is normally a chromosomal mosaic and is thus able to tolerate more variation in chromosome number than other species. There

are now many other instances of somatic variations being selected from cell cultures. Many of these are for resistance to various chemicals added to media and have their greatest significance as genetic markers for use in somatic hybridisation, a technique discussed above. Other areas where variants are of most interest is in selection for drought and salt tolerance and in herbicide resistance. In selecting for drought tolerance poly ethylene glycol is commonly added to media, while salt tolerance can be selected for by simply adding NaCl to the medium. Difficulties have been experienced in attempting to transfer the observed tolerance of cell cultures to measurable improvement in whole plants (Bright et al, 1986). Herbicide resistance to a number of different herbicides and classes of herbicide has been identified in a wide variety of plants (Malone and Dix, 1986).

This brief review of some of the uses of plant tissue culture has made little or no mention of a wide range of techniques, such as pollen culture, embryo culture as distinct from somatic embryogenesis, ovary culture and in vitro pollination, all of which rely on the same basic techniques of plant tissue culture.

Knowledge Of Plant Tissue Culture

All of the techniques above rely on there being a good understanding of the way the particular plant species or cultivar reacts to media constituents and culture conditions. A great deal of the information that has been published to date tends to be, to some extent, anecdotal. Reports are often published which relate to a single species or even to a single variety. This leads to apparently quite contradictory results being obtained under superficially similar circumstances or on closely related species. As noted by Hughes (1981), more research is required to establish the genetic and physiological basis for regulation of morphogenesis in plant tissue cultures overall. In many instances, there is a lot of published information about tissue culture of a particular plant species or variety without sufficient care having been taken to provide some cogent theory of why things occur as they do. There are any number of reviews, summaries and monographs that summarise the basic techniques and considerations for plant tissue culture generally. Bhojwani, Dhawan and Cocking (1986) list a total of 91 books that have been published on the topic of plant tissue culture. Only nine of these were published before 1970. Over 11,000 citations covering the period up to the end of 1985 are also listed. With this explosion of information, it is often difficult to discern the underlying patterns and rules governing the development of plant tissue cultures.

Micropropagation of Boronia Species

Information directly relating to the tissue culture or micropropagation of Boronia species is much less common. Lamont (1985) provided a brief summary of results of experiments using Boronia serrulata. This provided proliferation rates of up to 6 fold increase in shoots, over a six week culture period, but low survival rates for rooted plantlets. Boronia pilosa Labill. and Boronia edwardsii Benth. have also been successfully cultured (Williams and Taji, 1987; Williams, Taji and Bolton, 1985). Thus far, however, there do not appear to have been any references to the tissue culture of Boronia megastigma.

Growth in Tissue Cultures and its Measurement

In order to be able to understand the factors influencing growth and differentiation in tissue culture, it is important that the subtle differences in growth caused by interaction between various factors can be measured. Although there are substantial amounts of literature on the subject of plant growth and its measurement, very little of this work is devoted to the difficulties and specific requirements for the accurate measurement of growth and development in plant tissue culture. Of the references that do exist, almost all of them are on the topic of measurement in cell suspension cultures (King and Street 1973; Dixon, 1985).

Length of the Culture Period

One of the difficulties in attempting to compare growth rates in tissue cultures is that there does not appear to be any uniformity in the length of time over which the growth should be measured, let alone a uniform method of determining the growth. Bhojwani and Razdan (1983) tabulate the results of a wide range of clonal propagation references, with proliferation rates quoted from a three fold increase in 8 weeks (Strauss and Arditti, 1980) to 200 fold in 4 weeks (Takayama and Misawa, 1982). However, no mention is made by Bhojwani and Razdan to the method of determining this growth. There are hundreds of media listed in the various monographs and reviews on micropropagation, yet in few of these is there a section that specifically refers to ways in which optimum growth rate might be determined for clonal propagation schemes.

Measurement of Relative Growth

The two most common methods of measuring the effective growth in proliferation cultures are to determine the change in mass and to count or measure the number of new shoots produced by a culture. The measurement of mass is occasionally used to determine mean Relative Growth but is more commonly expressed as the percentage increase in mass per unit of time. Other growth indices have been developed by Staba, Nygaard and Zito (1984). This is a valid measure of growth but can give rise to a misleading

impression if the plant under study does not have a linear growth pattern (Singer, 1986). The second type of measurement, counting the number of new shoots produced (or roots in the case of a root initiation medium), has a great deal of relevance to micro propagation, since the objective is often the production of greater numbers of shoots and roots.

Before it can be decided what growth measure can be applied, it is necessary to determine whether growth is linear or logarithmic. Cell cultures have been shown (Street, 1977; King and Street, 1977) to follow a typical sigmoidal pattern. That is, there is usually a lag phase following initiation or inoculation of the culture then an exponential phase where growth is most rapid, strictly defined as when rate of increase in biomass per unit of biomass is constant (King and Street, 1977). This stage is followed by a period of declining growth, where growth firstly becomes linear and finally declines to a stationary phase with no measurable growth. It is reasonable to assume that many proliferation, callus and organ cultures should exhibit similar growth patterns. However the instances where such growth has been demonstrated are not common.

The Use of Relative Growth as Measure of Culture Growth

Murashige and Skoog (1962) showed that tobacco callus had a definite logarithmic growth pattern. However, they selected a growth period of four weeks for the determination

of mean relative growth rate on the basis that it extended past the end of the exponential growth phase. It would appear that data collected after the end of the exponential phase might cause erroneous conclusions to be drawn about which treatment has the highest growth rate. Hunt (1982) cites a number of references where comparisons of relative growth rate have been made between experiments and between species in which a value for maximum relative growth rate has been determined. In these instances maximum relative growth rate is best determined over a suitable time span within the period of logarithmic growth.

Difficulties of Measuring Relative Growth

The use of mass as a measure of growth has the advantages that it is precise and relatively easy to determine. It has the drawback of being destructive of asepsis. This difficulty has been tackled, in one instance, by the use of computer image analysis to provide a measure of the growth of a culture (Spomer and Smith, 1987). This must be providing a two dimensional measurement when growth, in most cases will be three dimensional. Thus, the measurements obtained might not be an entirely accurate representation of the growth occurring in all cases.

There is also a need for need some measure of initial mass to determine relative growth rate. However, initial mass may not accurately reflect growth potential. This final factor has been the subject of research by Singer

(1986), who noted that the equation for the determination of relative growth (R) is accurate only while the explant is growing exponentially. A number of authors (cited in Singer and McDaniel, 1986) have noted that growth rates in callus cultures decline with increasing initial weight, that is, small pieces grow more rapidly. Singer states that, for a callus suspension culture, the growth rate would be dependent, not on the total mass of the callus explant, but on the volume of the outer shell of cells in the callus piece that is actually in contact with the medium. Using this, an equation was derived that would most accurately measure the relative growth rate for such a system. However, experimental results by Singer and McDaniel (1986) showed that even this calculation did not give a satisfactory match to the observed growth rates for all the cell lines used. They note that other factors, such as the diffusion rate of nutrients into the centre of the callus piece, would effect the rate of growth of inner cells, which would in turn affect the overall growth rate. It should also be noted that one of the assumptions of the calculations is that the shape of the callus piece will be spherical, this cannot be guaranteed in practice.

Another difficulty that can arise from the use of weight as a measure of growth is that it is not necessarily representative of the usefulness of a treatment. It is often found that the increased weight is of no practical use. It may, for example, be the result of increased callus

growth at the expense of organogenesis. Similarly, there may be a change in the size, or moisture content in a culture, that does not represent a change in the number of cells or in their contents.

Alternative Measures of Development in Cultures

An alternative measure often used is to count the number of shoots initiated by a culture. This is of particular significance to micropropagation research where the objective is to find the most rapid and efficient method of multiplying shoots (Amin and Jaiswal, 1987). It has been suggested that counting the production of shoots and calculation of a shoot doubling time from this should become a standard measure for comparison of growth of different tissue culture systems (Flegman and Wainwright, 1981), but this practice has not been widely adopted. Counting of newly initiated roots is also commonly used to rate the efficiency of root initiation treatments, where factors such as weight are clearly not an appropriate measure.

Counting of shoots can also have a number of drawbacks as a measure of the relative usefulness of the medium. Most noticeable of these is the possibility that an increase in the number of shoots may occur concurrently with a decline in the mean size of the shoot (whether measured in terms of length or weight). Although this is not necessarily an undesirable feature it is none the less something that needs to be taken into account when assessing results.

Subjective Measurement of Cultures

Another popular method of determining growth and development in tissue cultures is to use some method of scoring individual cultures. This has the advantages that it is usually quick and easy to apply and is non-disruptive of the culture. It can also take account of a number of different factors at once, to produce an overall picture of the desirability or otherwise of the product of a particular treatment (Amin and Jaiswal, 1987; Sharma, Prasad and Chaturvedi, 1981). Another example of this is in the work of Aitken-Christie and Jones (1987), where a rating system was used in conjunction with fresh weight to determine the health of shoots in long term cultures of Pinus radiata. In this instance, the shoots being assessed were given numerical rankings "4 - green healthy shoots, vigorous growth; 3 - green and healthy shoots, slower growth; 2 - green shoots some yellowing and brown tips on needles and 1 - yellow to green shoots, some browning of entire needles."

The major difficulty with this sort of measurement is the problem of maintaining objectivity. This can be countered, to a large extent, by ensuring that measurements are taken and ratings given without reference to, and preferably without knowing, which treatment has been applied. The other difficulty with this type of measurement is that it is very difficult to standardise such measurements to allow comparison between different

experiments. 'Vigorous Growth', for example, is an entirely unmeasured concept as is 'Healthy' let alone 'Very Healthy'. This can be countered to some extent by reference to a standard treatment, as in Hiraoka and Kodama (1984). However such subjective measures are in common usage in micropropagation literature. As an example, out of a total of 296 references to micropropagation in Bhojwani and Razdan (Table 15.VI), 173 do not have an objective measure of propagation rate included in the table. This suggests that a large proportion of these references used subjective rather than objective terms to measure growth.

pH of Tissue Culture Media

The pH of media is usually measured and adjusted, if required, just prior to the addition of agar and sterilization. Dougall (1980) notes that, due to the low buffering capacity of most media, the pH may change significantly. Such changes can be expected to have effects on the metabolism of plant cell cultures. The amount of information on the behaviour of plant cell cultures, when pH is controlled is limited. Thorpe (1980) claims that the drift in pH during culture would probably be less in high salt media However, as noted by Dougall, the main buffering component in most media is phosphate, which commonly varies between 1 and 5 mM. This would be insufficient to influence the pH to any great extent (Dougall, 1980). The lack of

buffering capacity in media has been measured and highlighted by Chaleff (1983).

Most of the information relating to pH is highly specific to particular cases and refers to what is successful for a particular plant/media system, without attempting to explain why the needs for that plant are different from other plants. Bhojwani and Razdan (1983) note that pH is most commonly observed at between 5.0 and 6.0 and that pH below 5.0 does not allow for sufficient gelling of the medium, while pH above 6.0 gives a fairly hard medium. This may simply point to the need for different agar levels and may point to a means of significant savings in agar costs by raising the pH and decreasing the agar concentration. On the other hand, Skirvin (1981) notes that members of the Ericaceae grow best on Andersons medium at pH 4.5 and Stuart and Street (1969) describe a culture system for suspension cultures of Acer pseudoplatanus, where the optimum is apparently pH 6.4 - 7.2. It is interesting to note that Harris and Stevenson (1982) used a pH of 5.7 for agar media and pH 5.0 for liquid media in their work on grapes. This was apparently done on the basis that it would result in both media having a similar pH after autoclaving, although no evidence is offered to confirm this expectation.

Measurement and Adjustment of pH

It has been known for some years that the final pH of a medium after autoclaving can be quite different from that existing before autoclaving. A range of variables are involved in the autoclaving process and can contribute to this pH change. However, it is still standard practice in most plant tissue culture to quote only the pH measured prior to autoclaving, a measure that does not necessarily represent the true pH experienced by the explant when first placed on the medium. Skirvin et al (1986) showed that there are substantial changes in pH after autoclaving, both in the presence and absence of agar. They also showed that pH can change significantly after autoclaving and may continue changing for some weeks after, even in the absence of plant tissues.

Singha, Oberly and Townsend (1987) found that, in their cultures of pear and crabapple shoots, pH declined in the first few weeks and then increased over the next few. Although the concentration of a number of media constituents was monitored during this experiment, none varied in a way that might explain the pH variation that was observed, although NH_4^+ and NO_3^- were not among those monitored. This is perhaps surprising given that others have noted that pH change in the presence of plants is controlled by NH_4^+ and NO_3^- absorption (see section on nitrogen). It was also found in this experiment that although dry weight of the explants continued to increase linearly over the duration of the

experiment, the initiation of new shoots appeared to decrease approximately 2 weeks after the medium reached its lowest pH. The authors have concluded that the metabolic activity associated with the initiation of primary shoots from the explant in contact with the medium may be related to the decline in pH and that the lowest pH is associated with the cessation of primary shoot initiation.

It has been noted that in cell cultures of Nicotiana plumbaginifolia the cultures that commence growth at pH 4.0 or pH 6.0 both tend to change quite rapidly and after only a couple of days stabilised at approximately pH 5.0 (Connor and Meredith, 1984). This indicates that the cells have the ability to influence pH towards a preferred level, suggesting that the commonly assumed optimum pH of 5.8 for these cultures may not be correct. In experiments on sugarcane cell cultures, a similar change in pH was noted. During the first four days of culture, the pH of the medium changed from 6.12 to 4.94. It was pointed out by the authors that this represented a 27 fold increase in the concentration of H+ ions in the solution (Thom, Maretski, Komor and Sakai, 1981).

Behagel (1971) (as cited in Skirvin et al, 1986) discusses the dependence of post autoclave pH on the course of temperature during autoclaving. He notes that it is inevitable that there will be differences in post autoclave pH, due to the many chemical reactions, both temperature and pH dependent, that occur during media sterilization. Other

factors which Behagel claims could influence media pH include the type of autoclave, the position within the autoclave, the quality of mineral nutrients, the quality of water used in the medium and the duration of autoclaving.

Control of pH in Media

A number of unique systems have been proposed to influence or control the pH in culture. Cousson and Tran Thanh Van (1981), while studying the differentiation of flower buds, discovered that glass beads when added to media prior to autoclaving, prevented the characteristic decline in pH associated with autoclaving. However, the proffered explanation for this feature related to the dissolution of different types of glass under the high temperatures of autoclaving, this needs some further investigation.

Singha (1982) showed that pH drop associated with autoclaving could, to some extent, be decreased by the addition of agar but this did not entirely prevent it. It was not considered, in this instance, that pH decline would be exerting any significant effects on the growth being studied (Singha 1982). Given this result, the use of a higher pH for solid media by Harris and Stevenson (1982) would be likely to increase the differences between the two media rather than decrease them.

Despite the recognition by some authors of the changes in pH that can occur during autoclaving, little consideration has been given to looking at the actual mechanism by which the pH change occurs. Skirvin et al (1986) consider that it is inevitable that there will be pH changes due to the many chemical reactions that can occur during media sterilization but they do not offer any evidence or speculation on what those reactions might be.

pH Change During Culture

Dougall (1980) notes that there is often a pH decline during culture with media containing NH_4^+ as the sole source of nitrogen and that a rise in media pH occurs with NO_3^- as sole nitrogen source. He suggests that any changes in pH during culture can be related to the initial proportions of NH_4^+ and NO_3^- and to the proportions of these utilised by the plant. He is thereby suggesting that this is a mechanism by which plants are able to maintain an optimum pH that will allow maximum growth. There is now a significant amount of evidence that supports this theory, (see section on nitrogen nutrition). The effects of this will, to some extent, be influenced by the volume of media available to the explant. Small amounts of NH_4^+ or NO_3^- absorbed by

explants will not significantly affect the pH in a large volume of media. This is especially true in liquid systems, where diffusion is not a problem, although a pH gradient might be induced in solidified media if diffusion rates are sufficiently low. Singha, Oberly and Townsend (1987) mention the theory that the initial decline in the pH of an apple shoot proliferation medium might be due to acidification because of the accumulation of CO2 generated by intense respiration of the explant. The effects of selective absorption of NH4+ and NO3- can, to a large extent, be counteracted with the incorporation of buffers, such as succinate, phosphate, 2(N-morpholino) ethane sulphonic acid (MES) and calcium carbonate, in the medium, (Hamill and Cocking, 1986; Chaleff, 1983).

Effects of pH on Organogenesis

pH levels, as well as having implications for the availability of NO3- and NH4+ and many other medium constituents, have been found to be linked with organogenesis in a number of plant systems. Cousson and Tran Thanh Van (1981) have shown that flower bud formation from epidermal strips of tobacco is strongly influenced by the pH of the medium. They offer the hypothesis that pH may influence the initiation of flower buds by altering the rate of uptake of auxin, especially IAA. This has been suggested by Rubery and Sheldrake (1974), who have proposed that both undissociated IAA molecule diffusion and carrier

mediated IAA anion influx may be controlled by the pH gradient between the cytoplasm and the external medium. Adding further weight to this theory, is the work of Mutaftschiev, Cousson and Tran Thanh Van (1987), who showed that pH can influence the number of organs formed per plant as well as the proportions of root, vegetative buds and flowers buds in tobacco.

pH has also been found to exert a significant effect on somatic embryogenesis in soybean cultures (Lazzeri, Hildebrand and Collins, 1987), where it was found that the initial pH of the culture (measured after autoclaving) had little effect on the rate of embryogenesis. However, it did have a significant effect on the frequency of abnormal embryos. These occurred less frequently at low pH. However, when buffer was added to the medium it was found that low pH (pH 5.0) was inhibitory to embryogenesis. This might suggest that conditions of rising pH are most favourable to normal somatic embryogenesis.

Low pH (3.8 - 4.5) has also been found to enhance the growth of some parts of flower buds grown in culture, although overall the greatest number of flowers reached maturity at a much higher pH of 5.8 (Rastogey and Sawhney, 1987). These instances of the influence of pH on growth and development have been selected to show that there is still a great deal to be understood about the mechanism by which pH can have such diverse effects.

Nutrition of Plant Tissue Cultures

The nutrition of plant tissue culture media has quite naturally been the focus of considerable amounts of research over the years. The media must contain all the essential nutrients required by plants to sustain growth, in concentrations and forms that are accessible to the plant cells.

The Development of Media

Given the wide variety of plants and plant parts that are cultured in vitro, it is probably not surprising that there has been a wide variety of media developed, often containing only small differences in concentrations of one or two nutrients. Skirvin (1981) lists 65 different nutrient media in use for culture of fruit crops alone, a list that is by no means exhaustive. Some of the first media developed, such as that developed by White (1943), were derived from media used in the culture of algae. Although capable of sustaining growth the media did not give outstanding growth rates and were not universally applicable. In addition, the reliance on products such as casein hydrolysate and coconut milk were seen as deficiencies because the media was not totally defined. The formulation by Murashige and Skoog (1962), of a defined media for the rapid growth of tobacco callus, known as MS medium proved to be the starting point for a rapid expansion

in plant tissue culture research. This medium is still in widespread use and has become the basis for tissue culture systems involving a wide variety of plant species.

Macronutrients

Tissue culture media are generally subdivided into a number of groups of chemicals - macronutrients, micronutrients, carbohydrates, vitamins, other supplements and phytohormones. All mineral nutrients considered vital for plant growth must be included in the medium, for example nitrogen, phosphorus, potassium, calcium, magnesium and sulphur etc. It has been found that most variation occurs in the amount and source of nitrogen applied to the media (Bhojwani and Razdan 1983), although variations in other macronutrients are found to be of benefit in particular instances (Dougall 1980). The level of potassium in MS medium, for example, has been found to be inhibitory to some plants (Brand and Lineberger 1986). Most research has concentrated on the effects of nitrogen in culture media. Little consideration appears to have been devoted to possible interactions between nitrogen and other minerals such as phosphorus or potassium, or other mineral interactions.

Micronutrients, Vitamins and Carbohydrates

In the case of micronutrients, those mineral elements required in concentrations less than 0.5 mMol/L, most media include the levels used in MS medium. Very few instances are recorded of definite benefit derived from departing from this formulation. Carbohydrates are essential in all tissue culture media, even whole plants usually being unable to photosynthesise sufficiently to support growth without supplementation. The source and concentration of carbohydrates within the medium has been found to exert significant effects on the pattern of development of cultures (Hughes 1981). Vitamins are routinely included in media, even where no requirement for them has been established (Hughes 1981), although there is no doubt that in many instances they provide significant enhancement of growth (Staba 1980).

Other Media Constituents

There are, in many media, a wide range of additions that do not fit comfortably into the categories already mentioned. These include substances such as adenine, activated charcoal, amino acid and organic complexes such as casein hydrolysate, coconut milk, maize endosperm and yeast extract. These compounds have all been found to improve growth in particular cases, although the use of less well defined organic complexes is declining as the

understanding of tissue culture media improves (Staba 1980). It should be noted that addition of organic complexes can substantially alter the nutrient levels of the medium. It is reported by Thom, Maretski, Komor and Sakai (1981) that the addition of 0.1 % yeast extract to Whites basal medium gave a medium in which 70 percent of the nitrogen in the medium was available as organic nitrogen, as free amino acids, peptides or other organic compounds.

Agar is not usually considered as part of the nutrient medium but must not be ignored in the large proportion of tissue culture that relies on solid media. Bhojwani and Razdan (1983) have listed a number of instances where agar brand and quality have had an effect on culture development.

Plant Growth Regulators

The use of plant growth regulators in tissue culture is clearly established. The work of Skoog and Miller (1957) on tobacco pith callus established the basis for understanding role of plant growth regulators in controlling growth and development of plant tissue cultures. The determination of optimum hormone concentrations and combinations is still largely a matter for experimentation (de Fossard, 1981). Apart from cytokinins and auxins there are only occasional instances where other classes of compound have been shown to be of use. Giberellins are stated by Murashige (1974) to be generally inhibitory to the initiation of organs in plants although they may be of use to stimulate the growth and

development of organs previously initiated. Effectiveness of auxins tends to vary from plant to plant and may also be affected by factors associated with the composition of the medium. Use of auxin synergists, such as phloridizin, is finding favour in root initiation media for some species previously found to be difficult to root (Hammerschlag, Bauchan and Scorza 1987).

As noted by Krikorian (1982), it is misleading to assume that there is one way and one way only to achieve successful culture of any plant. There are often a number of ways of obtaining similar growth rates and development patterns through manipulation of media constituents and conditions. The choice of technique and media is often dependent on a number factors of not related directly to the efficiency of growth. A less than optimal level of one factor may change or reduce the requirement for another factor, thus improving the overall growth.

Nitrogen Nutrition of Cultures

As noted by Bhojwani and Razdan (1983), most tissue culture media contain both nitrate and ammonium, with nitrate often being the predominant form. The Murashige and Skoog (1962) MS medium is considered to be a high salt medium overall. In particular, it contains a higher level of ammonium than most media (20mM). On the other hand, Tabachnik and Kester's modification of Knops medium is one

of a number that contains no ammonium (Tabachnik and Kester 1977).

Bhojwani and Razdan (1983) claim that ammonium is often included in the medium not as a nutrient source but to act as a balancing ion to prevent excessive pH change. In contrast, it has been suggested that it is nitrate that is included to prevent extreme fluctuations in pH that would result from the use of ammonium as the sole nitrogen source (Ozias-Akins and Vasil, 1985). The work of Veliky and Rose (Veliky and Rose 1973) found that the optimum ratio of nitrate to ammonium was approximately 4:1 for carrot suspension cultures. Others have suggested a ratio of 2:1 nitrate to ammonium as more suitable (Hagimori, Matsumoto and Obi, 1982). However, it is probable that the optimum ratio varies between species cultivated and may be influenced by other media factors.

Nitrogen Nutrition and Media pH

Wetherell and Dougall (1976) first noted that the final pH of the medium was strongly affected by the ratio of ammonium to nitrate supplied in the medium. They also showed, using cell suspension cultures, that growth and embryogenesis of wild carrot tissues were markedly stimulated by the presence of reduced nitrogen sources in relatively low concentrations in the form of ammonium or as glutamine or some other forms of organic nitrogen. From this they concluded that reduced nitrogen is a requirement

for normal growth and embryogenesis. Similar conclusions had been reached with apple and pear callus cultures (Nitsch, Asahira, Rossini and Nitsch 1970).

Martin and Rose (1976) showed that in Ipomoea cultures the rate of ammonium usage increased with increasing pH, whereas the the utilisation of nitrate increased with decreasing pH. Through this mechanism plants were able to alter the pH of the medium. It was claimed by Gamborg and Shyluk (1970) that it was not possible for soybean callus cultures to grow on a medium containing ammonium as the sole nitrogen source without the presence of a Krebs cycle dicarboxylic acid. They suggested that the ammonium ions might be interfering with some of the Krebs cycle reactions, known to be sensitive to ammonium. Curtis and Smarrelli (1987) suggested that poor growth in soybean cell cultures using just nitrate as a nitrogen source might be due to inhibition of nitrate reductase.

Controlling pH Change in Media

It has since been demonstrated that many plant cultures are able to utilise ammonium as the sole nitrogen source provided the pH is not allowed to change. This may be accomplished by titration with suitable bases, such as NH4OH (Martin, Rose and Hui 1977) or with KOH and KHCO3 (Dougall and Verma 1978). Hamill and Cocking (1986), in working with nitrate reductase deficient protoplast cultures, found that growth on MS medium was poor and that the pH of the medium

declined sharply during the period of culture. However, the addition of buffers, such as succinate, phosphate, 2(N-morpholino)ethane sulphonic acid (MES) and calcium carbonate, was able to counteract the decline in pH to varying degrees and allow substantially more growth, approaching that of the parental strain containing nitrate reductase. This demonstrated that the inability of tobacco plants to grow on a nitrate free medium is most likely to be the result of pH changes in the medium rather than an innate inability to utilise ammonium.

Similar effects on the pH of nitrate free media supplemented with buffers were obtained by Chaleff and Kirby (Chaleff 1983, Kirby 1982). Hunault (1985) has suggested that asparagus cultures do not use nitrate supplied in the medium and that its role in the medium is purely to act on the pH of the medium. This claim is based on the observation that it is possible to obtain very similar rates of growth on media where buffers were supplied. It was found that MES was the best buffer but that the efficiency of various organic acids in the medium was not entirely correlated to their efficiency as buffers, suggesting some nutritional role for the buffering chemical. This is obviously unlikely to be the case for MES. A more likely suggestion is that buffers have effects on the transport and absorption of other constituents of the medium.

It would appear that in nitrate free media only part of the explanation for the poor growth can be attributed to pH

changes in the medium. Although the addition of a buffer, such as succinate, calcium carbonate or MES buffer, will greatly increase the growth of cultures, it has been claimed that succinate gives a much greater boost to growth than other pH controlling additives, even though the effect on pH is similar (Caboche 1985). It has been suggested that a major role for succinate and other organic acids is to provide a source of carbon skeletons for amino acid synthesis. Caboche's paper also shows that the response of cell cultures to nitrogen concentration is dependent on sucrose levels, with much higher levels of nitrogen required to give a similar response when the sucrose level has been raised. This highlights the fact that nitrogen interactions cannot be considered in isolation from other media factors, especially the osmotic potential of the medium. It is also possible that the nitrogen to carbohydrate ratio of the nutrient medium may be an important factor.

Experiments by Chaleff (1983) suggest that there is a definite need for the presence of a buffer in media with ammonium as the sole nitrogen source in rice callus cultures. It is claimed in this article that there is evidence that the organic acids used as buffers (succinate and a-ketoglutarate) are enhancing growth in some manner apart from their action as buffers. Before it can be conclusively claimed that prevention of pH change is not the only factor, some evidence of the lack of pH change should have been provided. As some of the media used in these

experiments were autoclaved and others the subject of filter sterilisation it would seem to be an elementary precaution to ensure that these differences in media preparation did not result in differences in the starting pHs of the various media. It is also possible that the action of the callus on medium may have caused the pH to vary through changes to absorption patterns. This factor could have easily been accounted for by measuring the pH of the medium prior to and at the conclusion of the experiment.

Effects of Nitrogen on Differentiation

There appears to be an increasing body of evidence to suggest that the form of nitrogen supplied to the medium can influence growth in ways other than those related to pH effects. In studies on Agave cultures it was found that it was essential to have some ammonium in the medium for shoot formation to occur and that the process of shoot initiation was sped up and enhanced by the inclusion of 1mM L-arginine (Robert, Herrera, Contreras and Scorer 1987).

Caboche (1987) found that in protoplast derived colonies of Nicotiana plumbaginifolia cells there was a marked influence by the source of nitrogen on the rate of shoot induction. It was found that, as well as there being an increased incidence of shoot induction with increasing concentrations of nitrogen, there was clear evidence that nitrate was promoting morphogenesis at a lower concentration than either ammonium or glutamine. He claims that an

approximately five fold increase in the concentration of ammonium is required to promote morphogenesis as efficiently as on media containing only nitrate. Caboche also suggests that, for this particular system, concentrations of ammonium above 20mM could be exerting cytotoxic effects. The claim that nitrate is more efficient in promoting shoot formation needs some qualification. In the figure accompanying these claims, the highest levels of shoot formation occur under the influence of ammonium. This occurs at a much higher ammonium concentration than the maximum rate of shoot formation for nitrate and only at low sucrose concentrations. Caboche suggests that the differences observed may be related to the secondary effects of nitrate assimilation on organic acid biosynthesis, an effect suggested by Haynes and Goh (1978). Alternatively, the improved rate of shoot initiation at low sucrose levels might suggest that the influence is due to differences in the rate of uptake of the two forms and that the sucrose level affects this by altering the osmotic potential of the medium.

The presence of ammonium is a definite requirement for initiation of somatic embryogenesis in alfalfa cultures, where it has been found that suspension cultures will not initiate embryogenesis unless exposed to at least six days of culture in the presence of ammonium (Walker and Sato 1981). It was also found that the degree of root development occurring in these cultures was significantly

inhibited by levels of ammonium that allowed substantial amounts of somatic embryogenesis. From this the authors concluded that there was evidence that there was an interaction between the exogenously applied ammonium ions and the growth regulators. This interaction was found to be controlling the pattern of morphogenesis in the cultures, even though there was a time separation between the application of the growth regulators and the ammonium.

In contrast, work on Brassica napus cultures has found that shoot initiation from epidermal strips is at its highest frequency when there is no ammonium in the medium and that the frequency declined with increasing substitution of ammonium for nitrate (Klimaszewska and Keller 1985). This effect was noted whether using ammonium citrate or ammonium chloride as the ammonium source, indicating that pH changes were unlikely to be involved in the mechanism. Experiments with wheat anther cultures have also found that nitrate levels are more important than ammonium. Feng and Ouyang (1988) noted that ammonium levels had little effect on the rate of initiation of callus in wheat anther cultures, which were found to respond to the nitrate level. Thus, it would appear that the importance of ammonium and nitrate in influencing organogenesis is very likely to be species dependant.

Dickens and van Staden (1988) have found that flower initiation in vitro can be affected by altering the level and source of nitrogen in the medium, in conjunction with

changes in day length, although it is suggested that at least part of this effect may be related to osmotic influences. Ammonium levels have also been demonstrated to have an inhibitory effect on callus growth in Pinus strobus, where media containing high levels of ammonium were found to give less growth than media containing a lower level of ammonium (Flinn, Webb and Georgis, 1986)

Ammonium Levels and Vitrification

Ammonium ion has been linked to vitrification in a number of species (Daguin and Letouze, 1986; Witrzens, Scowcroft, Downes and Larkin, 1988). In willow cultures an increased tendency to vitrification has been linked to increased levels of glutamate dehydrogenase activity. This is, in turn, correlated with the amount of ammonium supplied to the plant and the affinity constant for ammonium. It was found that decreasing the level of ammonium in the medium resulted in a lower frequency of vitrification. Witrzens et al (1988) noted that altering the balance of ammonium to nitrate or increasing the total nitrogen level had no effect on the rate of shoot initiation in sunflower cultures. It was found, though, that substituting inorganic nitrogen with amino acids did give some improvement in initiation in some clones as well as a decreased frequency of vitrification.

Nitrogen Nutrition in Boronia megastigma

B. megastigma has previously been noted to be sensitive to the intensity and source of nitrogen applications in field plantings and glasshouse pot trials. Reddy (1987) used container grown boronia and plants in solution culture to establish that boronia showed increased growth using ammonium as a sole nitrogen source over that obtained using nitrate. This was thought to be related to the nitrogen status in the plants normal forest habitat. It was also noted that increased levels of nitrogen had a significant effect on the development of the plant by causing an increased number of axilliary shoots to develop into lateral shoots. The ability of the plant to utilise nitrate was found to be inhibited by the low level of nitrate reductase found in the leaves. It was also noted that at low nitrogen levels the plants had a tendency to accumulate other minerals leading to toxicity symptoms.

Adenine in Culture Media

As noted by Wareing and Phillips (1978), adenine was the first compound to be described as having activity as a cytokinin. Its discovery was made during investigation of the growth requirements of tobacco callus cultures. However, it has very low activity in comparison to kinetin, benzyladenine (BA), zeatin and other cytokinins, the majority of which are adenine derivatives. It has fallen

out of use as a source of cytokinin activity in plant tissue cultures. Murashige lists a number of instances where adenine has proved beneficial to tissue cultures, however these relate primarily to research conducted prior to the widespread use of benzyladenine and kinetin (Murashige 1974).

Even though adenine is no longer being used as the primary source of cytokinin activity, it is none the less included in many plant tissue culture media. Many of these media are based directly upon Murashige and Skoog MS medium (Murashige and Skoog 1962) but others are more distinct media where adenine is specifically added, although there may not be any reference to the reasons for its inclusion in the medium. These media, such as that proposed for raspberry (Pyott and Converse 1981) or broccoli (Anderson and Carstens 1977), already contain a cytokinin and are substantially different from MS media. It must be assumed, therefore, that there are advantages for its inclusion distinct from the high levels of cytokinin already included in this medium. Hughes (1981) does not include adenine in the section of her review relating to cytokinins but includes it in the section titled "Other organic compounds" and cited Murashige (Murashige 1974) for a review of the effects of adenine. However, the references cited by Murashige relate to the effect of adenine as a weak cytokinin. Reinert (1973) suggests that adenine and some amino acids, such as tyrosine, may act to augment the

effects of kinetin on the organogenesis of tobacco callus. In one example, where adenine has been shown to beneficial to plant growth, it is claimed to be acting as a cytokinin synergist (Mante and Tepper 1983). There are also instances where the effects of adenine have been found to be detrimental. It was found to inhibit root formation in carnation, as did kinetin, suggesting cytokinin activity (Pennazio 1975). It was also found to decrease the rate of survival of newly established grape shoots (Harris and Stevenson 1982).

Possible Roles For Adenine

It is well known that adenine is the main breakdown product of cytokinin metabolism through the enzyme cytokinin-oxidase, which cleaves the side chain to produce free adenine (Letham and Palni 1983). In soybean callus cultures, utilising C14 labelled benzyladenine, zeatin and kinetin, the callus was found to rapidly accumulate quite large amounts of adenine. In the case of BA, over 25% of the applied radioactivity was detectable as adenine after 48 hours (Forsyth and Van Staden 1986). However, it was shown in these experiments that adenine did not itself exhibit any significant growth promoting properties on the soybean callus, suggesting that in this system there is no significant synthesis of cytokinins from adenine. Studies on immature caryopses of maize have shown that, although adenine was metabolised very rapidly, none of the labelled

adenine was detectable in free cytokinins in the plant. This suggests that free adenine is not used as a precursor of cytokinin synthesis in this plant (Van Staden and Forsyth 1986).

There is evidence from some plant species that suggests that cytokinins are sometimes synthesised from adenine bases but the evidence is apparently questionable (Letham and Palni 1983). The report by Chen, Ertl, Leisner and Chang (1985) showed, however, that when C14 labelled adenine was supplied to pea and carrot tissues there was significant incorporation of the radioactivity into cytokinins. This might suggest that adenine, where it has been demonstrated to have an effect, is related to the synthesis of cytokinins. A positive effect from the inclusion of adenine in tissue culture media need not be derived solely from its possible incorporation in cytokinins. As a purine it plays a central role in a large number of plant biochemical reactions. Adenine can, for example, inhibit de novo purine synthesis in Catharanthas roseus because of the plants ability to scavenge excess adenine and convert it to adenine nucleotides (Hirose and Ashihara 1984). It is not certain to what extent this use of adenine would add to the growth of plants, although it might decrease the requirement for nitrogen.

Vitamins

All plant tissue culture media contain vitamins but the number of vitamins included and their concentrations vary widely. It seems to be generally accepted that thiamine is the vitamin most often required (Murashige 1974, Skirvin 1981). Dougall (1980), however, notes that even it has not been found essential in all cultures but that it is beneficial in most. The two other most commonly used vitamins, pyridoxine and nicotinic acid, are also water soluble or B group vitamins, although the evidence for their absolute requirement is less common.

There are a number of instances where various other vitamins have been added to media to improve growth or organogenesis. The most complex mix is that of Staba (1969), which contains ten different vitamins. However, the difficulties of adding vitamins that are not water soluble and may be heat labile preclude their use in most micropropagation schemes, although it is used in some media (Parliman, Evans and Rupert 1982; Skirvin and Chu 1979). Dougall (1980) lists a number of instances where pantothenic acid requirements have been tested, none of which showed a positive requirement for this vitamin. Vitamin E (dl-alpha tocopherol acetate) has been shown to to be of importance in the promotion of callus growth in clover and soybean (Oswald, Smith and Phillips 1977).

The amino acid glycine is also routinely included in media and is routinely referred to in discussions on

vitamins. Its inclusion in Murashige and Skoogs medium, based on the experience of White (1943), has led to its widespread use without serious question as to what role it is fulfilling in the medium. On occassions when glycine has been tested as a medium supplement it has often been found to be unneccessary (Hagimori, Matsumoto and Obi 1982). Evans, Sharp and Flick (1981) note that it has been found to slightly increase the yield of some cell cultures but Gamborg and Shyluk (1981) and Skirvin (1981) fail to mention it at all. This is surprising, especially since Skirvin lists 25 different media which contain glycine as an ingredient. Ascorbic acid is occasionally added to media, however this is usually intended to act as an anti-oxidant rather than having a nutritional value to the culture (Murashige 1974).

Although many of the most commonly used media contain the same three vitamins, the concentrations and proportions vary quite substantially. Murashige and Skoog (1962) included these three vitamins at levels which have become standard for many tissue culture systems, even where other aspects of the medium have been changed. However, there is not any discussion by Murashige and Skoog on why those particular levels should be used, apart from noting that they were based on Whites solution. It should be noted however that the vitamin level for White's solution quoted in Murashige and Skoog appears to differ from that quoted by White (1943). In the case of the medium developed for

tissue culture of citrus by Murashige and Tucker, as described by Skirvin (1981), the only difference between this medium and that of Murashige and Skoog (1962) is in the concentrations of the three vitamins, thiamine, nicotinamide and pyridoxine, which are increased by factors of 100, 10 and 20 respectively. This medium is used extensively in tissue culture of a number of citrus species (see Table 1 in Skirvin, 1981).

Despite the almost universal use of vitamins in culture media there is very little information available about why vitamins need to be added to media. Dougall (1980) suggests that the main reason for including vitamins of any type in media might be to minimise stress on explants or cultures. He suggests that the stress might result from low expressed capacity to synthesise vitamins, leakage of vitamins into the medium or general stress due to transfer to a fresh medium. He points out that, in most instances where vitamins have been shown to be needed, the cell cultures have been under some form of stress. The use of vitamin E in clover and soybean callus has been linked with the inhibition of fatty acid oxidation as it has in mammals (Oswald, Smith and Phillips 1977). The use of vitamins such as vitamin E needs to be considered very carefully. It may be an indication of some other deficiency of the media or culture conditions rather than a genuine deficiency of this vitamin in the plant cells.

Sucrose and its Role in Tissue Culture

As noted by Dougall (1980) and others, plants are able to use a number of carbohydrates as sources of energy but sucrose and its component monosaccharides give the best growth rates, as in, for example, apples (Chong and Pua, 1985). Other carbon sources have been applied in particular cases. Many Rosaceous plants, for example, are apparently able to grow as well using sorbitol as the source of carbohydrate (Skirvin, 1981). It is perhaps not surprising that fructose and glucose should be considered satisfactory substitutes in many cases since it has been found that most, if not all, sucrose is hydrolysed to produce these sugars, after which uptake readily occurs. The results of Ball (1953) should also be considered when discussing carbohydrate nutrition. He found that up to 30% of the supplied sucrose in a medium was hydrolysed during autoclaving and that this resulted in significantly different growth patterns from those obtained where the sucrose had been filter sterilised.

The only other sugar that is in use almost universally is myo-inositol, which was orginally added to cultures after its discovery as one of the active components of coconut milk. Gamborg and Shyluk (1981) claim there is no absolute requirement for it but Hughes (1981) claims it has been shown to be essential for a number of culture systems and species. It is now included in media almost without question. There appears to be little doubt that the

influence of myo-inositol is not to act as a source of carbohydrate. It is more likely to be because of its involvement in the synthesis of phospholipids, pectins, and cytoplasmic membrane systems (Loewus and Loewus, 1983). It also plays an important role in the area of bound auxins, where the IAA-inositol glycosides are linked to a number of important metabolic processes (Hughes, 1981; Cohen and Bandurski, 1982). This does not explain why supplementation of myo-inositol is needed in media, particularly in proliferation cultures, where plant metabolism is similar to that in whole plants.

The necessity for supplying a source of carbohydrate to cultures is quite clear. Attempts to induce cultures to become photoautotrophic by simply omitting sucrose from the medium have been largely unsuccessful (Langford and Wainwright, 1987). It has proved possible to induce photoautotrophism under conditions of high light intensity and enhanced levels of CO2 inside the culture vessel (Husemann 1985). Such conditions do not provide entirely satisfactory growth rates experimentally and are not suitable for large scale propagation. It has been observed in carrot root cultures that, over a period of four weeks, nutrition changes from entirely heterotrophic to become mixotrophic, where photosynthesis plays a significant role. This can extend to becoming autotrophic if the supplied sugars are exhausted (Bender, Pauler and Neumann, 1987).

Sucrose levels are, in the main, kept at 30 g/L and with very few exceptions are within the range 20 to 50 g/L (Skirvin 1981; Escobar, Villalobos and Villegas, 1986). It has been noted that roses tend to have a higher rate of CO2 usage when grown at lower sucrose levels but that this corresponds with lower fresh weight and an increased incidence of vitrification (Langford and Wainwright, 1987). Care needs to be taken in interpreting the effects of varying sucrose levels because of the action of osmotic potential on the availability of other nutrients.

Sucrose and Osmotic Factors

Much of the research conducted on the effects of sucrose in media is directed not at the effects of carbohydrate source and concentration but at the effects sucrose may be having on the osmotic potential of the medium. Sucrose is by far the largest component of most media. In Murashige & Skoog it is approximately 70%, by weight, of the solutes of the medium (assuming agar at 8 g/L) and assumes even greater importance in low salt media or in liquid cultures. Sucrose, is therefore, the most important factor in influencing osmotic potential of media, a factor which, in many cases has been found to influence growth and differentiation of cultures significantly.

One of the difficulties is that researchers often do not attempt to distinguish between the osmotic and nutritional effects. High sucrose levels have been used to

slow the growth of garlic cultures to improve long-term storage. This result has been attributed to a purely osmotic effect (El-Gizawy and Ford-Lloyd, 1987). In studying the initiation of haploid embryo formation in the leguminous tree Peltophorum pterocarpum it was found that sucrose levels of less than 10% allowed too much callus development at the expense of embryo development but that at higher sucrose levels caulogenesis declined and allowed more androgenesis to occur (Lakshmana Rao and De, 1987). It is suggested by the authors that, in this case, the high concentration of sucrose is acting primarily as an osmoticum rather than as a triggering agent for embryo development, although no further explanation is offered as to how osmotic changes might act to affect embryogenesis.

Morphogenetic Effects Of Sucrose

Increased sucrose levels above the optimum for shoot proliferation have been found to strongly promote root initiation in an number of species (Maene and Debergh, 1985; Ziv, 1981). In some cases it has been found that it is not possible to duplicate the effect using mannitol. This shows that in these the influence of sucrose is unlikely to be due solely to an osmotic effect (Maene and Debergh, 1985). Mannitol has, however, been shown to have a beneficial effect on rice callus cultures. Addition of 3% mannitol or sorbitol significantly increases the ability of the callus to regenerate shoots after long term (1400 days) culture, an

effect that could not be duplicated by raising the sucrose level (Kavi Krishor and Reddy, 1986).

There is clear evidence that carbohydrates exert influence on the induction of flower initiation. This is apparently not related to altered osmotic values but rather to the effects of increased free sugar levels in the shoot apex during induction to flower (Dickens and Van Staden, 1988).

The interaction between carbohydrate levels, nitrogen levels and the osmotic potential of the medium is an area that has gained increasing attention over the last few years. It is clear that alterations in the sucrose level do have important effects on the osmotic potential of the medium but how much these changes affect growth is yet to be determined.

Root Initiation and Post-Culture Survival

The ultimate aim of a micropropagation is to produce healthy, viable cloned plants in soil. Much of the research in plant tissue culture is concerned with attempting to find the most efficient, effective method to clone plants through axillary proliferation, adventitous shoot production, or somatic embryogenesis. Much less research appears to be devoted to the difficulties associated with initiating roots on those shoots and with establishing healthy plants in soil.

Krikorian (1982) extensively reviewed the general techniques for cloning higher plants through tissue culture without including a section on the techniques and difficulties of root initiation and establishment on soil. It is clear that a plant cannot really be claimed to have been successfully cloned until it can be established in the ground. Much of the information that is available relates to individual species. Many of the reviews of the techniques consist of a list of individual examples of different techniques that have been successfully applied, without any attempt to establish underlying principles (Hughes, 1981; Skirvin, 1981; Bhojwani and Razdan, 1983).

Root Initiation in Non-sterile Media

It has been found that many plants can be successfully rooted without the use of a separate root initiation medium. The choice of whether to use a root initiation medium or to

root plantlets directly in a non-sterile medium would appear to depend very much on the plant in question. Many plants will form roots readily, without the need for added hormone treatments, and so may be planted into a mistbed or similar non-sterile medium directly from the proliferation phase of culture. Some authors (Hussey 1980; Debergh and Maene, 1981; Pyott and Converse, 1981) claim that where possible this should be the preferred method, primarily because of its simplicity and reduced cost. Others claim that rooting under non-sterile conditions can offer better root initiation and faster growth (Brand and Lineberger, 1986).

Constantine noted that if 'ex vitro' root initiation is to be attempted then the shoots produced for this must carry sufficient endogenous reserves to carry them through the rooting process and the change from heterotrophic to autotrophic growth (Constantine, 1986). It has been claimed that root initiation in vitro can result in poor vascular connection between the root and stem due to the interferance of callus growth (Rancillac, Faye and David, 1982). However, in many species such ex vitro rooting is not possible either because the plantlets do not root easily or because very small plantlets are produced. The problems of very small explants can be overcome by the use of a 'singulation medium' (Debergh, 1987) to allow shoots to extend and develop. However, this defeats some of the benefits of direct rooting. In such cases, a root initiation phase is needed. If the necessary alterations to

the balance of hormones are made, a rooted plantlet can be produced. With the cessation of proliferative growth it is often the case that the root initiation phase also allows time for the plantlets to become much larger, making separation into individual plantlets much easier and improving the rate of survival.

Factors affecting Root Initiation in Culture

Clearly the most important factor controlling the initiation of roots is the change in hormone balance, something that is highly specific to the species and variety of plant being grown and should mostly be determined by direct experimentation. The balance of relatively high auxin to cytokinin levels, first established by Skoog and Miller (1957), is still found to be the case in most situations. In addition, a number of other features have been shown to play a significant part in initiation of roots in some species. Bhojwani and Razdan (1983) list a number of instances where a reduction in salts concentration or additions and omissions of media components have improved the rate of root initiation in particular plants.

Nutritional Factors

In the case of roses it was shown (Hyndman, Hasegawa & Bressan, 1982a) that lowering the concentration of nitrogen in the medium had a marked effect on the rate of root initiation, an effect that was not found when the

concentration of other salts was altered. The ratio of NO3 to NH4 was also found to be influencial. A ratio of 3:1 (NO3:NH4) was found to be the most beneficial and was not corelated with the final pH of the medium, suggesting that there is a positive role for NO3 in rhizogenesis in roses (Hyndman, Hasegawa & Bressan, 1982b). It was also claimed that raising the sucrose concentration in the medium enhanced root initiation quite independently from the influence of nitrogen. This effect was found to be solely due to the influence of sucrose and could not be mimiced by other sugars. This implies that changes in the osmotic potential of the medium were not responsible for the observed changes.

The phenolic glycoside, phloridizin, a component of apple sap, and its breakdown product, phloroglucinol, has been added to cultures of apples to improve root initiation (Jones, 1976, Welander and Huntrieser, 1981). It is suggested that the phenolic compound is acting as a synergist to auxin. However, it appears that the effects of phloroglucinol on root initiation are not consistant and may be genotype specific (Jones and Hopgood 1979; Zimmerman and Broome, 1981). Other compounds that have been used as auxin synergists include chlorogenic acid, rutin and quercitin (Hammerschlag, Bauchan and Scorza, 1987).

Time in Culture

A number of researchers have found that root initiation is affected by the time that the plants have been in culture. Gupta Mascarenhas and Jagannathan (1981) found that Eucalyptus citriodora would not initiate roots until after it had been subcultured at least three times. The time in culture has also been found to be important in rose cultures (Bressan, Kim , Hyndman, Hasegawa and Bressan, 1982) and in peach cultures (Hammerschlag, Bauchan and Scorza, 1987). In some apple cultivars at least 30 subcultures are required to achieve reasonable levels of root initiation (Sriskandarajah, Mullins & Nair, 1982; Howard and Marks, 1987).

Culture Conditions

Culture conditions are important both in influencing root initiation as well as post culture survival. Light conditions have been shown to influence root initiation in apples, where a decrease in light intensity was found to be beneficial (Howard and Marks, 1987), and in roses, where similar results were achieved (Khosh-Khui and Sink, 1982). However, other work on roses found that increasing the light intensity resulted in more root initiation (Bressan, Kim, Hyndman, Hasegawa & Bressan, 1982). Bressan et al also found that exposure of plantlets to cool night temperatures enhanced the initiation of roots.

Post Culture Survival

Murashige (1974) noted that "a successful tissue culture method of propagation must result in re-establishment in soil of a high frequency of the tissue culture derived plants" and further noted that the preparation of propagules for re-establishment in soil was an area of research that had been neglected. He proposed that this area be considered a completely separate stage in the micropropagation process. Ziv (1986) suggests that one of the main problems is that the tissue culture conditions that promote rapid growth and maximum shoot proliferation often result in the formation of abnormal plants, most notably by causing poor development of cuticular waxes, loss of stomatal function and abnormal leaf development. Root initiation usually occurs in the same growth conditions as is used for shoot proliferation. Derbergh (1987) claims that high humidity in the culture vessel results in the development of a non-functional plant vascular system and that this is the main cause of the low survival rates noted by other authors.

Cultured plants often have poor vascular connection between the shoot and root, making them vulnerable to wilting (Grout and Aston, 1978). It has been found that there are significant differences between the leaf anatomy of plum plants grown in vitro and those grown in glasshouse conditions (Brainerd, Fuchigami, Kwiatskowski and Clark,

1981). This is combined with a stomata frequency in the tissue cultured plants found to be 50% of that in glasshouse grown plants. Overall, it was found that tissue cultured leaves lost moisture at up to three times the rate of glasshouse grown leaves. Tissue cultured leaves have also been found to be almost totally lacking epicuticular waxes, a factor which was found to be a significant contributor to the excessive water loss noted in tissue cultured plants (Fuchigami, Cheng and Soeldner, 1981). This may also contribute to the susceptibility to attack from phytopathogens involved in 'damping off'.

Vitrification

According to Debergh (1987), many plants showing poor growth and low survival should be considered to be vitrified, even if the normal visible symptoms of vitrification are not present. Vitrification was first described by Debergh, Harbaoui and Lemeur (1981) and has become the standard term to describe the water soaked appearance of plant structures that is often found in a percentage of cultures and is frequently described as being associated with excessive moisture in cultures (Debergh, 1983; Ziv 1986). It is associated with very poor growth and a greatly reduced post culture survival rate (Bhojwani and Razdan, 1983). Vitrification can be induced in existing plant organs but requires new plant structures to develop to allow reversion to normal cell types to occur (Bottcher, Zoglauer and Goring 1988). Alternative explanations for the

causes of vitrification have been suggested including excessive cytokinin levels (Leshem, Werker and Shalev, 1988) and ammonium levels in media (Dagouin and Letouze, 1986).

The Role of Humidity in Vitrification

Much of the research on this problem has been directed towards attempting to decrease the humidity within the culture vessel. This would allow the plant to develop under conditions more closely aligned with those encountered in vivo. Ziv (1986) discusses a number of possible modifications that might result in better survival. It can be argued that most of these have an effective, if not direct, consequence of lowering the relative humidity within the culture container. Debergh claims that most of the difficulties encountered in achieving a suitable success rate in establishment can be traced to excessive humidity within the tissue culture flask. He suggests that vitrification is a physical manifestation of the effects of high humidity. He suggests that the humidity should be lowered within the flask during the root initiation phase. This would result in improvements in all those features associated with low survival, such as lack of leaf waxes, non-functional stomata and abnormal leaf and vascular development (Debergh, 1987).

Many authors propose increasing the concentration of agar in the root initiation stage as a method of increasing the survival. This results in the appearance of fewer

vitrified shoots, apparently as a result of changes to the osmotic potential of the medium. Similar results can be obtained using increased sucrose or other carbohydrate concentrations (Ziv 1986). However, not all species respond to osmotic potential in this way. Debergh, Harbaoui and Lemeur (1981) found that increased sucrose concentration had little effect on vitrification but that increasing the agar concentration did result in a significant decrease in the frequency of vitrified plants.

Others have found that the incidence of vitrification in apples can be reduced by the inclusion of phloridzin in the medium. This apparently acts to help restore the ability to synthesise lignin, a deficiency that has been linked with vitrification in apple cultures (Phan and Hegedus, 1986). Witrezens, et al (1988) found that phloridzin was also found to control vitrification in sunflower cultures, as did the other phenolic glycosides, naringin and esculin hydrate. These authors also found that the incidence of vitrification could be reduced by substituting a mixture of amino acids for some of the inorganic nitrogen in the medium.

The work of Barghchi (1987) found that high humidity in flasks was the cause of shoot tip necrosis in Pistacia species through preventing calcium transport in the plant vascular system. It has also been found that covering flasks with PVC film reduced growth and development of cultures as well as the rate of flower bud initiation, an

effect that could well be related to reduced transpiration rates within the flask (Dickens and Van Staden, 1988). The work of Jackson et al (1987) discusses in detail some features of the gaseous phase of tissue culture flasks with respect to the concentrations of ethylene and carbon dioxide and the possible negative effects these gasses might have on development of cultures but fail to consider the effects of relative humidity.

The solution proposed by Debergh (1987) is to apply a temperature differential to the flask by actively cooling the bottom of each flask. He found that if the temperature of the bottom of the flask was at least 5 deg less than the growth chamber then water vapour tended to condense on the agar rather than on the sides of the flasks and relative humidity of less than 75% could be achieved giving significant improvements in survival. An alternative method of preventing desiccation damage in plants lacking epicuticular waxes has been suggested by Selvapandiyan et al (1988), who have successfully applied either glycerol, paraffin wax or grease to the leaves of plantlets at the time of transplantation. This greatly improved the percent survival of tissue cultured plants in a number of species.

Root Initiation in Boronia

Boronia has been propagated from cuttings with variable amounts of success (Legget and Menary 1980). Lamont (1985) was able to achieve up to 75 % root initiation in cultures

of B. serrulata but only 50% post culture survival. Root initiation could only be achieved by use of liquid media and filter paper bridges. The presence of agar apparently inhibited the formation of roots.

Summary of the Literature Review.

There are large gaps in the knowledge of factors controlling the development of plant cells and organs in culture. Although there is great deal of information which suggests uses for tissue culture in all facets of agriculture. There is a persistant problem that many of the techniques proposed are still only applicable to a limited number of species. Many of the more exotic techniques available in plant tissue culture are yet to be applied to those species in which they would be of most use, such as the major agronomic crops and woody perennials. Part of the reason for this is undoubtedly due to the lack of knowledge of the precise factors controlling morphogenesis. There is a lack of understanding of how medium constitiuents interact within the medium to affect not only the growth rate of the plant but also the pattern of development aand organogenesis. One difficulty is that the effects are, on occasions imprecisely measured. Until more knowledge is gained much of the research in tissue culture will continue to rely on an empirical approach, referred to as 'in vitro gardening' by Yeoman (1986).

Before any of the techniques can be applied to boronia the factors which have the most influence over growth and development must be identified. Once identified an understanding should be gained of the mode of action to facilitate prediction of growth under different conditions.

III. MATERIALS AND METHODS

Media

The standard media used in all experiments is based on a modification of Murashige and Skoogs (MS) medium first used for tobacco callus (Murashige and Skoog, 1962). This has been modified by replacing the NH_4NO_3 and KNO_3 with 1400 mg/L KCl and variable amounts of NH_4NO_3. This is, in effect, MS medium modified to be nitrogen free, to which NH_4NO_3 can be added as required. All macronutrient media constituents were made up in individual stock solutions, to prevent the possibility of precipitation. This system allows for easy preparation of test media by omitting or varying any one particular component under study. Fresh stock solutions of hormones were made up before each experiment, all dissolved in minimal 1N NaOH. Analytical grade chemicals were used for all mineral nutrients and sucrose. All other chemicals used in media preparation, such as the hormones, vitamins and other organic media constituents were supplied by Sigma.

Proliferation Media

G5 Medium - Basal medium + 500 mg/L NH_4NO_3
G8 Medium - Basal medium + 800 mg/L "
G10 Medium - Basal medium + 1000 mg/L "
G12 Medium - Basal medium + 1200 mg/L "

Root Initiation Media

R3, R5, R8, R10, R12 and R16 media all contain basal medium without Adenine hemi-sulphate and with 300, 500, 800, 1000, 12000 and 1600 mg/L NH_4NO_3 respectively.

Table 1

Basal Media - Based on that of Murashige and Skoog (1962) with modifications

	(mg/L)		
KH_2PO_4	170	$MnSO_4.4H_2O$	22.4
$MgSO_4$	370	$ZnSO_4.7H_2O$	8.6
NaFeEDTA	40.7	H_3BO_3	6.2
KCl	1400	KI	0.83
$CaCl_2.2H_2O$	440	$Na_2MoO_4.2H_2O$	0.025
		$CuSO_4$	0.025
Glycine	2.0		
Thiamine	0.1	Proliferation Media	
Pyridoxine	0.5	IAA	4.0
Nicotinic acid	0.5	BAP	1.5
Myo-Inositol	100	Root Initiation Media	
Adenine sulphate	80		
Sucrose	30,000	NAA	4.0
		Kinetin	0.15

The pH was normally adjusted to pH 5.8 by adding 1 N NaOH or 0.1 N NaOH when media volumes were small or critical or where the required adjustments were slight.

Agar

Difco Bacto agar was used throughout. It is an agar used by many researchers, although it is noted by some to have its faults (Debergh, 1983; Bhojwani and Razdan 1983). Agar concentration was 10 g/L for all experiments in 50 ml

flasks and nominally 11.1 g/L for root initiation experiments and for the 'Growth Period' experiment. This was obtained using a scoop containing 0.4 g/L (+ or - 0.02 g/L) to measure dry agar into individual flasks.

Flasks

All experiments were carried out in autoclavable polycarbonate sample containers with translucent lids, made by Bunzyl plastics. Two sizes were used. 250 ml cylindrical sample containers (70 mm in diameter) were used for root initiation media and for pre-treatment media. For these, dry agar and 36 ml of medium were dispensed into flasks prior to autoclaving. All other experiments used 50 ml sample containers (30 mm in diameter), in which individual explants were grown. For these flasks, the media was first heated to approximately 100 deg C to dissolve agar then allowed to cool slightly before dispensing 8 ml into flasks and autoclaving it.

Autoclaving of Media

All media were autoclaved at 121 deg C (15 psi) for 15 minutes but using three different types of autoclave with quite different modes of operation.

'Type 1' Autoclave - An American Steriliser Company 'Cyclomatic Control' model, relying on a pressure valve to control the pressure and hence temperature of the autoclave.

'Type 2' Autoclave - An American Steriliser Company 'Athena' model, which is fitted with thermometers linked to a steam valve to control the temperature

'Type 3' Autoclave - a Namco pressure cooker sitting on a gas ring, pressure controlled by a weight valve.

The 'Type 1 and Type 2 autoclaves are both insulated and fitted with a steam jacket. Both of these features add to the time taken to cool to room temperature. The type 3 autoclave has the advantage that it heats and cools very quickly.

Origins of Cultures

Apart from the 'Clone Trial' and two root initiation experiments, all experiments have been conducted on the University of Tasmania clone HC4. This is a clone selected for its commercial oil and harvest characteristics, which make it a desirable type for large scale propagation. The cultures of HC4 used were established from shoot tips and had been grown for approximately four years. The cultures have all been through numerous subcultures during this time. Any abnormal cultures have been discarded, including those showing signs of vitrification. All other clones used are from the University of Tasmania selections undergoing evaluation for potential commercial use. The cultures have all been established for more than twelve months and have undergone numerous sub-cultures.

Selection of Hormone Levels

Hormone levels were arbitrarily maintained at 1.5 mg/L BAP and 4.0 mg/L IAA for proliferation experiments and 0.15 mg/L Kinetin and 4.0 mg/L NAA for root initiation experiments. These hormone levels are believed to be close to the optimum for use with the clone HC4 and are quite well suited to other clones. They have been found to be well suited to large scale micropropagation, but do not necessarily represent the levels to obtain maximum proliferation or root initiation, as some compromise is needed to allow for ease of handling.

Pretreatments

Unless stated otherwise, all experiments used five week old cultures grown under standard growth conditions, using standard G5 medium.

Growth Conditions

All experiments were grown under standard light and temperature conditions, mostly in Conviron growth cabinets. These cabinets were programmed to provide days of 16 hours at 22 deg C and a light intensity of 80 to 100 uE and an 8 hour night at 18 deg C. The 'Root Initiation Conditions' experiment was also conducted in these cabinets. The remaining root initiation experiments were grown in a growth room, where temperature was controlled with a commercial air conditioner, under similar temperature and photoperiod

conditions but with a light intensity of 70 to 95 uE. In practice, it has been found that the temperature control of this room is less precise than that of the commercial cabinets, particularly when temperature or light changes occur in the cabinet.

Measurement

In experiments where relative growth has been determined the starting weight of each explant was determined by tareing the weight of the flask on a Mettler top pan balance, to within 10 mg, adding the explant to the flask and reweighing. At the conclusion of the experiment each explant was removed from its flask, all adhering agar was removed by scraping and it was then wiped with tissues to remove any surface moisture. The explant was then weighed on the same top pan balance. In experiments where dry weights were determined, the explants were then dried at 60 C for 48 hours and then reweighed using a Mettler precision balance, to within 1 mg.

After fresh weight had been determined, the number of shoots were counted for each explant. For the purposes of counting, any part of the explant that had the appearance of being a vegetative shoot, visible to the naked eye, was counted. In practice, this usually meant any shoots above 1 mm in diameter were counted. There were, on occasions considerable difference in the size and appearance of shoots

within and between treatments. Because of the complexity of this task and to ensure a reasonable degree of accuracy of counting a sub-sample of explants were routinely recounted. Accuracy was found to be within + or - 10% on all occasions.

Nodes were counted as a measure of the amount of extension growth occurring in the explants. A node was counted as being the appearance on a shoot stem of a pair of leaflets of any description, apart from the pair of leaflets surrounding the terminal bud. This included any leaflet pairs on side branches of large shoots.

pH Measurement

Where the pH of spent media was measured in 50 ml flasks, it was done by inserting the combination electrode directly into the spent media after the explant had been removed. It was found that this gave a reproducible result and it was not necessary to stir the media. In the 250 ml flasks, the media was mixed thoroughly using a spatula in the flask before measuring.

Methods Relating to Individual experiments

Comparison of Autoclaves

In order to compare the pH change associated with different types of autoclave, flasks of media from a single batch of media were autoclaved in the three autoclaves in

use. The medium used was a batch of R10 media, the pH of which was adjusted to 5.84 prior to dispensing in the normal manner. Twelve flasks were selected at random for each treatment. This represented a full load for the Namco pressure cooker. For the other autoclaves, the treatments were distributed randomly among loads of approximately 120 flasks per autoclave, in three trays, then autoclaved as normal. In each case the autoclaves were allowed to cool overnight before removing the media from the autoclave, as is normal practice.

Source of pH Variation

Various media components were autoclaved alone and in combination in order to identify some of the sources of pH change occurring during autoclaving. The medium used in this experiment was R10, autoclaved in the Type 2 autoclave. Agar was added, in this instance, by weighing into individual flasks at the rate of 10 g/L. Each flask contained 40 ml of medium. The starting pH of each treatment was adjusted to 5.82 prior to dispensing to individual flasks.. The treatments were distributed randomly in two trays within the autoclave. After autoclaving and cooling overnight the pH was determined.

Table 2

Treatments for 'Source of pH Variation' experiment

Standard medium with agar
Standard medium without sucrose, with agar
Zero medium (distilled H2O) with agar
Zero medium with sucrose and agar
Standard medium without agar
Standard medium without sucrose, without agar
Zero medium
Zero medium with sucrose

10 replicates per treatment.

pH Response

To measure the response of cultures to different initial pHs two different methods were employed on different media.

G5 Medium

Media was prepared from a standard batch of G5 medium. Aliquots of 200 mls were adjusted to the desired pH and then heated to dissolve the agar. 8 ml aliquots were then dispensed into 50 ml flasks and autoclaved in the Type 1 autoclave. Following autoclaving it was found that the pH change, when measured from a sub-sample of three flasks, was substantially more than anticipated from previous experience (all were found to be within + or - 0.05).

20 replicates per treatment.

G8 Medium

The media was prepared from a single batch of standard G8 medium. Each treatment consisted of 300ml aliquots of this medium that were adjusted to one of a series of pHs prior to the addition of agar, then autoclaved in the Namco pressure cooker. While still hot, the media was dispensed

into pre-autoclaved 50ml flasks using sterile 10 ml dispensers in the laminar flow cabinet. The media was allowed to cool in the laminar flow cabinet before capping to prevent the condensation of vapour in the flasks. Once all the media had been dispensed and allowed to cool, the pH for each treatment was tested in 5 flasks per treatment. Seven treatments that had a pH in the desired range were selected for use in this experiment. This method of media preparation has the advantage that there was found to be very little pH variation (+ or - 0.02) within treatments. 22 replicates per treatment.

Growth Period Experiment

The experiment was designed to determine the growth curve of cultures. In this experiment the explants were cut to a predetermined size, rather than being weighed. The pieces had the approximate dimensions of 4mm x 4 mm x 6 mm, with the longer dimension being the height of the explant. In this experiment the individual shoots on the explant were not trimmed off. 75 flasks (250 ml) were started, each containing four explants. For each treatment, six flasks were selected at random and measurements made on the explants.

Explant Trimming

In most experiments involving proliferation medium, the explants were prepared to a standard size by trimming off excess callus growth and removing any developing shoots that

had begun to extend above the level of the main proliferation. This ensured evenness of initial explants. In order to assess the effect that this trimming had, an experiment was designed to compare the growth of explants where trimming did not occur with the growth of the standard, trimmed explant. This comparison was made over three nitrogen levels. All other growth conditions were standard.

Table 3

Treatments for the 'Explant Trimming' Experiment

Treatment 1	G5	medium - untrimmed explants
Treatment 2	G5	medium - trimmed explants
Treatment 3	G8	medium - untrimmed explants
Treatment 4	G8	medium - trimmed explants
Treatment 5	G12	medium - untrimmed explants
Treatment 6	G12	medium - trimmed explants

20 replicates per treatment.

Explant Orientation

The explants used in this experiment were cut in the usual manner and trimmed to remove excess shoots. Explants tend to have a distinct orientation, in that most of the shoots initiate on the upper side of a proliferation. Thus, even after explants have been dissected from a proliferation and trimmed to remove any large shoots, it is usually clear which is the correct orientation. In this experiment, explants were randomly assigned to be placed in the medium in either their normal orientation or inverted so as to expose the trimmed shoots to the agar. The growth and

development of each treatment was measured as previously described. 24 replicates per treatment.

Explant Size

To assess the effect of initial explant size on the subsequent growth rate, explants were cut to give a range of sizes and were transferred to standard G5 medium. A smaller number of explants with the same size range were put onto G8 medium.

Media Constituents

Nitrogen Trial

The experiment was designed to measure the response of the cultures to different levels of nitrogen, supplied as NH_4NO_3. It compared the response of cultures using two different pre-treatments, to avoid the possibility that the low nitrogen level of the standard G5 pre-treatment medium might influence the growth response of the cultures. This was achieved by growing half of the explants on a pre-treatment medium for five weeks, with nitrogen levels equivalent to that of the treatment media. In effect, this meant that half the explants were exposed to the nitrogen treatment for ten weeks, while the other half were grown on G5 medium for five weeks followed by the nitrogen treatment, for another five weeks. For this reason, the measurement of the two halves of the experiment were designated Week 5 and Week 10 measurements.

One of the difficulties encountered with this experiment was the extent to which the altered growth habit of some of the high nitrogen treatments restricted the exact duplication of starting material for the two halves of the experiment. In the standard pre-treatment half of the experiment, the starting material was from a culture of a single age and standard type and all explants were cut to a similar size and trimmed of excess shoots, as was standard practice in all experiments. However, when material was being prepared from the variable pre-treatments it was found that the material from the very high nitrogen level pre-treatments were of a substantially different growth habit. Had this material been trimmed in the usual manner to remove shoots it would have left explants that were entirely unrepresentative of the pre-treatment. For this reason, it was decided that for this part of the experiment the explants would not have any shoots trimmed off.

Treatment levels were selected to range between 200 mg/L and 1600 mg/L at 100 mg/L intervals. However, due to a combination of poor growth rates and contamination on pre-treatment media, there was insufficient plant material available to complete the 200, 1300 and 1500 mg/L treatments, so these treatments were omitted. The pH of the media were adjusted to 5.8 prior to autoclaving in the Type 1 autoclave, but was not checked after autoclaving. In addition, the media for the week 5, 400 mg/L NH_4NO_3

treatment was accidentally spilled during preparation and a second batch prepared and autoclaved separately. There were twenty replicates for each treament.

Clone Trial

This experiment was designed to compare the response of different clones to nitrogen levels in the medium. A series of media were prepared with seven levels of nitrogen supplied in the form of NH4NO3 supplied at 400, 600, 800, 1000, 1200, 1400 and 1600 mg/L. Four clones were included in this trial - HC4, HC15, HC17 and HC129. All other conditions were as previously described. The pH for each treatment was adjusted to 5.8 before autoclaving in the Type 2 autoclave. 15 replicates per treatment.

Nitrogen Source

This experiment was designed to look at the growth of cultures on media using NH4 and NO3 as the sole nitrogen sources. This was compared with the growth on media having NH4NO3 as the nitrogen source. Two levels of NH4NO3 were used, as detailed in Table 4. Treatments with and without adenine were included to measure the extent to which this chemical might be used as an alternative nitrogen source.

All media were prepared by adding the required treatment to aliquots of nitrogen free medium prior to adjusting the pH to 5.8. Agar was then added, melted and autoclaved in the Type 1 autoclave. At the conclusion of the experiment, the explants were weighed and measured in

the usual way. The pH of the spent medium was assessed by combining the medium from six flasks and blending well before measuring with a pH meter.

Table 4

Treatments Levels for 'Nitrogen Source' Experiment

1) 10 mMol N as NH4 - supplied as (NH4)2SO4

2) 10 mMol N as NO3 - supplied as NaNO3

3) 10 mMol N as 5 mMol NH4 + 5 mMol NO3 - supplied as NH4NO3

4) 20 mMol N as 10 mMol NH4 + 10 mMol NO3 - supplied as NH4NO3

The two levels of were adenine 0 and 100mg/l.

24 replicates per treatment

Buffered Media

In this experiment, succinate was added to media, to measure its effect on pH buffering when NH4, NO3 or NH4NO3 were used as the nitrogen source. A single lot of standard proliferation medium was prepared, omitting only NH4NO3 and KCl and made up at x1.25 concentration.

Three media were prepared from this:

: Standard G8 medium, by adding 800 mg NH4NO3 and 1490 mg KCl per 800 ml of medium.

: Nitrate free medium, by adding 1320 mg (NH4)2SO4 and 1490 mg KCl per 800 ml of medium.

: Ammonium free medium, by adding 1010 mg KNO3 per 800 ml of medium.

All media were adjusted to pH 5.0 after preliminary tests indicated that this would result in a post autoclaving pH of 4.8 under these conditions (unpublished data). Buffer was prepared by titrating a quantity of 100 mM succinic acid with 100 mM potassium succinate to produce a mixture of pH 4.85. The pH of the water to be added was also adjusted to pH 4.85 using 0.1 N KOH. Two 400 ml lots of each of the media were autoclaved. To these 100 ml of autoclaved buffer or water was added and mixed thoroughly, while still hot. The media was dispensed into pre-autoclaved 50 ml flasks using sterile 10 ml dispensers in the laminar flow cabinet. They were allowed to cool in the laminar flow cabinet before capping, to prevent the condensation of vapour in the flasks.

The pH of each treatment medium was determined at the time the explants were transferred to the medium, using a sample of three flasks. Extra flasks were added to the growth cabinet to measure the pH of the media without explants. At weekly intervals, three flasks from each treatment were removed to measure the pH of the flasks and the weight of the explants. The pH of the media without explants was estimated after one, three and five weeks from a sample of three flasks per treatment.

Growth measurements were made on 24 replicates/treatment.

Adenine

The experiment was designed to test the effect of adenine on the growth and development of cultures at two nitrogen levels (500 and 1000 mg/L NH_4NO_3) in the presence and absence of adenine (80 mg/L). To check for the possibility of more long term effects from adenine, pretreatments where adenine was withheld for either one or five sub-culture periods, were included. This gave ten treatments in all, on four different media. The pH of the media, measured after autoclaving, was 4.86, + or - 0.10, for all treatments. See treatment table (Appendix 2) 30 replicates per treatment.

Vitamins

The effects of vitamins on growth were determined using vitamin formulations obtained from four different media and a control of zero vitamins. The medium used was standard G5 medium, prepared as normal, without vitamins.

The treatments are from media as reported by Skirvin (1981).

Treatment 1 - Murashige and Skoog 1962.
2 - Murashige and Tucker 1969.
3 - Miller 1967.
4 - Gamborg and Eveleigh 1968.
5 - Zero Vitamins.

All other conditions were standard. The pH was adjusted to 5.8 before autoclaving in the Type 1 autoclave.

Table 5

Vitamin Levels

	Treatment				
	1	2	3	4	5
THIAMINE (mg/L)	0.1	10.0	0.1	10.0	0
NICOTINAMIDE (mg/L)	0.5	5.0	0.5	1.0	0
PYRIDOXINE HCl (mg/L)	0.5	10.0	0.1	1.0	0
GLYCINE (mg/L)	2.0	2.0	0	0	0

24 replicates per treatment.

Sucrose

The experiment was designed to test the response of cultures to the effects of different levels of sucrose at two different nitrogen levels. Five levels of sucrose were used: 10, 20, 30, 50 and 80 g/L. They were compared at two levels of NH_4NO_3: 500 and 800 mg/L. All other conditions were standard. The pH was adjusted to 5.8 before autoclaving in the Type 1 autoclave.

Methods - Root Initiation Experiments

Measurement and Handling of Plants

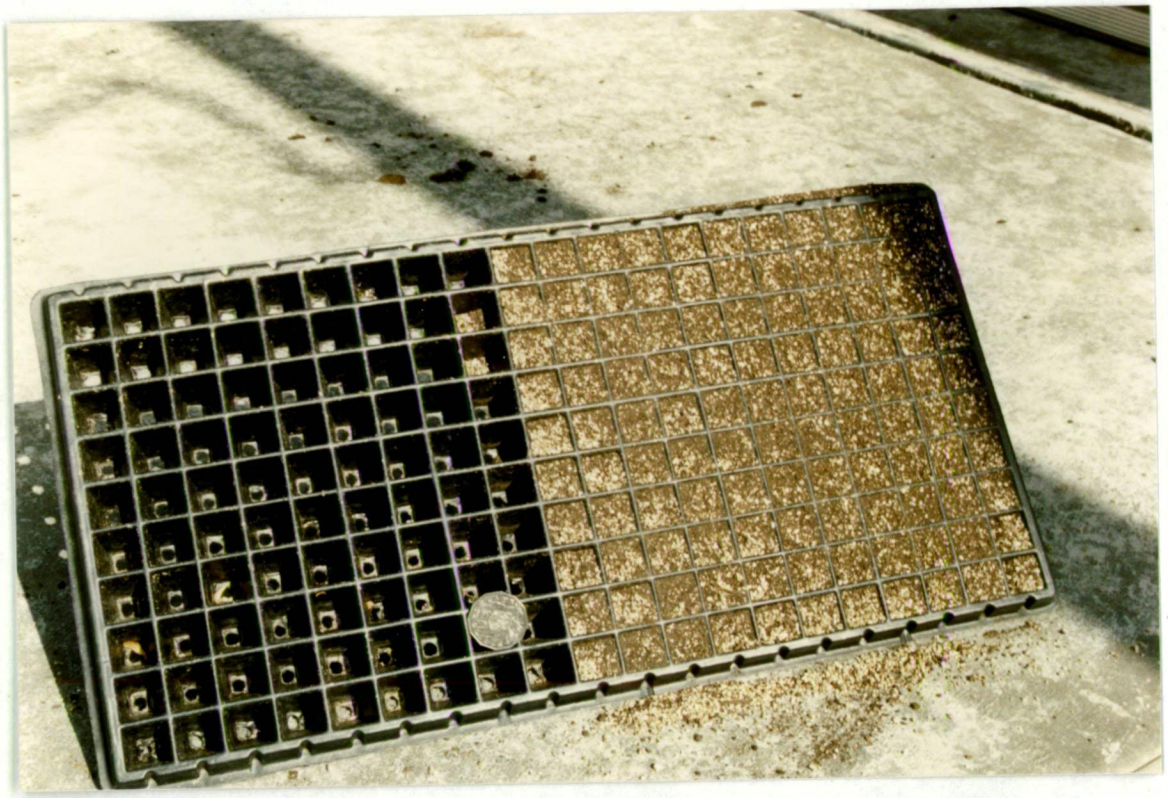
All root initiation experiments were conducted using 36ml of media in 250ml flasks. Media was prepared and dispensed as previously described. Explants suitable for use in root initiation experiments were prepared by trimming shoots to a length of between 10 and 25 mm, with one actively growing shoot per explant and normally eighteen such explants per flask. At the completion of each

experiment, prior to removal of the plantlets from the agar, the rate of root initiation was assessed, by counting the number of explants with roots visible in each flask. The plantlets were then planted into Celpak trays, a tray containing 200 cells measuring 23 x 23 x 37 mm (see Fig 1). These were filled with a potting mixture composed of equal volumes of composted Eucalyptus Bark, obtained from Clark's Milvale Nursery, sieved through a 5 mm sieve, and Boral horticultural grade vermiculite. Added to this was 50 g Iso Butyl Di Urea (IBDU) per 10 kg of potting mix.

The height of the plantlet above the soil was measured,to an accuracy of + or - 5 mm, in a random sub-sample of the plantlets, after planting out.

All plantlets were grown on a heated mistbed, with a continuous bottom heat of approximately 22 C and intermittent overhead water misting. Trays were subject to routine fungicide treatment, consisting primarily of fortnightly applications of Previcur fungicide, at the rate of 1.5 ml/L applied to each square metre of trays. Other fungicides were applied as required.

Fig 1 Celpak Trays Used in Root Initiation Experiments



Root Initiation - Nitrogen Experiment

The effects of nitrogen level in root initiation media was investigated and compared for three different nitrogen levels in pre-treatment proliferation media. There were three nitrogen pre-treatments, where explants were grown on standard media containing 500, 800 and 1200 mg/L NH_4NO_3 for a period of five weeks prior to transfer to root initiation media. Five different nitrogen levels were applied to the root initiation media: 300, 500, 800, 1200, and 1600 mg/L NH_4NO_3, giving a 3x5 factorial experiment, 24 replicates/treatment.

A rating for root development was devised, in order to be able to obtain some measure of the time taken for the roots to start to grow. The method was designed to give a crude measure of the number of plants with a significant amount of root development, without disturbing the plant. Each of the Celpak trays containing plantlets was examined by turning onto its side so that the drain holes at the bottom of each cell could be seen. The percentage of cells at which any live root material could be seen was recorded to give an estimate of the number of plants with significant root development in that tray, 136 explants/treatment.

Root Initiation - Growth Conditions Experiment

In this experiment, explants were grown on G10 media for five weeks prior to transfer to R12 media. The treatments in this experiment consisted of four different sets of growth cabinet temperature conditions. The light

conditions, of a 16 hr day and an 8 hr night, were constant for all cabinets.

The temperature conditions were:

Treatment 1: Constant Temperature of 22 deg C.

Treatment 2: Day temperature of 22 deg C, and night temperature of 15 deg C.

Treatment 3: Constant temperature of 22 deg C, with one 1 hour period of 15 deg C immediately prior to the start of the light period.

Treatment 4: Constant temperature of 22 deg C, with two 1 hour periods of 15 deg C immediately prior to the start of the light period and immediately after its conclusion.

All treatments were measured after five weeks growth, transferred to Celpak trays and grown under standard conditions, 180 explants/ treatment.

Root Initiation Time Experiment

Explants were held on root initiation medium for increasing periods of time, in order to measure the effect of this on root initiation and on subsequent plantlet survival. The plant material used in this experiment was grown on G12 medium prior to transfer to R10 root initiation medium. Approximately 100 flasks of rooting medium were prepared and eighteen explants transferred to each. Thirty flasks were chosen at random, at the end of each time period, and all the explants were planted randomly in three

Celpak trays. Initial height was determined by measuring a sub-sample of plantlets after planting. The trays of plantlets were then transferred to a mistbed and grown under standard conditions. A sample from each tray was measured at the conclusion of the experiment to estimate growth.

Root Initiation - Adenine Experiment

To measure the effect of adenine on root initiation, the rate of root initiation was compared on media with and without adenine sulphate (80 mg/L). R10 root initiation media was used. Both media were adjusted to pH 5.2 prior to autoclaving. The explants were of clone HC223, from five week old cultures growing on G10 medium. Approximately 20 explants were placed in each flask and grown under standard conditions.

Root Initiation Media and Sucrose

In order to measure the influence of sucrose on the initiation of roots in culture, a series of media with increasing levels of sucrose were prepared. Treatments applied were 30, 45, 60, 75 and 90 g/L sucrose. The media were adjusted to pH 5.2 prior to autoclaving. Explants of clone HC223, from five week old cultures growing on G10 medium were used. Approximately 20 explants were placed in each flask and grown under standard conditions for four weeks before measuring. 22 flasks/treatment, 40 explants/treatment for height estimation.

IV. RESULTS AND DISCUSSION

Autoclave Comparison

As can been seen from the table of results, there is a significant pH decline in pH in all three autoclaves, from the starting point of 5.84. The pH decline is significantly less in the case of the Namco pressure cooker, most probably because of the faster cooling rate of this smaller un-insulated vessel. Probably of as much significance as the mean pH change is the range of pH's measured in all three autoclaves. The wide range of pHs is probably due to deficiencies in the heat distribution and cooling pattern. This would be especially apparent in the two larger autoclaves, where the steam must penetrate through several layers of flasks and where an insulating effect may be occurring during cooling.

Table 6

pH of Media after Autoclaving in Three Different Autoclaves

Autoclave	Mean pH	Std Dev	pH Range
Type1	5.16	0.172	4.80 - 5.35
Type2	5.20	0.247	4.80 - 5.42
Namco	5.45	0.156	5.15 - 5.60

Source of pH Variation

It would appear from this experiment (Table 7) that there is not a single cause of pH decline in autoclaved media. It is more likely to be a result of the action of both sucrose and the mineral nutrients that cause the changes. Agar does not add to the pH change and acts to decrease the effect of some of the other changes through a buffering effect. This experiment is also a good example of the variation between runs of the autoclave, since the pH decline of the full medium was less in this instance than in the 'Autoclave Comparison' (this experiment was run on the Type 2 autoclave).

Table 7 The effect of media constituents on pH. (Mean pH and Std. Dev.)

	With Agar	Without Agar
MS Salts	5.34 0.081	5.14 0.295
MS Salts, No Sucrose	5.43 0.098	5.32 0.096
No Salts With Sucrose	5.86 0.247	4.80 0.284
No Salts, No Sucrose	5.93 0.414	5.83 0.453

The standard deviations for all treatments containing agar are less than those for the corresponding treatments without agar indicating that the buffering effect of agar is reducing the amount of variation within treatments, as well as increasing the mean pH.

Although sucrose does cause the pH to change when it is autoclaved in water alone, the change is not significant in the presence of agar. This indicates that the buffering action of the agar is sufficient to absorb most of the pH change associated with autoclaving of sucrose. Removing sucrose from the medium also results in less pH decline. The conclusion to be drawn from this is that the main cause of the pH drop is in reactions between minerals and other constituents of the medium and from interaction between the minerals and the sucrose.

From the results of these two experiments it can be seen that the common method of recording the pH of the medium is likely to be quite misleading. Merely quoting the pH of the medium before autoclaving cannot be relied on to give an accurate estimate of the pH the plants are likely to encounter at the start of the experiment. Since the medium, agar and sucrose interact, it would not be surprising if the media formulation and even agar brand and quality affect the pH. It would appear likely that Harris and Stevenson (1982) were quite in error to use a higher starting pH to reduce the difference in pH between solid and liquid medium, since this would be likely to accentuate the pH differences between the liquid and solid medium.

If the pH of the medium is to be quoted, and it should be, then a far more useful measure would be to measure the pH of a sample of flasks from each run of the autoclave, even though this is wasteful of media.

As it appears that there are substantial differences in the final pH obtained from different runs using the same autoclave, it cannot be assumed that any differences observed between the two large autoclaves are necessarily the result of design or operational differences. It is quite possible that each run may give substantially different final pHs.

pH Response Experiments

G5 Medium

A large pH drop associated with autoclaving is not unexpected but the fact that it was so substantial for this experiment is surprising (Table 8). Comparison of these pHs with those measured during the autoclave test experiment highlights the degree of variation that can be experienced between different autoclave runs.

Table 8

pH of Media Before and After Autoclaving and After Five Weeks Growth - G5 Medium

Pre Autoclaving pH	Post Autoclaving pH	Spent Media pH
4.5	4.46	4.72
5.0	4.65	4.72
5.5	4.71	4.66
5.8	4.90	4.82
6.0	4.92	4.71
6.5	5.26	4.75
7.0	5.75	5.11

Fig 2

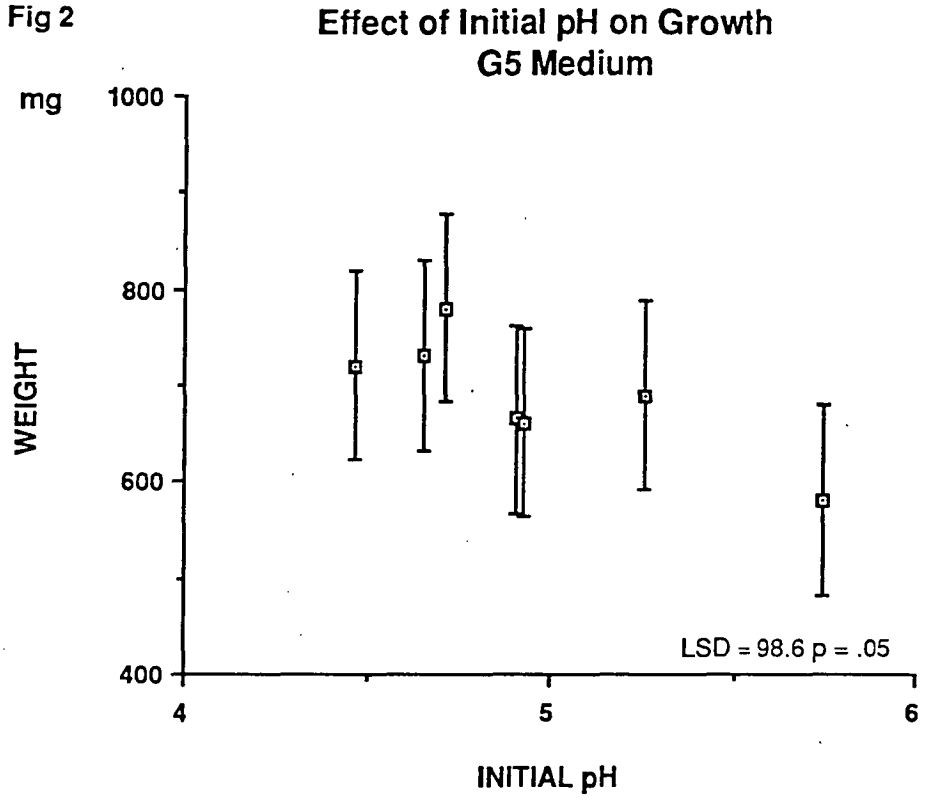

Effect of Initial pH on Growth
G5 Medium
mg
1000
800
600
400
WEIGHT
LSD = 98.6 p = .05
4
5
6
INITIAL pH

Fig 3

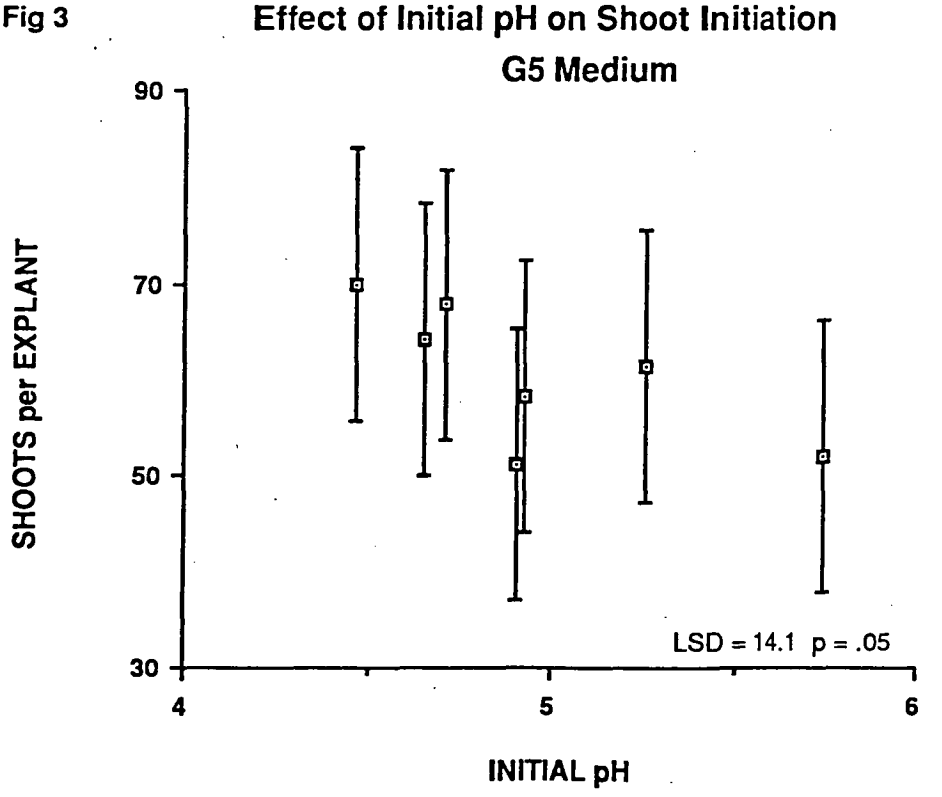

Effect of Initial pH on Shoot Initiation
G5 Medium
90
70
50
30
SHOOTS per EXPLANT
LSD = 14.1 p = .05
4
5
6
INITIAL pH

Fig 4

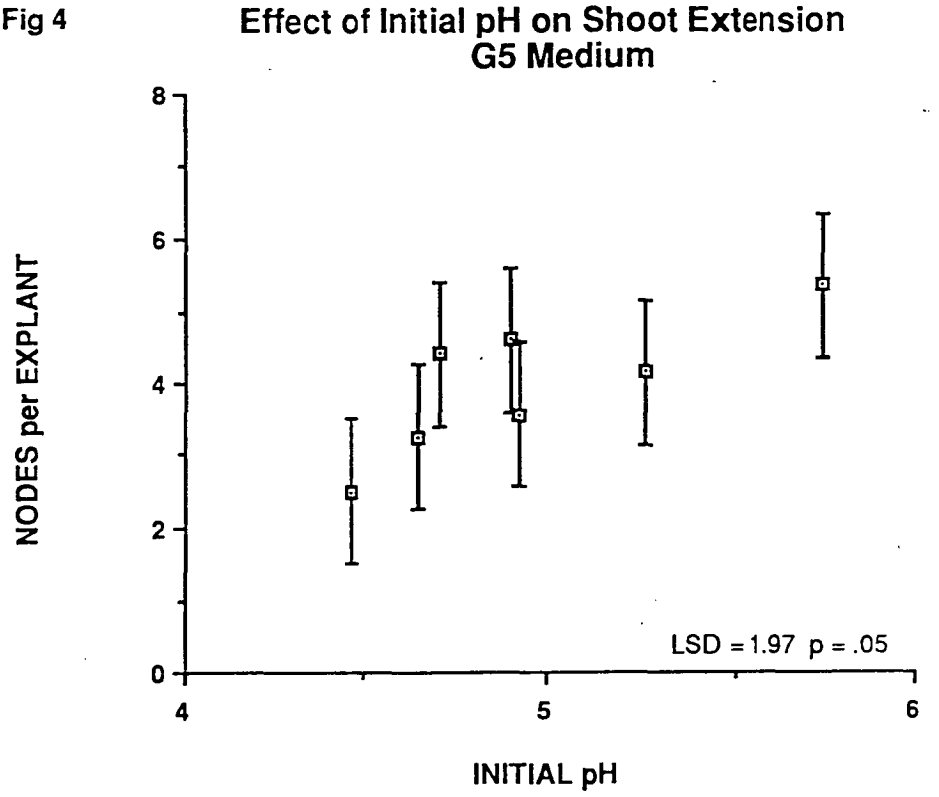
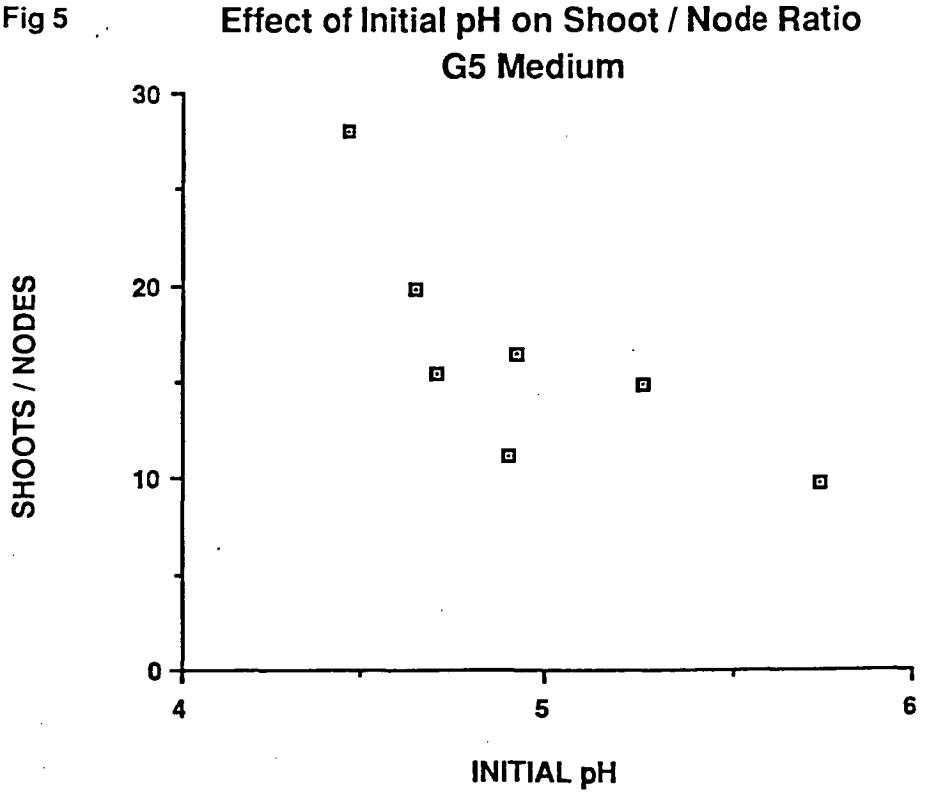

Effect of Initial pH on Shoot Extension
G5 Medium
NODES per EXPLANT
8
6
4
2
0
LSD = 1.97 p = .05
4
5
6
INITIAL pH

Fig 5


Effect of Initial pH on Shoot / Node Ratio
G5 Medium
SHOOTS / NODES
30
20
10
0
4
5
6
INITIAL pH

The preference for a pH in the range 4.65 to 4.82 is clearly demonstrated by the final pH of the media. Only the treatment with the highest initial pH has not dropped to this optimal level after five weeks growth. There appears to be a relationship between the initial pH of the medium and the growth of the explants, although the regression equation for the data is not significant with an RSqu value of 0.08 for weight and less for shoot intiation and shoot extension. In particular, it would appear that there is a trend for weight and shoot initiation to decline with increasing initial pH (Figs 2 & 3) and for node production to increase. (Fig 4)

G8 Medium

The increase in pH observed during autoclaving in this experiment is surprising (Table 9). It is probably due to the rapid cooling of the medium in the laminar flow cabinet, rather than being allowed to cool slowly in the autoclave, as is normally the case. This indicates that the length of time the heat is applied is more important in causing pH to decline, than the temperature that is reached.

The optimum pH for G8 medium appears to differ from that of G5 and is in the range 4.40 to 4.60, assuming that the final pH of the medium does represent the optimum for growth. The mean growth for different initial pHs is statistically different for two of the treatments, however,

there does not appear to be a definite trend in this measure over the range of pHs applied (Fig 6).

Table 9

pH of Media Before and After Autoclaving and After Five Weeks Growth - G8 Medium

Pre Autoclaving pH	Post Autoclaving pH	Spent Media pH
3.8	4.18	4.47
4.0	4.47	4.40
4.2	4.64	4.44
4.4	4.88	4.45
4.6	5.05	4.43
4.8	5.10	
5.0	5.17	4.58
5.2	5.25	
5.4	5.35	
5.6	5.42	
5.8	5.54	4.44
6.0	5.67	

* not all treatments selected for use.

Differences in the rate of shoot initiation are also statistically significant for the same two treatments. In addition, there appears to be some trend towards decreased shoot initiation with higher initial pH (Fig 7), although this trend is not clearly expressed. It is difficult to be certain of the differences in shoot extension due to the variability of results (Fig 8). The graph of shoot to node ratio gives a clear trend of decreasing number of shoots per node with increasing initial pH (Fig 9)

Fig 6

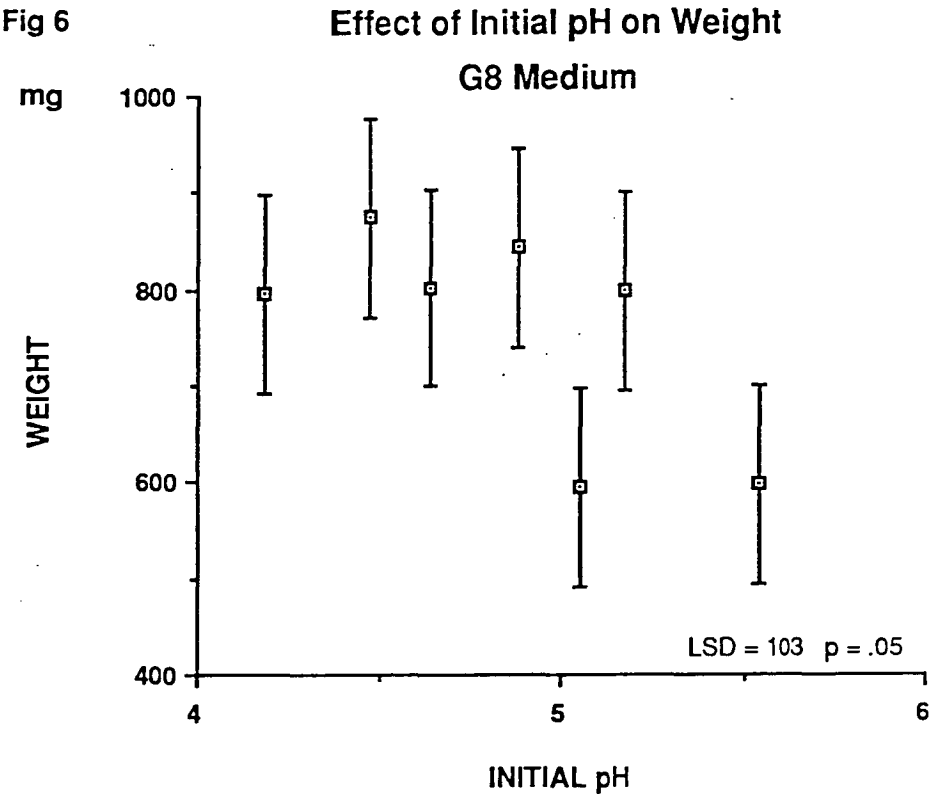

Effect of Initial pH on Weight
G8 Medium
mg
1000
800
600
400
WEIGHT
LSD = 103 p = .05
4
5
6
INITIAL pH

Fig 7

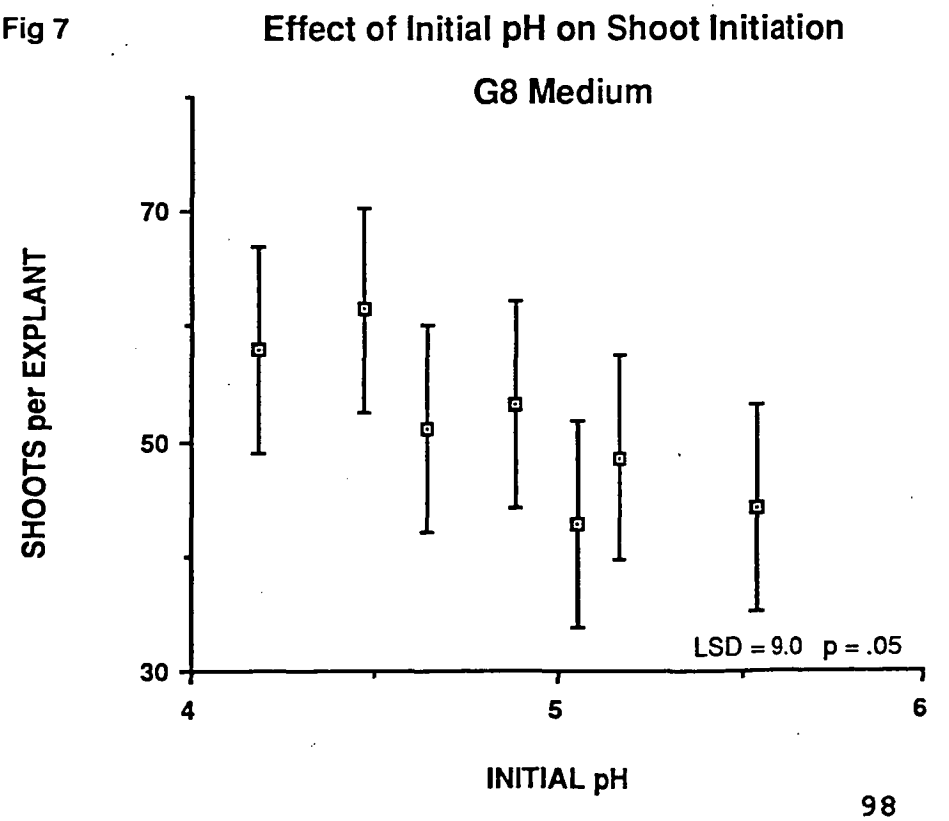

Effect of Initial pH on Shoot Initiation
G8 Medium
70
50
30
SHOOTS per EXPLANT
LSD = 9.0 p = .05
4
5
6
INITIAL pH

Fig 8

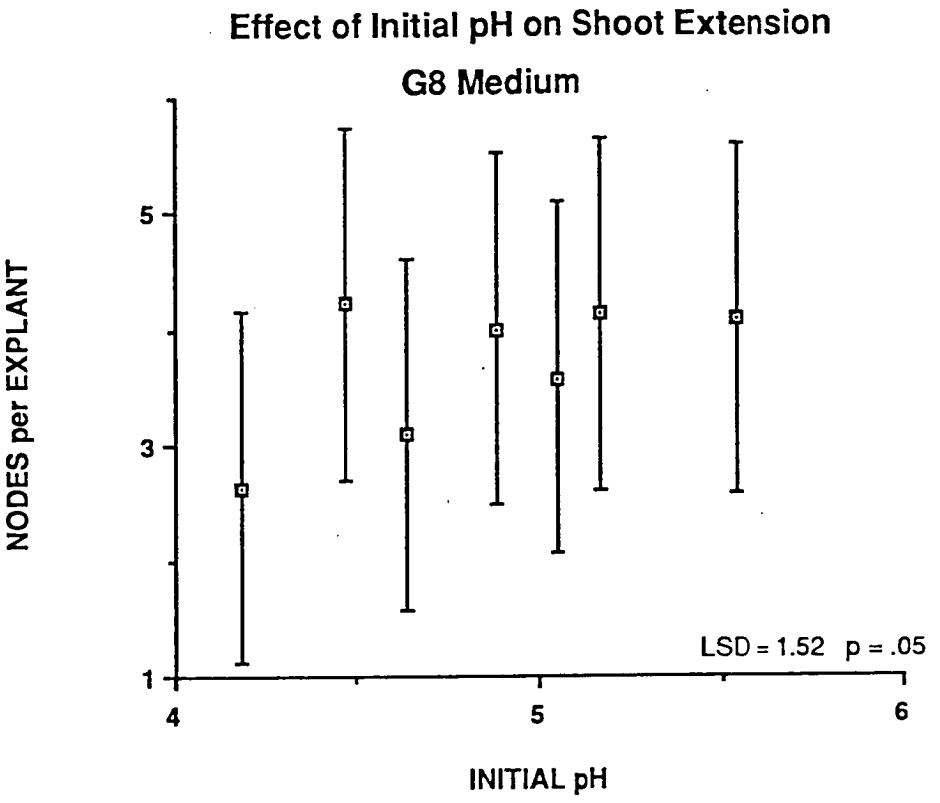
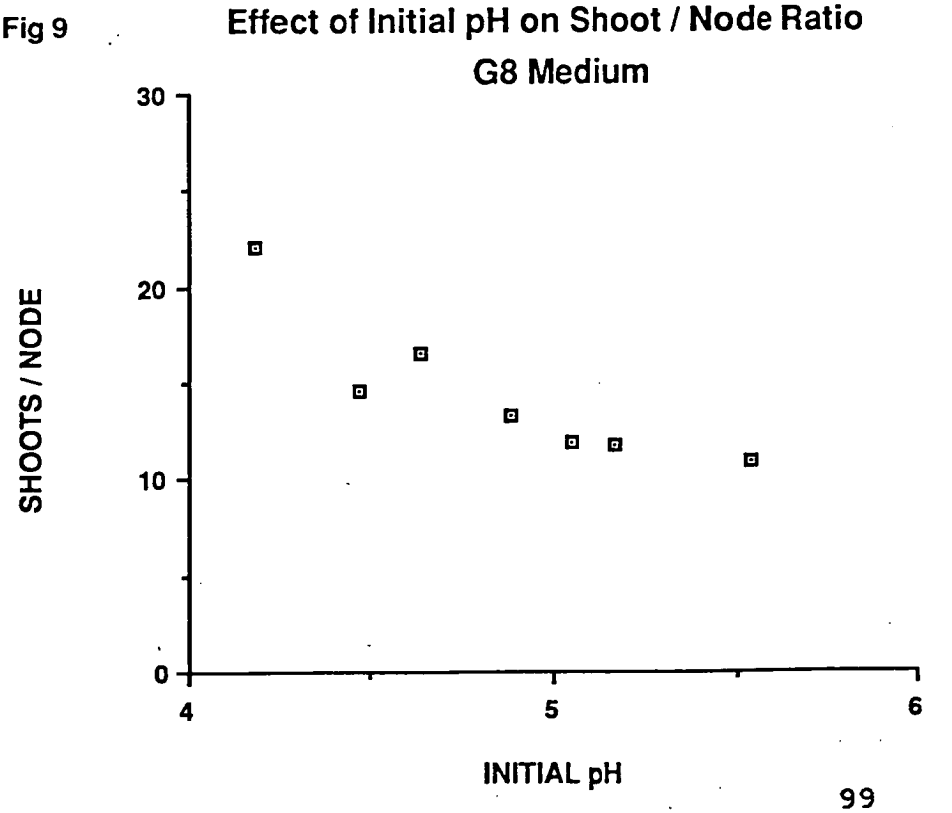

Effect of Initial pH on Shoot Extension
G8 Medium
NODES per EXPLANT
5
3
1
LSD = 1.52 p = .05
4
5
6
INITIAL pH

Fig 9


Effect of Initial pH on Shoot / Node Ratio
G8 Medium
SHOOTS / NODE
30
20
10
0
4
5
6
INITIAL pH

Effects of Initial pH

The influence of initial pH on the ratio of shoot initiation to shoot extension in both G5 and G8 medium is probably the best indication of the effects of initial pH. The lack of control over the initial pH in the first experiment is a factor which decreased the accuracy of the results. In the second experiment, the initial pH was accurately controlled and yet there was still substantial variation within treatments. This makes it difficult to determine the nature of the relationship , if any, between initial pH and growth and development. It is quite likely that there would be an optimum initial pH for cultures but the relationship between initial pH and growth of cultures is still to be accurately defined. One of the difficulties appears to be the rapidity with which the explant is able to alter the pH of the medium toward one apparently more favourable.

It is not clear from these experiments whether changes to the physical characteristics of the medium, caused by different pHs, have any effect on the growth of the explant. The setting qualities of the agar are quite noticeably affected by low pHs. pHs below 4.0 do not allow the agar to set sufficiently to prevent the explants form sinking to the bottom of the flask. On the other hand, pHs above 5.5 give quite a hard medium at the agar concentrations being used

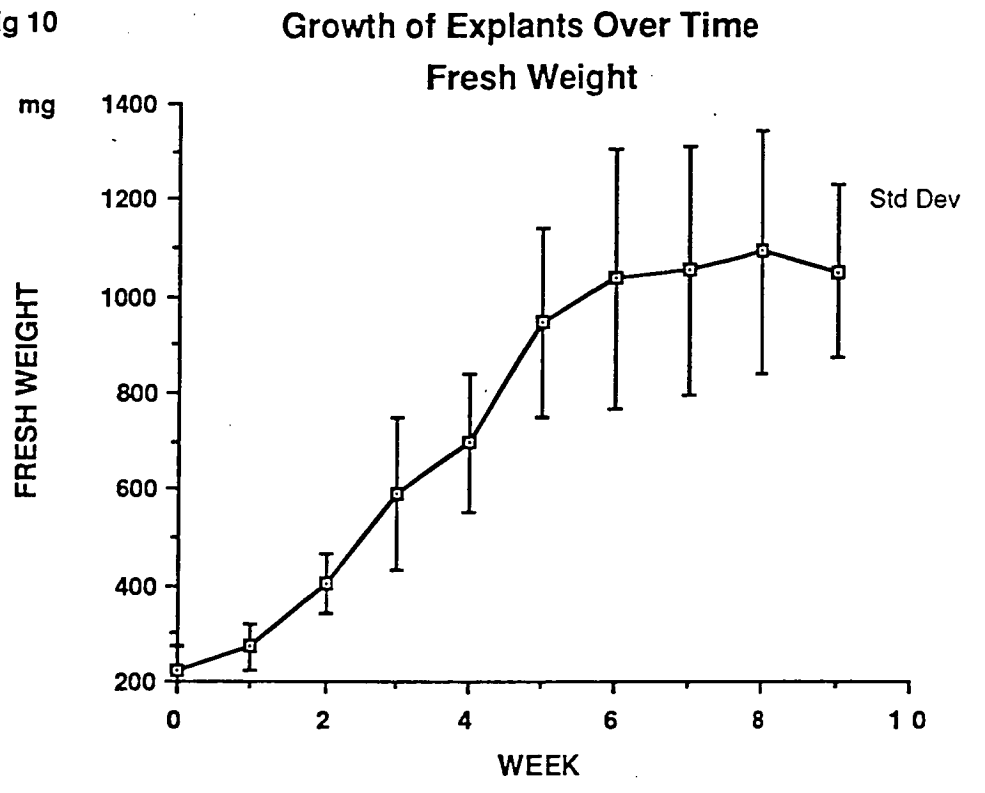
and may result in more cracking of the agar as the explant grows. Within this range, the changes in setting quality are more subtle.

The results of these and the other experiments in which pH is a factor highlight the need for measurement of pH after the media has been autoclaved. Although most autoclaves are capable of good timing and temperature control, a number of other factors may interact to cause variations in the pH of the autoclaved medium. These may have quite significant effects on the growth of cultures. Simply quoting a pH measured before autoclaving may be quite misleading and ought not to be encouraged.

Growth Period Experiment

The results from this experiment indicate the exponential nature of growth in this culture system. When plotted on a logarithmic scale, all four measures appear to have a linear increase for the first five weeks of growth (Figs 10, 11, 12 & 13). After this, growth begins to taper off quite quickly, whether measured as weight, shoots per explant or nodes per explant, although the tapering off is less pronounced for dry weight. This might indicate that shoot extension and shoot initiation are the first factors affected by media depletion, while some growth is still occurring, but that this late growth is not reflected in fresh weight gain because of decreasing tissue moisture levels. Slowing down of shoot initiation and shoot

Fig 10


Growth of Explants Over Time
Fresh Weight
mg
1400
1200
1000
800
600
400
200
FRESH WEIGHT
Std Dev
0
2
4
6
8
10
WEEK

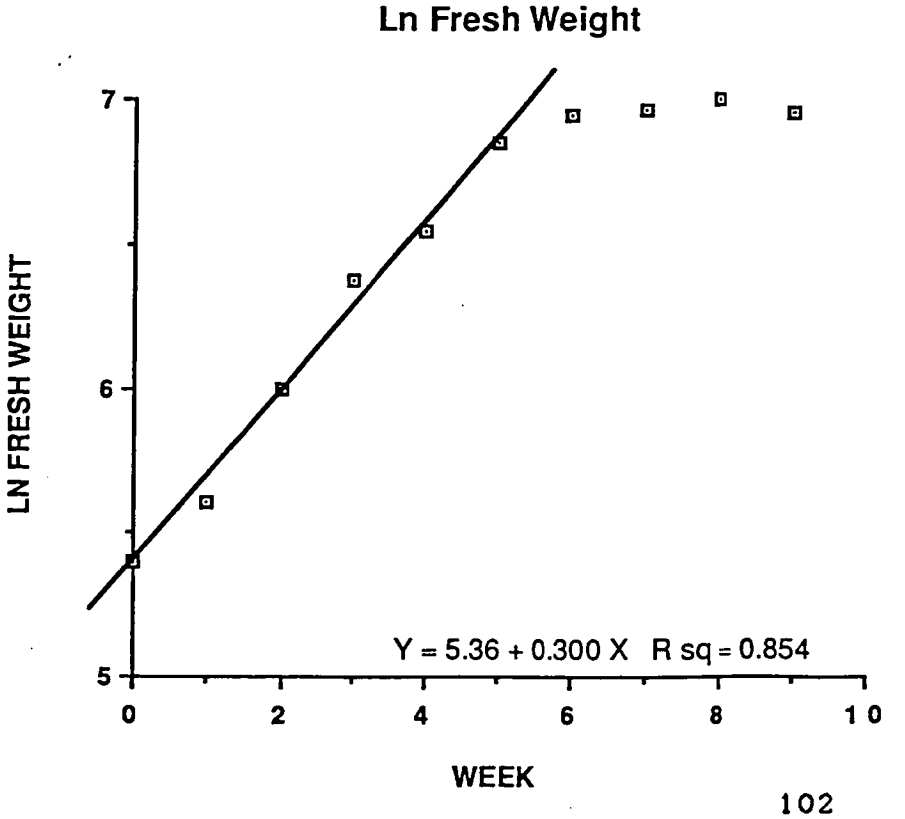
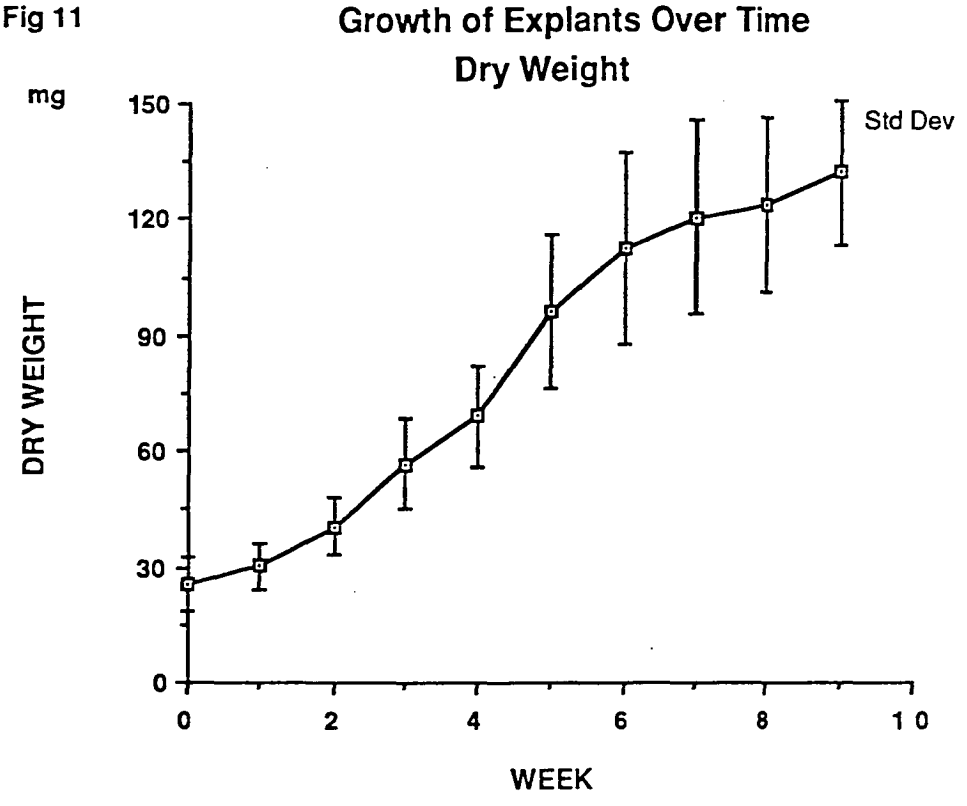

Ln Fresh Weight
7
6
5
LN FRESH WEIGHT
Y = 5.36 + 0.300 X R sq = 0.854
0
2
4
6
8
10
WEEK

Fig 11


Growth of Explants Over Time
Dry Weight
mg
DRY WEIGHT
150
120
90
60
30
0
0
2
4
6
8
10
WEEK
Std Dev

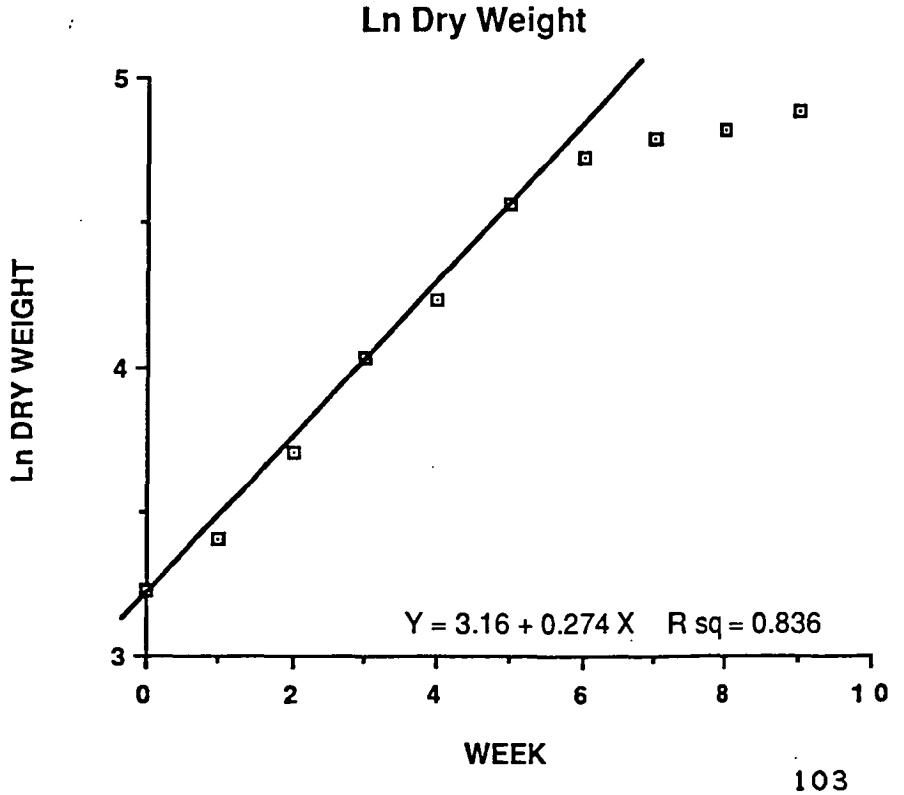
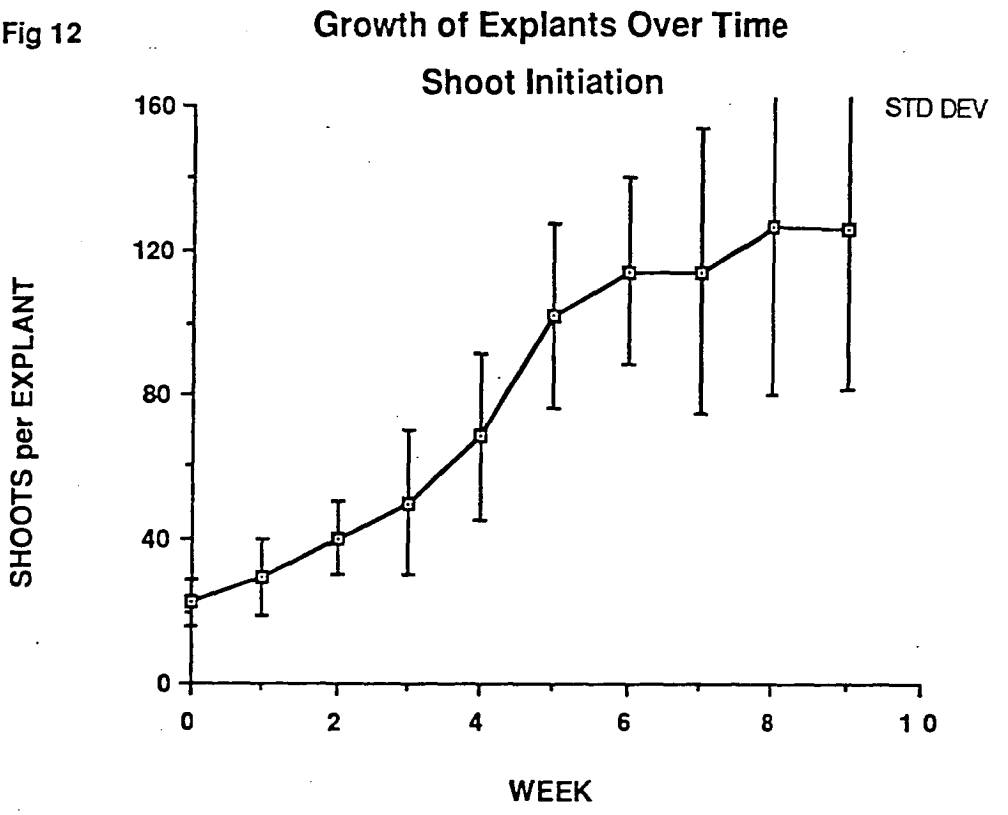

Ln Dry Weight
Ln DRY WEIGHT
5
4
3
0
2
4
6
8
10
WEEK
Y = 3.16 + 0.274 X R sq = 0.836

Fig 12


Growth of Explants Over Time
Shoot Initiation
STD DEV
SHOOTS per EXPLANT
160
120
80
40
0
0
2
4
6
8
10
WEEK

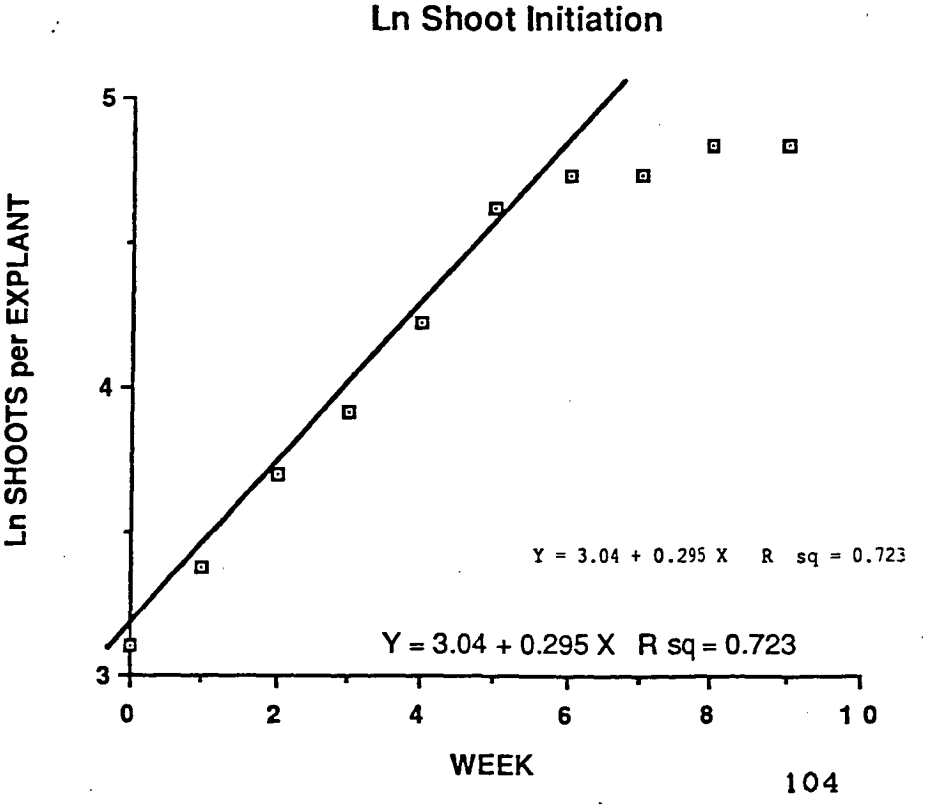
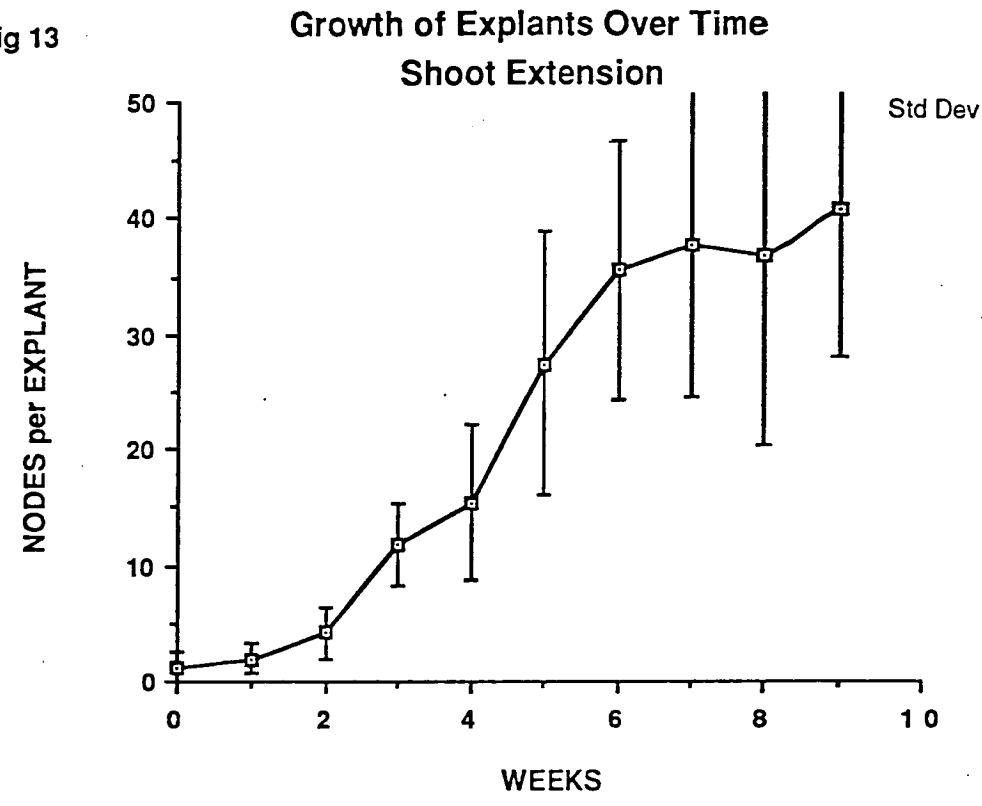
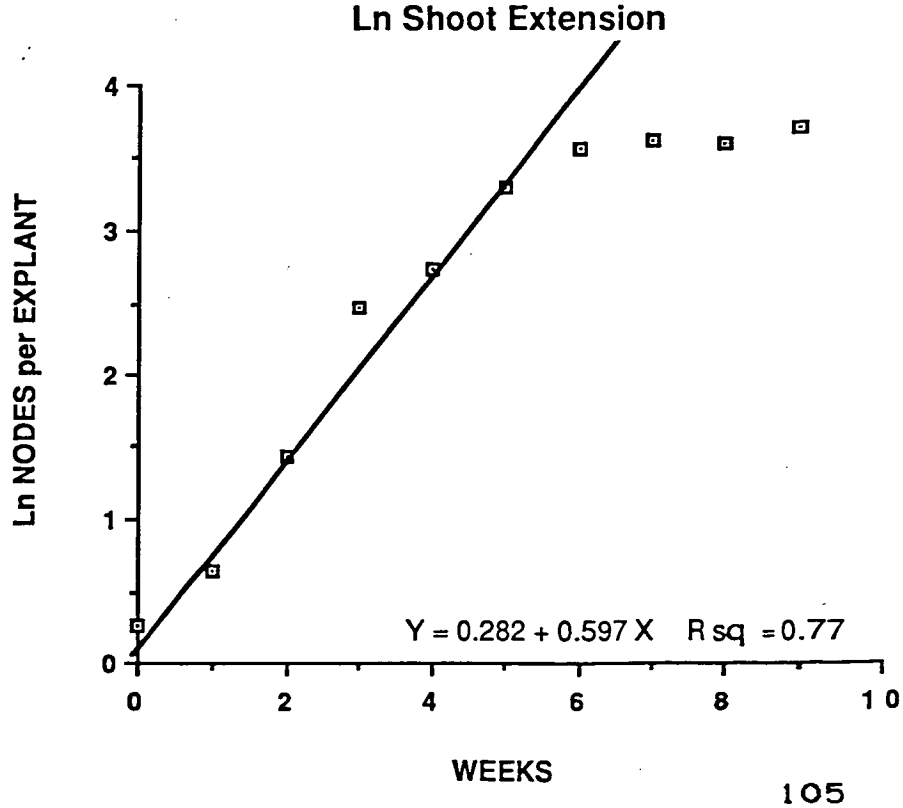

Ln Shoot Initiation
Ln SHOOTS per EXPLANT
5
4
3
Y = 3.04 + 0.295 X R sq = 0.723
Y = 3.04 + 0.295 X R sq = 0.723
0
2
4
6
8
10
WEEK

Fig 13


Growth of Explants Over Time
Shoot Extension
Std Dev
NODES per EXPLANT
50
40
30
20
10
0
0
2
4
6
8
10
WEEKS


Ln Shoot Extension
Ln NODES per EXPLANT
4
3
2
1
0
Y = 0.282 + 0.597 X R sq = 0.77
0
2
4
6
8
10
WEEKS

extension might indicate that one of the first factors to become depleted in the medium is the plant hormones.

There did appear to exist in these measurements a lag phase, where significant growth did not occur. Probably the most significant contributor to this lag phase would be the time taken for the explant to recover from damage received during preparation and transfer of the explant. This damage might arise from cutting the explants, slight drying out of plants and crushing during transfer.

One of the difficulties with this experiment is the wide variation that was measured at each time period, particularly for the T-0 measurements. It is clear from this that selection of explants on the basis of size did not give an accurate enough determination of their initial weight and hence their growth potential. This large variation in initial size was reflected in very large standard deviations in later weeks.

Although the methods used in this experiment differed somewhat from those in later experiments, the results should still be comparable. The larger flasks used contained 32 mls of medium, which was available to be used by four explants, giving the same volume of medium per explant. A resulting difference is that the surface area of the medium and the volume of air in the flask per explant would be slightly higher in this experiment. This might be expected to result in a slightly faster rate of desiccation of the medium in the larger flasks. This would mean that, if

anything, there would be a longer logarithmic growth period for smaller flasks.

It should be noted that, in this experiment, the explants were not trimmed to remove shoots. This resulted in the values obtained for shoot extension being much higher than in many of the later experiments. However, the selection of a five week growth period for all experiments should ensure that all measurements are made while the explants are still undergoing vigorous, logarithmic growth.

Explant Trimming

The clear result from this experiment is that trimming has very little effect on the growth rate of the explants but does have a very significant effect on the amount of shoot extension occurring in each explant (Figs 14,15 & 16). The differences in the amount of shoot initiation do not appear to be significant. However, an analysis of variance is not applicable because of the heterogeneity of the variance. This can be related to increased nitrogen levels.

These results give an indication of the importance of endogenous phytohormone production in the growth and development of the explant. The auxins produced in the actively growing, extending shoot tip quite clearly influence the pattern of shoot initiation and development but do not influence the total weight of the explant. Instead, they have their effect by influencing the pattern

Fig 14

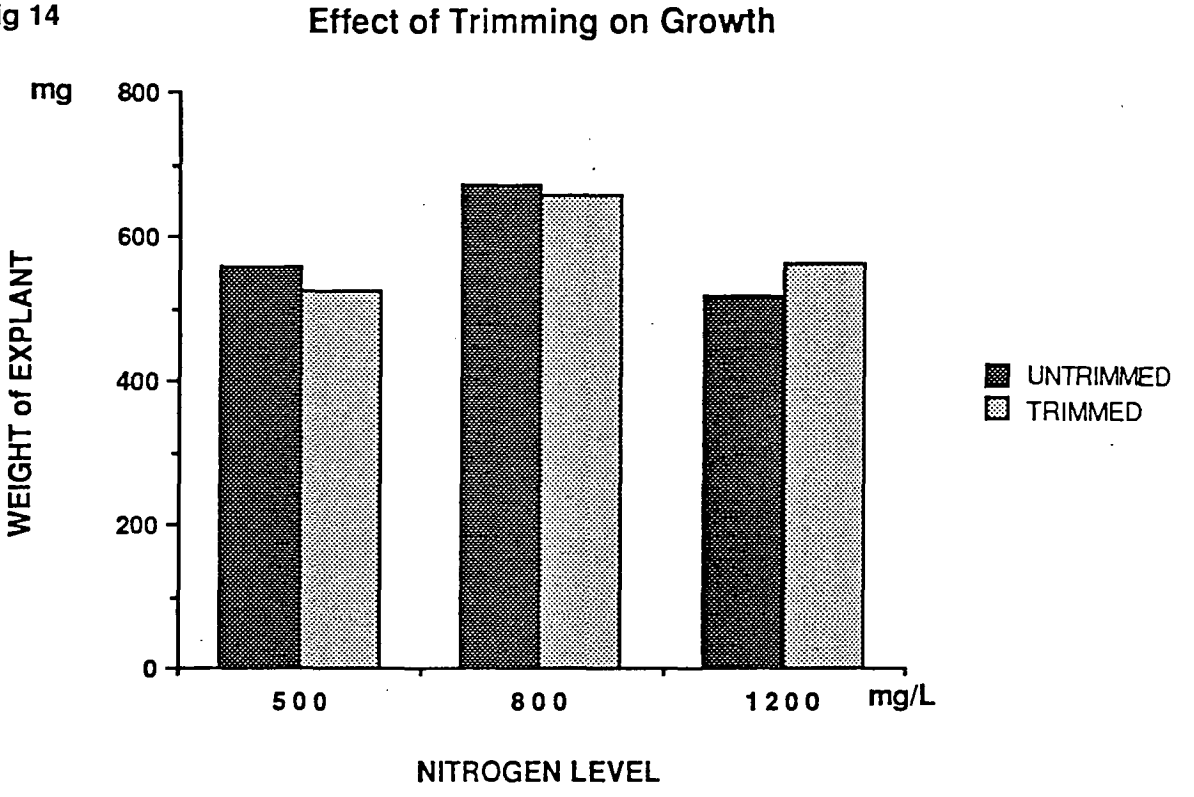

Effect of Trimming on Growth
mg
800
600
400
200
0
WEIGHT of EXPLANT
500
800
1200
mg/L
NITROGEN LEVEL
UNTRIMMED
TRIMMED

Fig 15

Effects of Trimming on Shoot Initiation

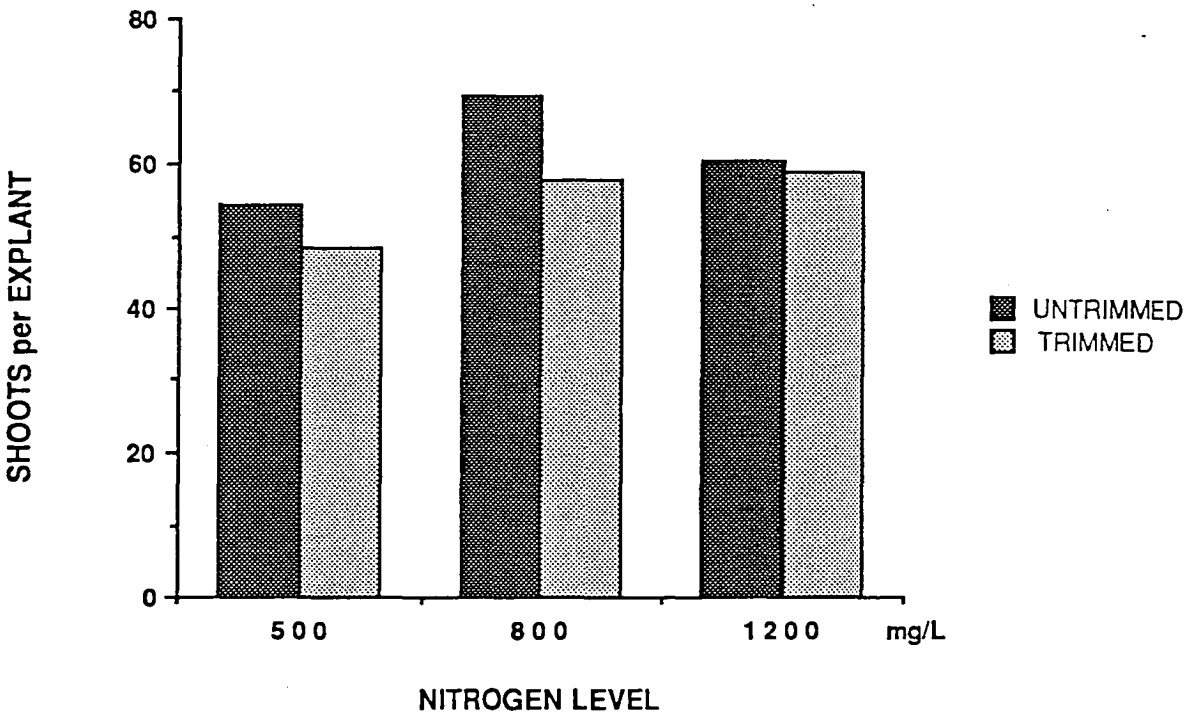
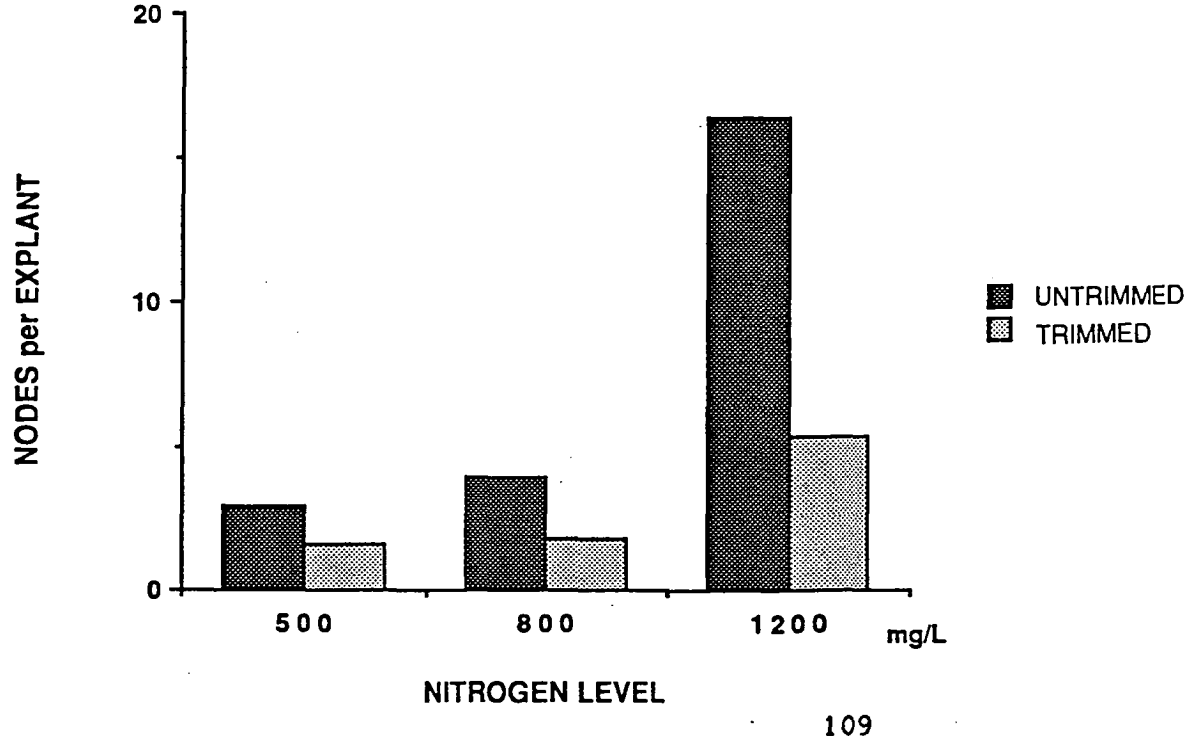


Fig 16

Effects of Trimming on Shoot Extension



of growth rather than the rate of growth. This is important because of the increased control over explant uniformity that can be achieved by trimming off extending shoots from explants at the start of experiments. The results are also useful for the nitrogen trial. It shows that the growth obtained in the two halves of that experiment can be compared while the shoot initiation and shoot extension measurements should not be compared, because they are probably measuring two different effects.

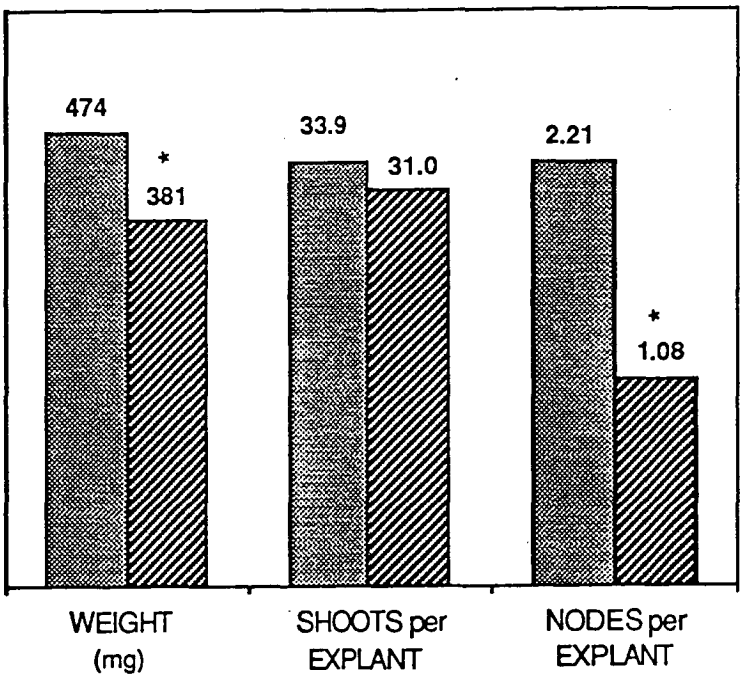
Explant Orientation

The results of the orientation experiment (Fig 17) show that it is important to take some care to ensure that explants are placed onto the agar correctly in order to ensure maximum growth. If this is not done, a decrease in growth of approximately twenty percent can be expected, (Fig 17). This could presumably add significantly to the variation within treatment.

The change in the development pattern (slight change in shoot initiation and large change in shoot extension) indicates that the orientation affects not just the growth of the explant overall but also the pattern of development. This effect is probably due to disruption of intercellular transport patterns in the explant. The explants are a mixture of callus and shoots and presumably the vascular

Fig 17

Effect of Explant Orientation on Explant Growth and Development



systems within shoots and newly initiating shoots are disturbed to some extent if the flow of nutrients and exogenous hormones is reversed.

Explant Size Experiment

The results of this experiment clearly indicate that there is a link between the size of the initial explant and the Relative Growth of that explant. This has the effect that small explants appear to have a much higher Relative Growth than larger explants. Relative growth (calculated as Ln w1 - Ln w0) should be independant of the starting weight, since it is designed to give a measure of growth that is independant of the starting weight.

It would appear from the graphs of the data (Fig 18, 19), that measurement errors have an effect on the measurement of growth when very small initial explants are used. On both media there is a tendency for the very small explants to result in a growth rate that is markedly below the trend. When the data points with initial weights below 30 mg are excluded, there appears to be a substantial improvement in the fit of the regression (Fig 20,21). Although the regression equations for the two media have slightly different slopes, the differences are not significant using the appropriate T test (Steel and Torrie 1980).

The conclusion that must be drawn from this experiment is that Relative Growth can not be used to compare growth between treatments because it assumes growth rate is

independent of the size of the starting material. In this experiment, it has been shown that growth rate is in fact inversely proportional to starting weight. This must have the effect of decreasing the accuracy of measurements, since it is not practical to try to cut all explants to a single weight.

Fig 18

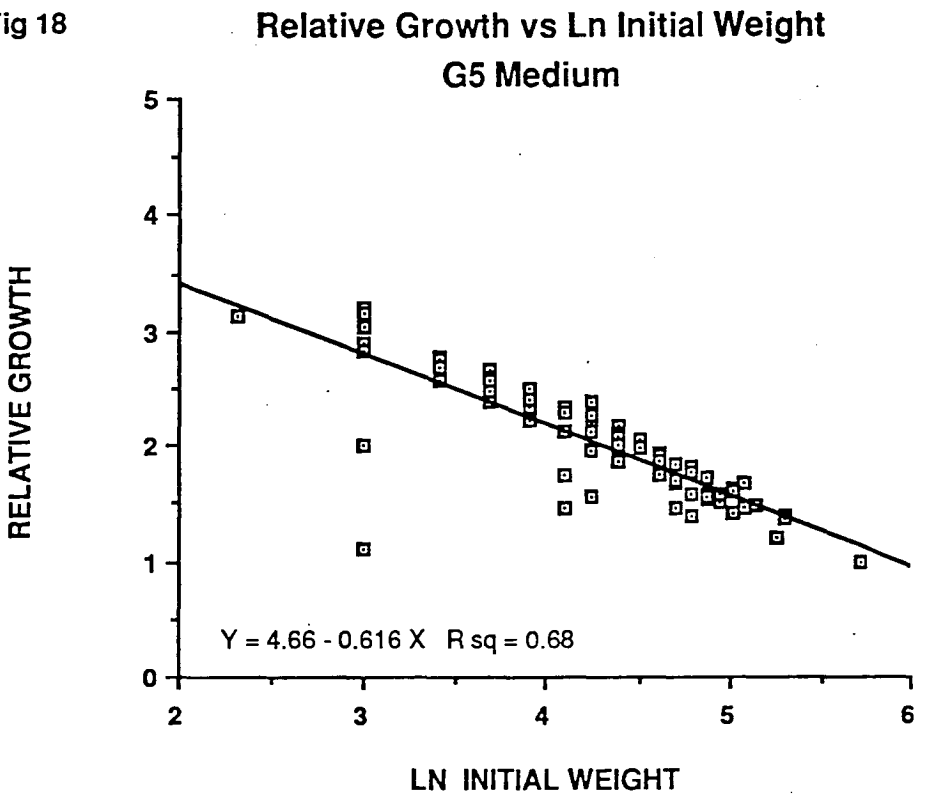

Relative Growth vs Ln Initial Weight
G5 Medium
RELATIVE GROWTH
LN INITIAL WEIGHT
Y = 4.66 - 0.616 X R sq = 0.68
0
1
2
3
4
5
2
3
4
5
6

Fig 19

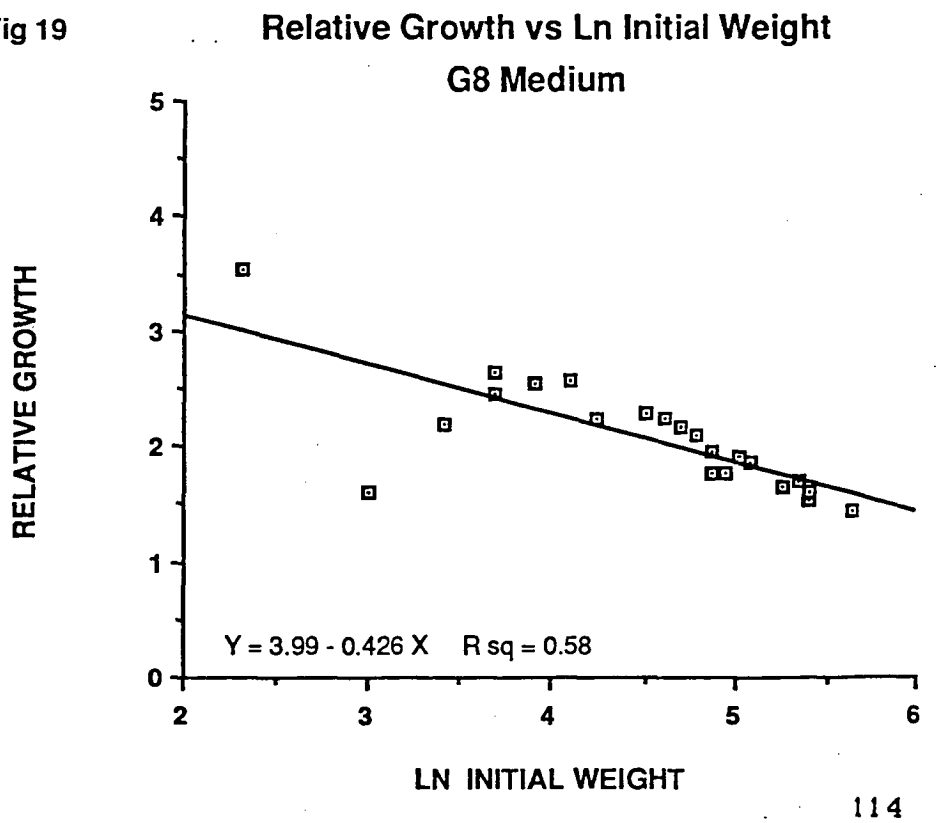

Relative Growth vs Ln Initial Weight
G8 Medium
RELATIVE GROWTH
LN INITIAL WEIGHT
Y = 3.99 - 0.426 X R sq = 0.58
0
1
2
3
4
5
2
3
4
5
6

Fig 20

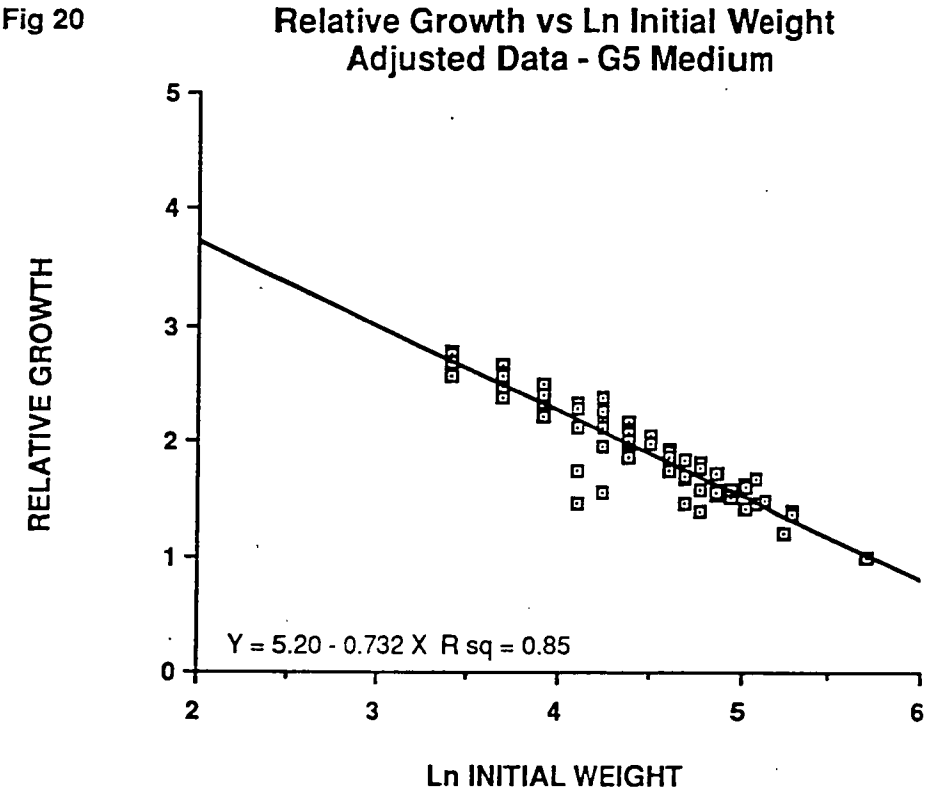
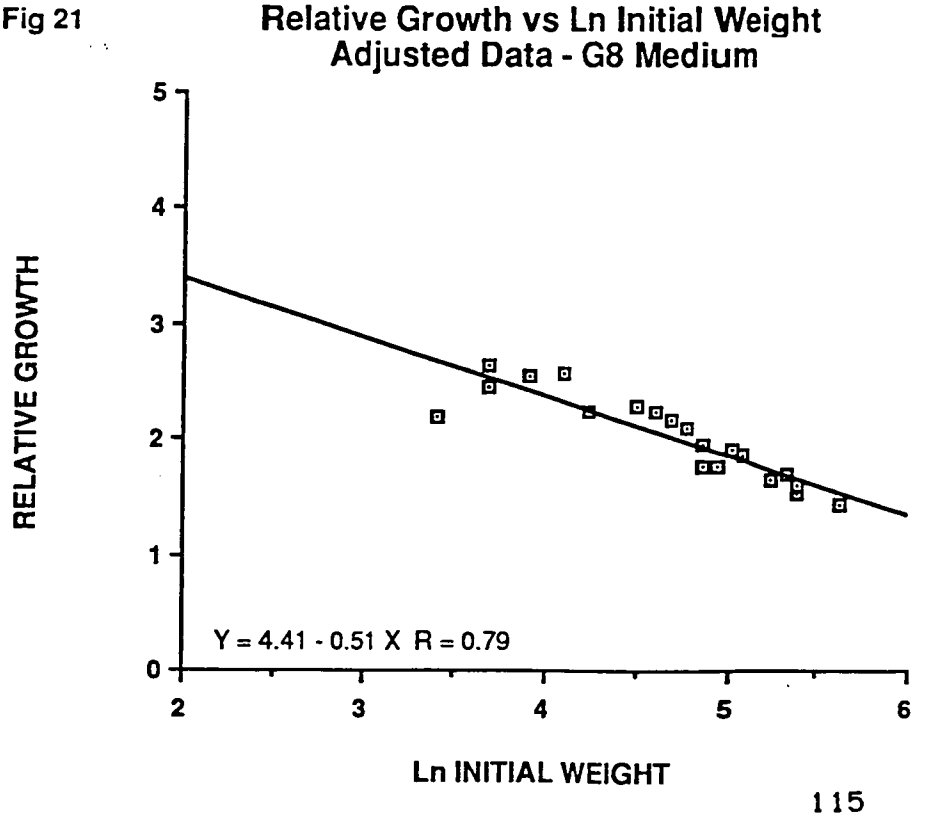

Relative Growth vs Ln Initial Weight
Adjusted Data - G5 Medium
RELATIVE GROWTH
Ln INITIAL WEIGHT
Y = 5.20 - 0.732 X R sq = 0.85
0
1
2
3
4
5
2
3
4
5
6

Fig 21


Relative Growth vs Ln Initial Weight
Adjusted Data - G8 Medium
RELATIVE GROWTH
Ln INITIAL WEIGHT
Y = 4.41 - 0.51 X R = 0.79
0
1
2
3
4
5
2
3
4
5
6

Media Constituents

Nitrogen Trial

This experiment is one that suffers from the problem of heterogeneity of variance. The heterogeneity appeared to be associated mostly with increased nitrogen levels. Attempts to decrease the heterogeneity, by use of a log transformation of the data, did not increase the precision. The results for shoot initiation are less obviously heterogeneous but standard deviations have been plotted on the graphs, rather than using LSD values, for the sake of consistency. (See also Appendix 1)

Despite the large variation associated with some of the means, there is a clear trend towards increasing growth with increasing nitrogen levels (Fig 22, 23). It would appear that there is an optimum level, at about 1200 mg/L, above which growth begins to decline. This pattern is found in both halves of the experiment, although there is much less consistency in the pattern for week 5 measurements. Part of the inconsistency may have been caused by the quite high growth noted for the 400 mg/L treatment, which was prepared as a separate batch of medium. The fact that the peak for the week 10 data is slightly higher is consistent with the effects of a low nitrogen pre-treatment medium slowing the rate of growth in the week 5 measurements.

Fig 22

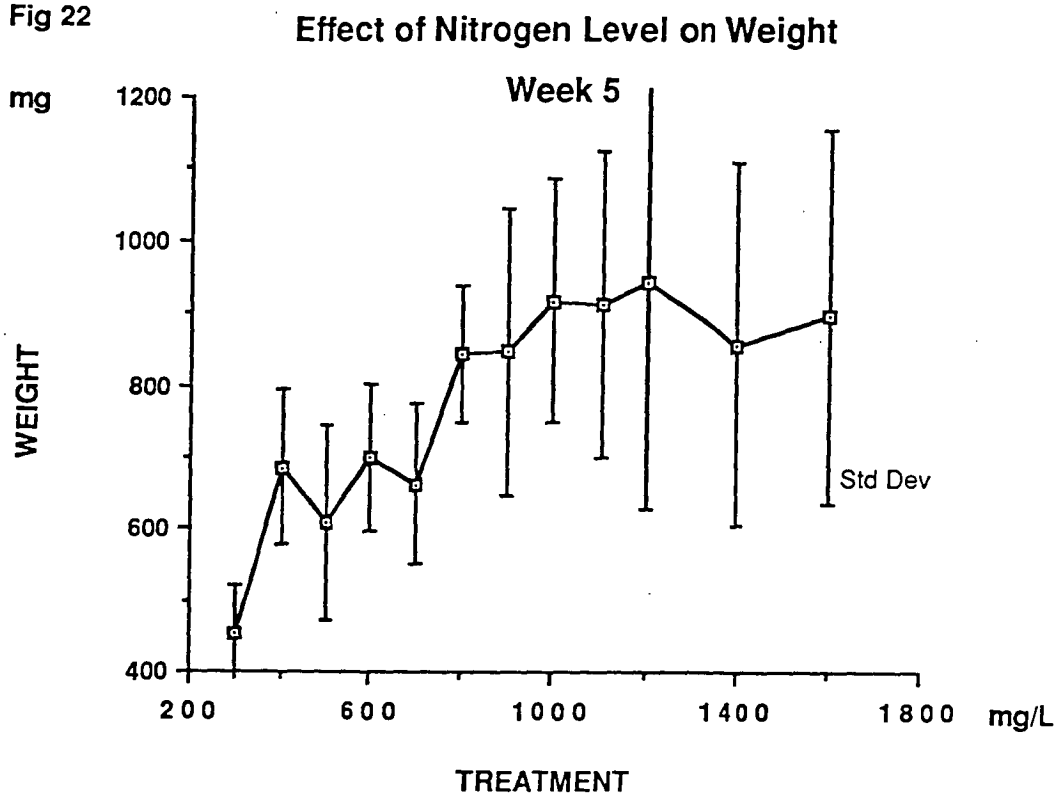
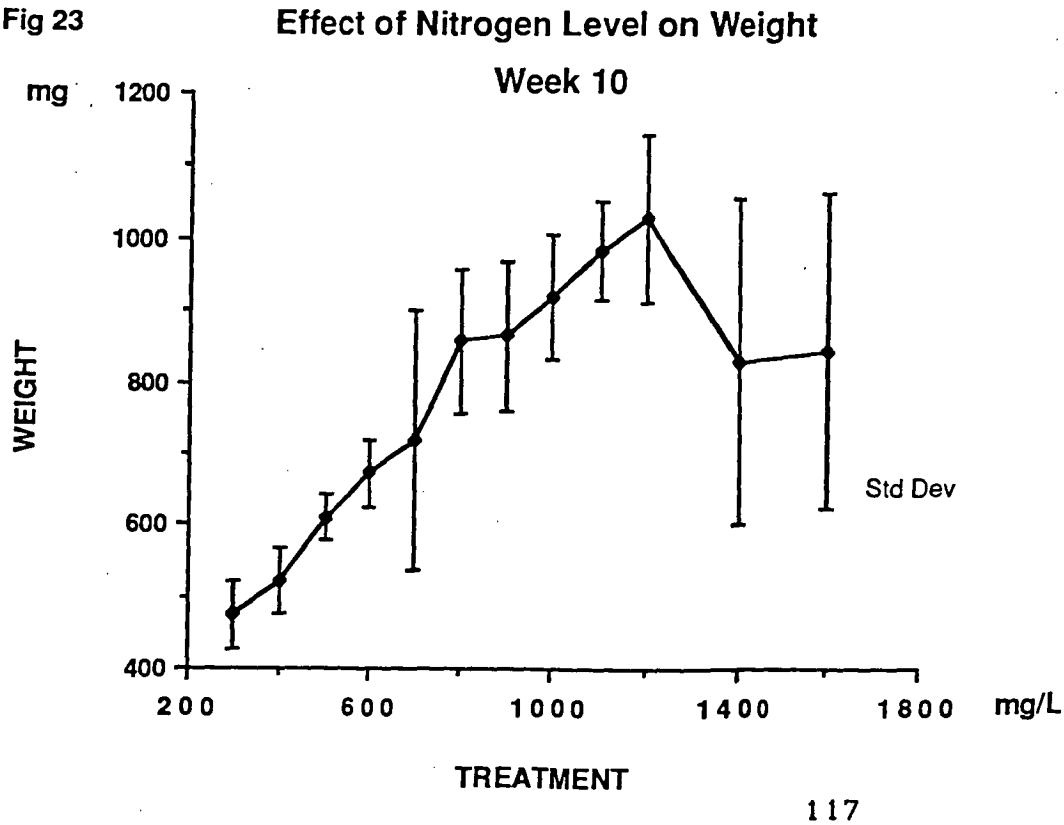

Effect of Nitrogen Level on Weight
Week 5
mg
1200
1000
800
600
400
WEIGHT
Std Dev
200
600
1000
1400
1800
mg/L
TREATMENT

Fig 23


Effect of Nitrogen Level on Weight
Week 10
mg
1200
1000
800
600
400
WEIGHT
Std Dev
200
600
1000
1400
1800
mg/L
TREATMENT

Shoot initiation follows a slightly different pattern (Fig 24, 25). In the week 5 measurement the pattern of growth would appear to indicate a slight increase in the rate of shoot initiation with increasing nitrogen. However, the inconsistent pattern over the range of nitrogen levels make it difficult to determine where the optimum level may be. For the week 10 measurement, the pattern of development is much more obvious. There appears to be a distinct bi-modal pattern, with one peak level of shoot initiation occurring at 500 mg/l and a second, much greater peak, occurring at 1200 mg/L. There is no immediately apparent reason why shoot initiation should decline so clearly and substantially in the 600 - 800 mg/L range, nor does it appear to have occurred at all in the week 5 measurement.

It would seem that the different methods of preparation for the two halves of the experiment have some effect on the rate of shoot initiation, since there is substantially less shoot initiation at high nitrogen levels in the week 5 measurement than in the week 10 measurement. The results of the previous trimming experiment would suggest that trimming should have less effect on shoot initiation at high nitrogen levels. This is where the greatest difference occurred in this experiment, suggesting that the differences may be more related to the effects of the low nitrogen pre-treatment medium. This means that the low rate of shoot initiation that occurs in the G5 pre-treatment is carried through to affect growth in the next culture cycle.

Fig 24

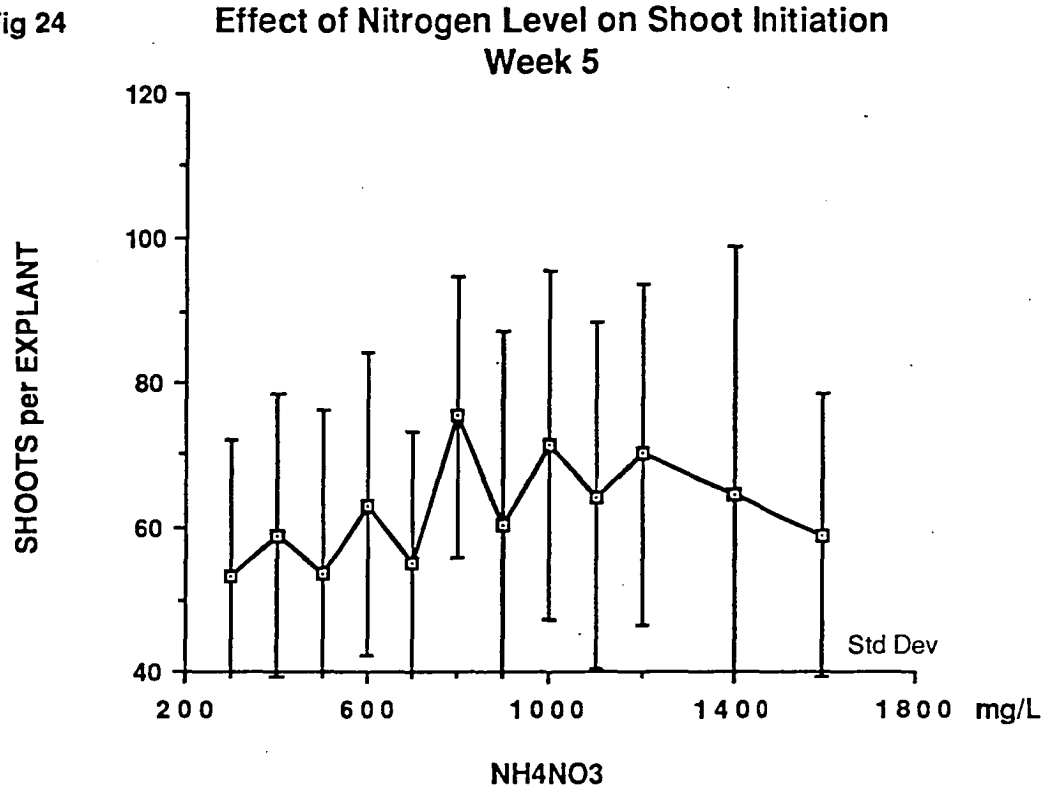
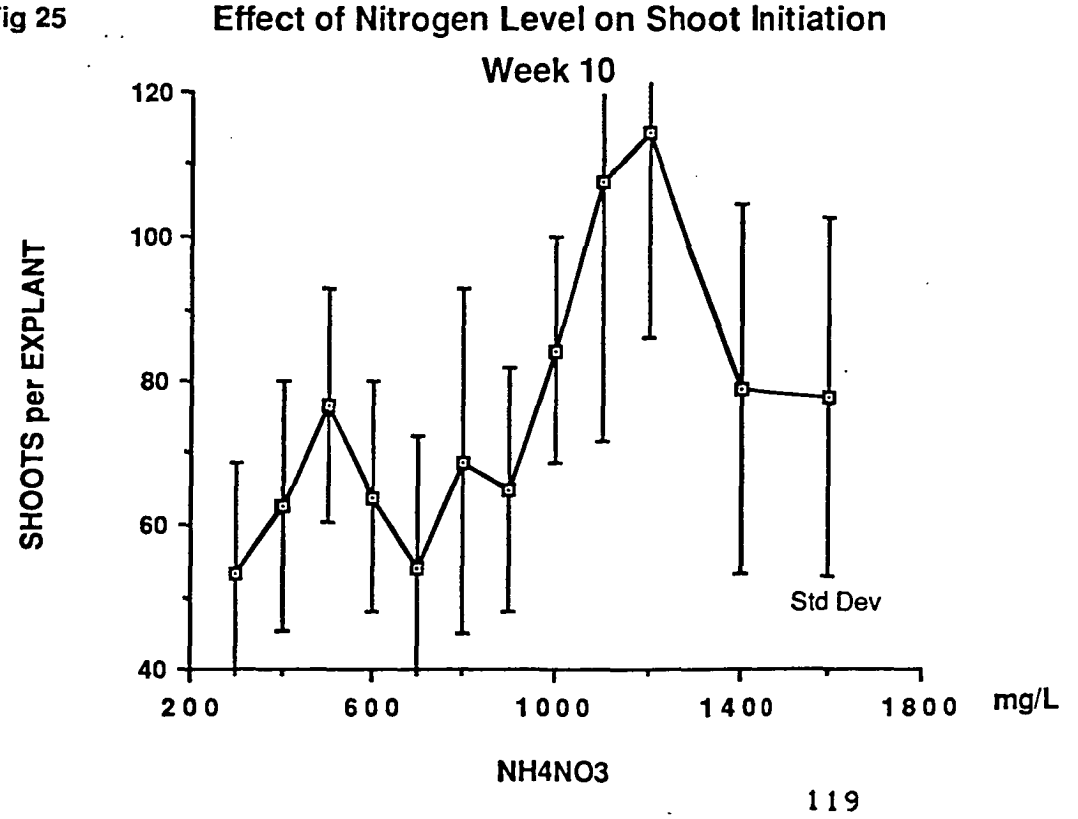

Effect of Nitrogen Level on Shoot Initiation
Week 5
SHOOTS per EXPLANT
120
100
80
60
40
200
600
1000
1400
1800
mg/L
Std Dev
NH4NO3

Fig 25


Effect of Nitrogen Level on Shoot Initiation
Week 10
SHOOTS per EXPLANT
120
100
80
60
40
200
600
1000
1400
1800
mg/L
Std Dev
NH4NO3

Shoot extension (Fig 26, 27) continues to increase up to the 1600 mg/L level for the week 5 measurement. The week 10 measurement again shows a bi-modal pattern of growth, with a distinct low point in shoot extension occurring at 1100 mg/L NH_4NO_3, then increasing sharply again. It appears to not have reached its peak at 1600 mg/L NH_4NO_3 indicating that shoot extension continues to increase well after the other growth indicators have begun to decline. There is no obvious reason why shoot extension should have a bi-modal pattern like this but it may be significant that the low point in shoot extension occurs at about the same NH_4NO_3 level as the peak for Weight and Shoot Initiation.

The effects of the different preparation for the two halves of the experiment can be seen most readily in the week 10 measurement of shoot extension (Fig 27), where there is a substantial increase in the amount of shoot extension growth. This might have been expected from the results of the 'Trimming' experiment.

The overall effects of changes in nitrogen level are well illustrated by the graphs of mean weight per shoot (Fig 28, 29). The trend for the week 5 measurement is for there to be an increase in the mean weight/shoot over the range of NH_4NO_3 levels used, whereas the week 10 measurement shows a definite optimum level. This information obviously has important practical implications. It is important not

Fig 26

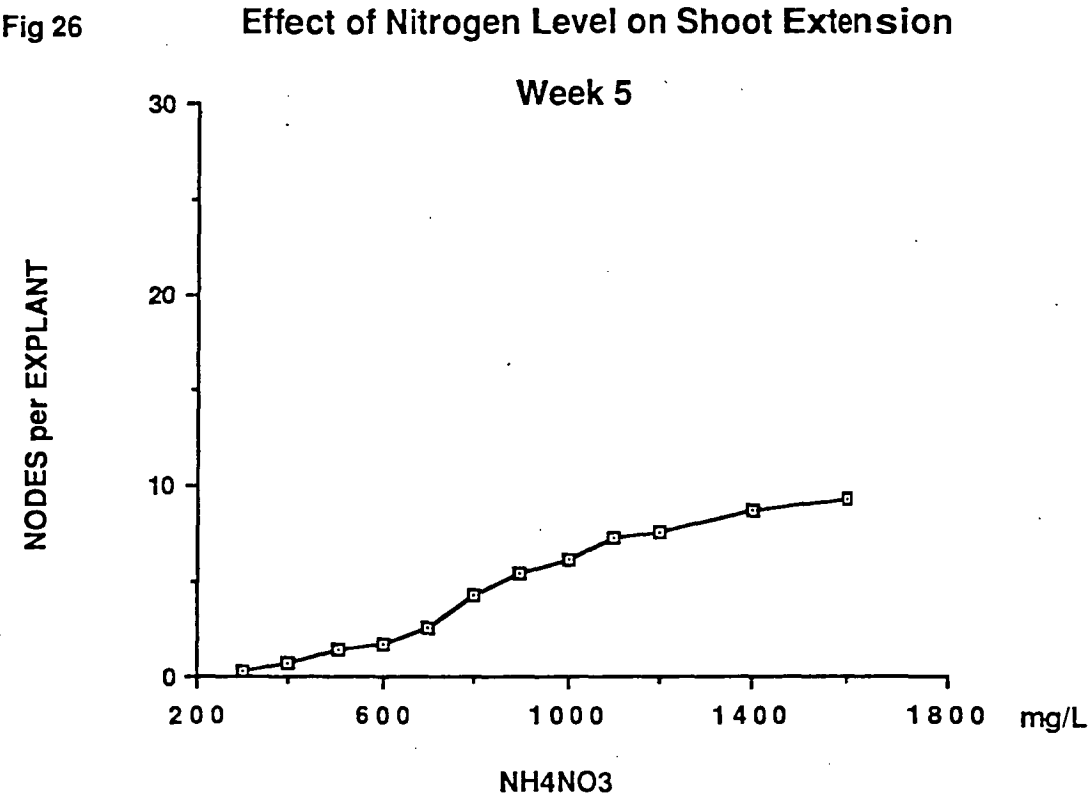

Effect of Nitrogen Level on Shoot Extension
Week 5
NODES per EXPLANT
30
20
10
0
200
600
1000
1400
1800
mg/L
NH4NO3

Fig 27

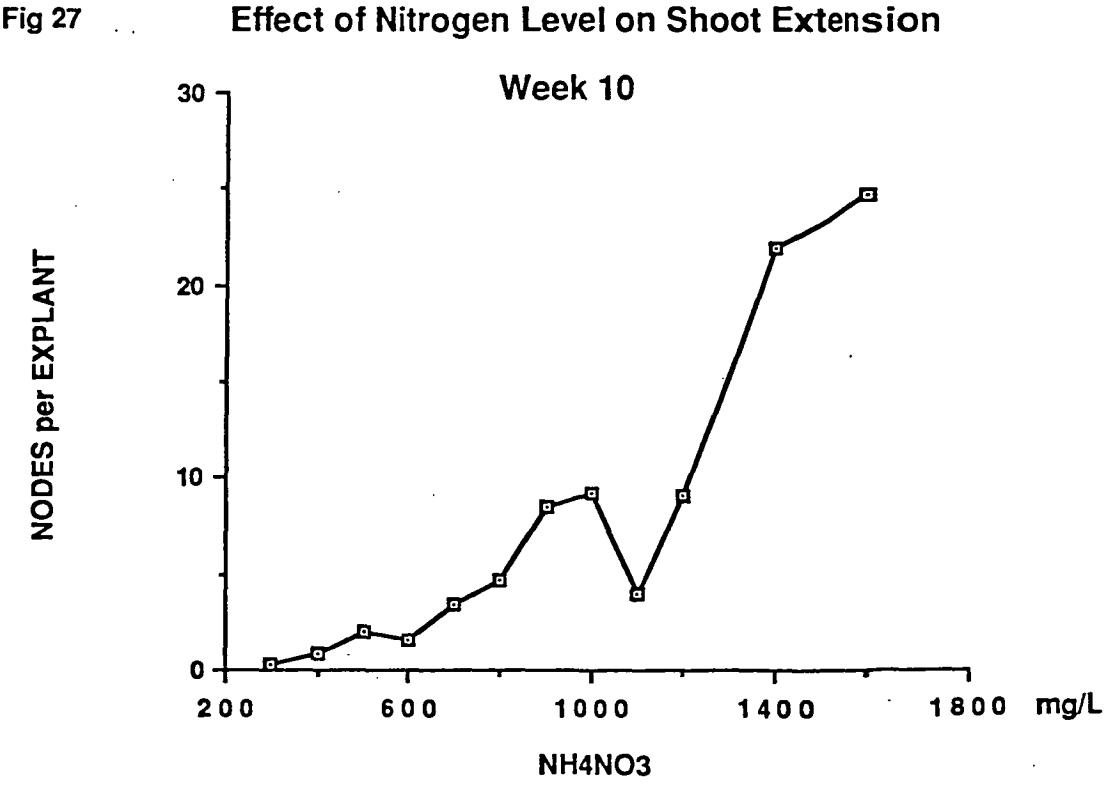

Effect of Nitrogen Level on Shoot Extension
Week 10
NODES per EXPLANT
30
20
10
0
200
600
1000
1400
1800
mg/L
NH4NO3

Fig 28 Effect of Nitrogen Level on Weight to Shoot Ratio

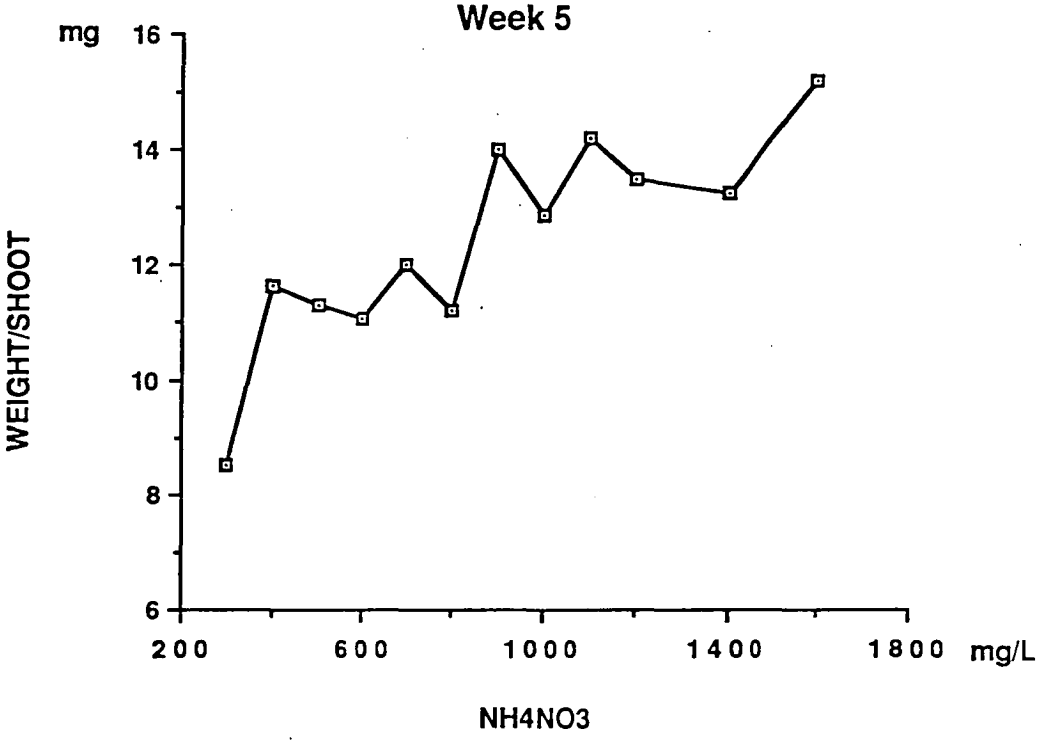
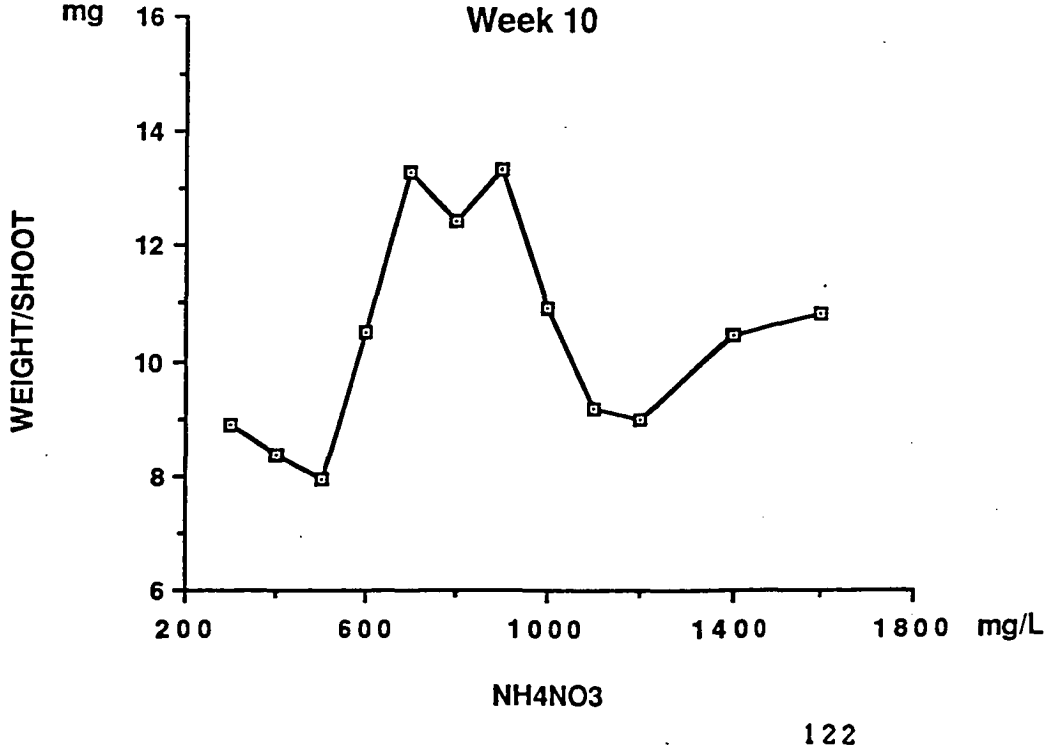


Fig 29 Effect of Nitrogen Level on Weight to Shoot Ratio



only to try to obtain the maximum level of proliferation but also obtain a shoot of sufficient size that it is easily handled without damage. Some care needs to be taken in interpreting this graph, as it might easily be concuded that 700 mg/L NH_4NO_3 gave the highest weight per shoot, in the week 10 measurement. This, however, occurs at a low rate of shoot initiation so is probably not the optimal level.

The basic objective of this experiment was to determine the growth characteristics of the clone over a range of nitrogen levels, while keeping all other growth parameters constant. By designing this experiment to run over ten weeks, it was hoped to be able to separate any possible pre-treatment effects, thus eliminating one source of potential variation. It has been shown, quite clearly, that the pre-treatment medium and trimming of the explants both substantially affect the growth and development of the cultures and interact with the effects of different nitrogen levels.

It is also clear that nitrogen is influencing the balance between proliferative growth, and extension growth resulting in changes in the appearance of the shoots. It is apparent that the standard conditions that have been adopted for most experiments have the capacity to distort the normal pattern of growth of cultures, by superimposing effects of the pre-treatment medium and preparation of explants over effects of the treatment medium.

The level of nitrogen has some considerable effects on the clone outside those effects that might normally be associated with an increase in nitrogen levels, such as increase in weight and increasing shoot initiation. It is difficult to identify how the nitrogen level might cause such sharp changes in growth pattern, such as the increase in shoot extension, when at nitrogen levels where growth and shoot initiation are adversely affected. The decline in shoot initiation and shoot extension are also difficult to explain but it might be speculated that rates of uptake of other media constituents, possibly hormones, are somehow affected by the rate of absorption of NH_4NO_3.

Further study may be required to look at how nitrogen concentration affects hormone levels within the explant under different nitrogen levels. Study of the influence of nitrogen levels on the rate of absorption of other media constituents may also provide useful information.

Clone Trial

The results of the clone trial are largely self explanatory and need little comment. A high degree of variability within treatments, a small number of replications (15) and the tendency for increased variance with increasing nitrogen levels rules out analysis of variance, although in most cases the trends are quite apparent. See Appendix 1.

All four clones quite obviously have different responses to the levels of nitrogen supplied. The growth responses measured give an indication of the types of growth occurring (Fig 30, 31, 32 & 33). In particular, it is worth noting the high rate of shoot initiation in HC 129, occurring at a moderate weight per explant, giving large numbers of very small shoots. In contrast, the growth of HC 15 is very slow and did not appear to respond to changes in nitrogen level very much at all. The growth observed for HC 17 appeared to be substantially less than has been observed in previous cultures, where it has been noted to have a high growth rate at high nitrogen levels (unpublished data). Reasons why this growth rate should not be observed in controlled experiments are not obvious, although there was observed to be tendency for explants to be slightly vitrified. This is a factor that has not been recorded by any of the measures used in this study. The growth of HC4 was also substantially less than observed in

Fig 30

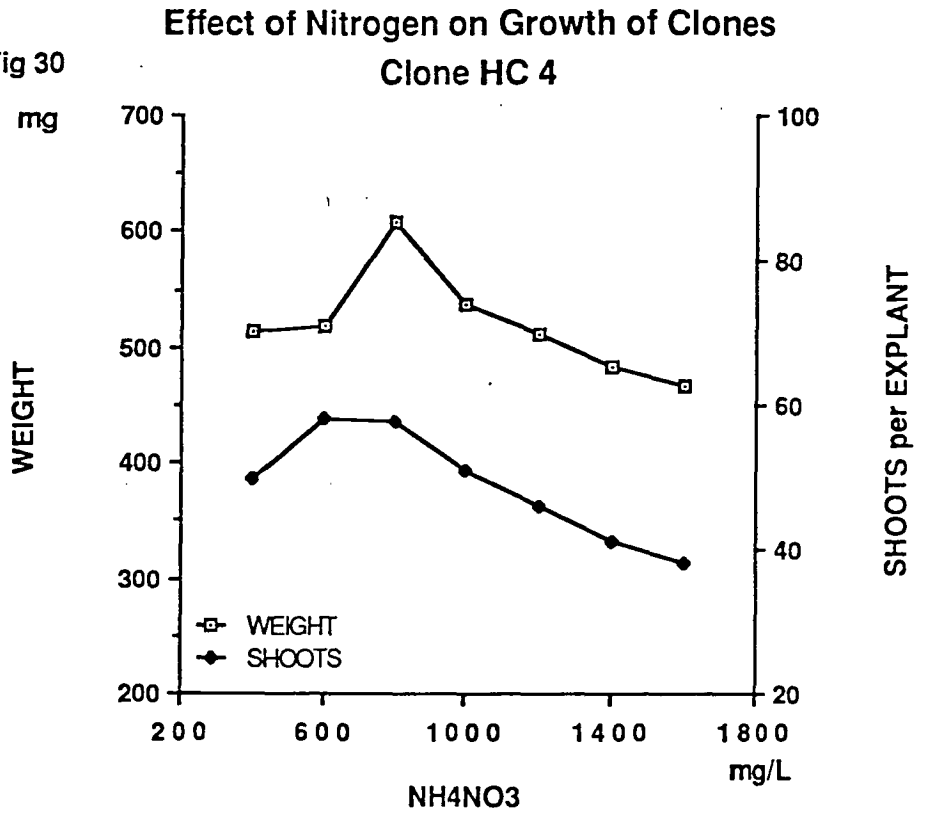

Effect of Nitrogen on Growth of Clones
Clone HC 4
mg
700
600
500
400
300
200
WEIGHT
100
80
60
40
20
SHOOTS per EXPLANT
WEIGHT
SHOOTS
200
600
1000
1400
1800
mg/L
NH4NO3

Fig 31

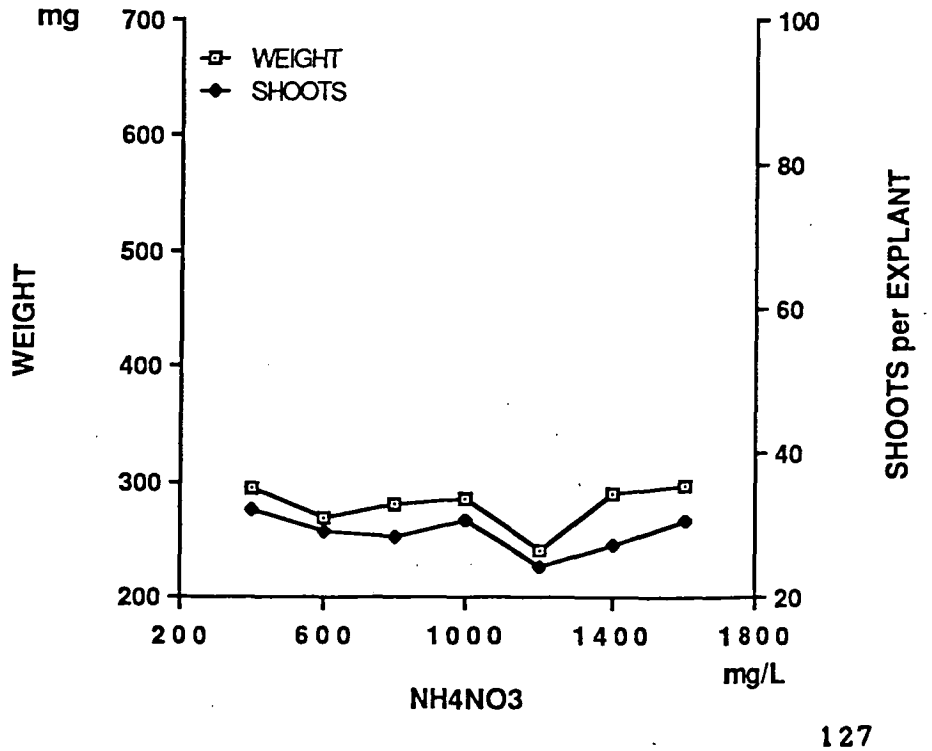

Clone HC 15
mg
700
600
500
400
300
200
WEIGHT
100
80
60
40
20
SHOOTS per EXPLANT
WEIGHT
SHOOTS
200
600
1000
1400
1800
mg/L
NH4NO3

Fig 32

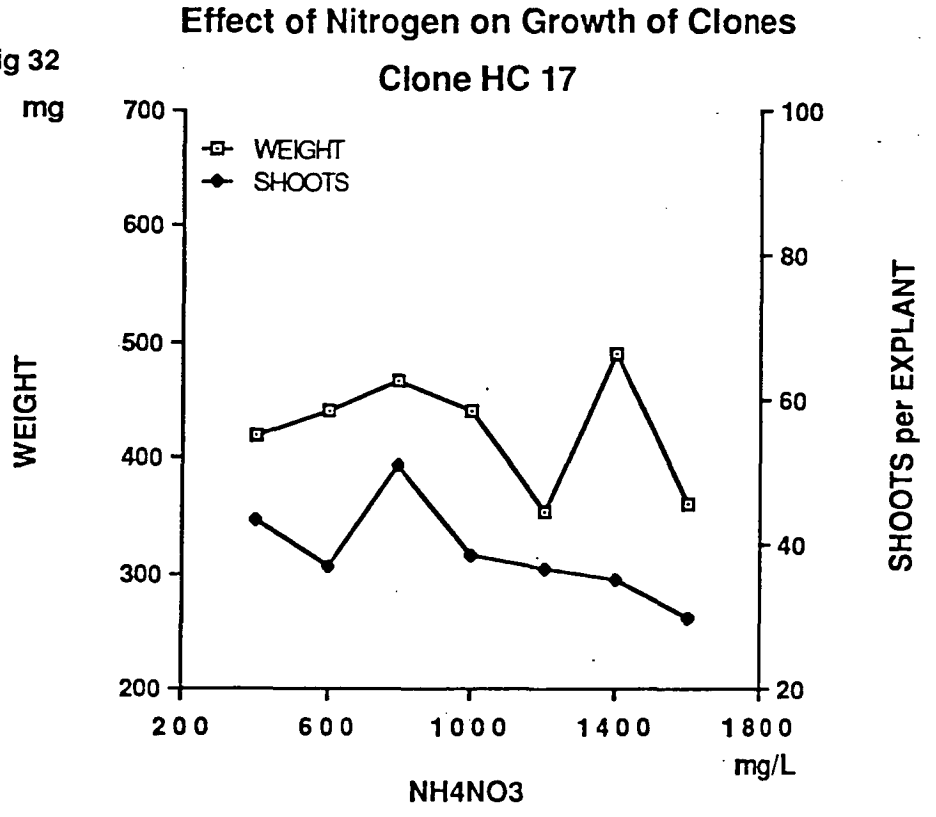
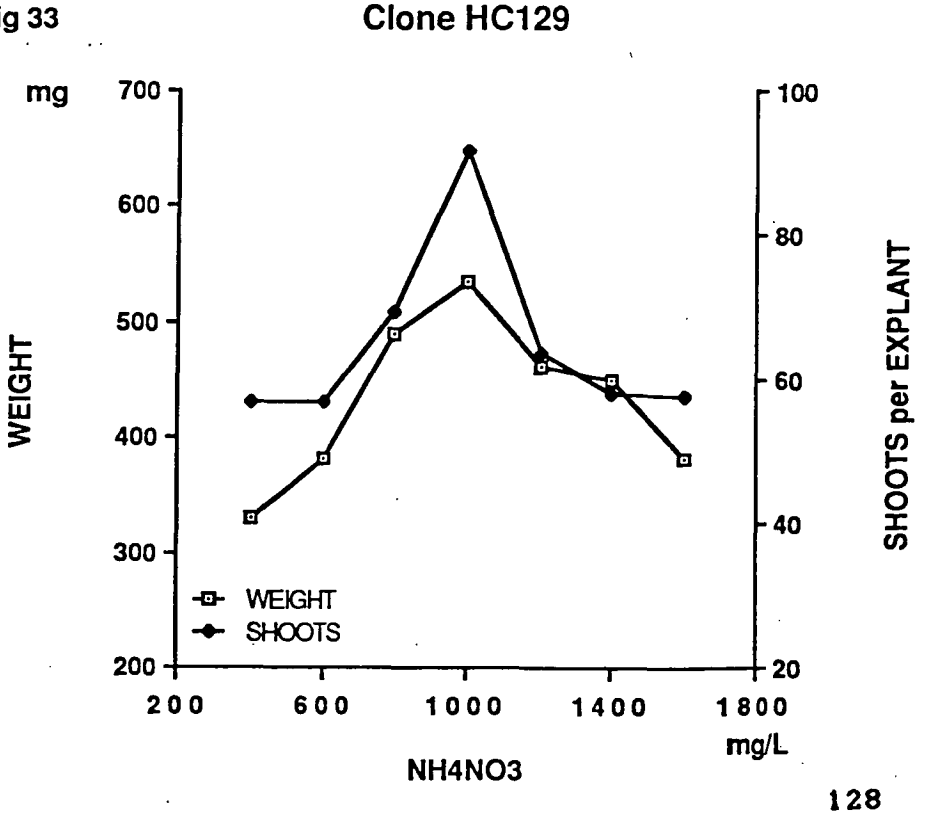

Effect of Nitrogen on Growth of Clones
Clone HC 17
mg
WEIGHT
SHOOTS
700
600
500
400
300
200
100
80
60
40
20
WEIGHT
SHOOTS per EXPLANT
200
600
1000
1400
1800
mg/L
NH4NO3

Fig 33


Clone HC129
mg
700
600
500
400
300
200
100
80
60
40
20
WEIGHT
SHOOTS per EXPLANT
WEIGHT
SHOOTS
200
600
1000
1400
1800
mg/L
NH4NO3

most other experiments, particularly at high nitrogen levels despite the fact that all conditions were consistent with other experiments.

The results from these experiments are important because they detail some of the wide variations in growth response that are observed in cultures of selected clones. They highlight the fact that the media and conditions developed for any one clone do not necessarily mean that all clones well be able to grow to the same extent under those conditions. This makes gaining an complete understanding of the factors controlling growth in cultures even more important. Such an understanding would allow adjustments needed to make media suit different clones easier to determine.

Nitrogen Source

Once again, an analysis of variance is not applicable because of the heterogeneity of variance. It is interesting, though, that there is considerable increase in the standard deviations associated with the adenine treatments, indicating that the inclusion of adenine greatly increases the variation within treatments.

Table 10

Effect of Nitrogen Source on Growth and Development

Treatment		No Adenine		Adenine 100 mg/L	
		Mean	Std Dev	Mean	Std Dev
10 mMol NH4	Weight	107	17.8	170	199.1
	Shoot Init	9.00	4.68	13.1	6.47
	Shoot Ext.	0.00	0.00	0.00	0.00
	Final pH	3.52		3.68	
10 mMol NO3	Weight	125	22.6	143	32.7
	Shoot Init	9.00	4.64	10.5	6.98
	Shoot Ext.	0.21	0.51	0.17	0.48
	Final pH	6.89		6.73	
5 mMol NH4NO3	Weight	393	98.9	453	120.1
	Shoot Init	44.9	18.4	60.8	25.21
	Shoot Ext.	0.63	0.87	1.71	1.30
	Final pH	4.54		4.51	
10 mMol NH4NO3	Weight	657	98.6	625	192.8
	Shoot Init	67.0	72.8	75.2	32.4
	Shoot Ext.	4.87	2.07	5.37	3.69
	Final pH	4.55		4.46	

The cultures are clearly unable to maintain growth in media that contain only ammonium or nitrate as the nitrogen source. The poor growth occurring with the nitrate and ammonium alone treatments is associated with changes to the pH of the medium as these major components are absorbed by the cultures. However, it is not clear whether the growth stops because of the pH changes. Growth may be able to continue at these extreme pH's but has ceased because of the imbalance in nitrogen source.

Fig 34

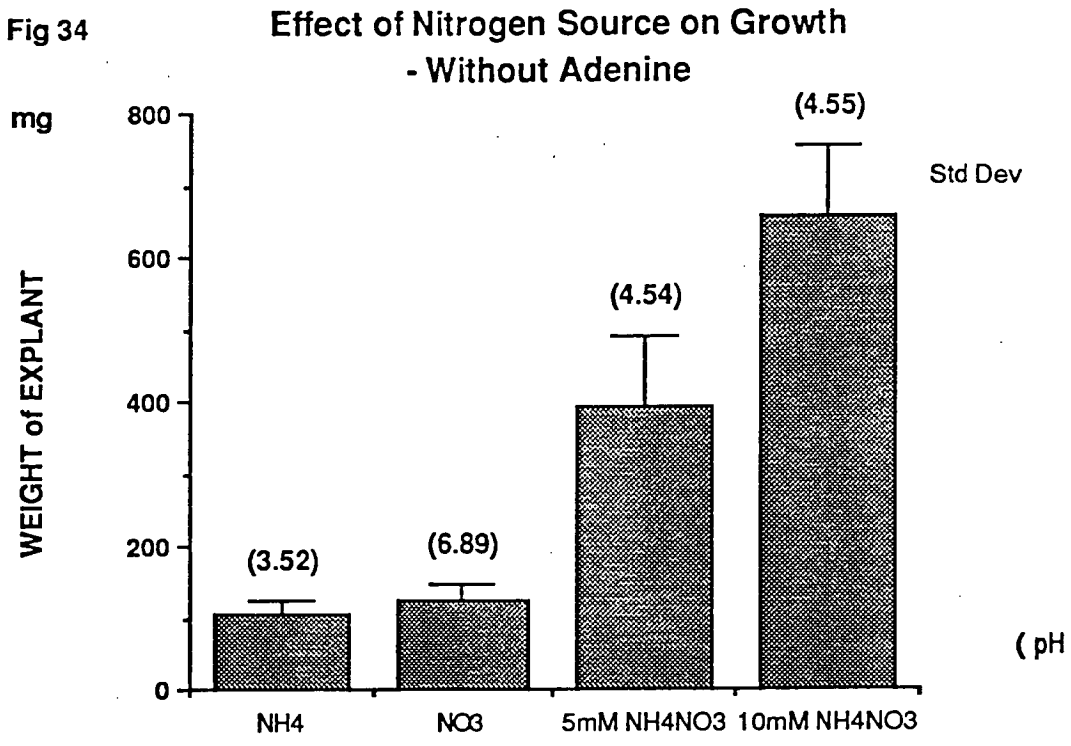

Effect of Nitrogen Source on Growth
- Without Adenine
mg
WEIGHT of EXPLANT
800
600
400
200
0
(3.52)
(6.89)
(4.54)
(4.55)
Std Dev
NH4
NO3
5mM NH4NO3
10mM NH4NO3
(pH)

Fig 35

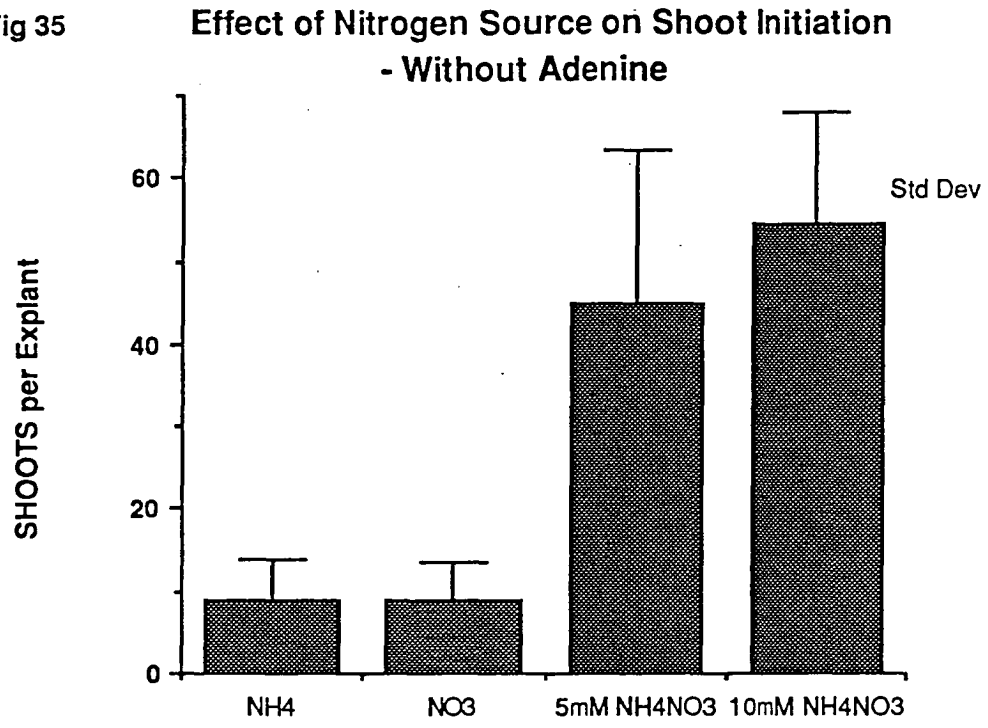
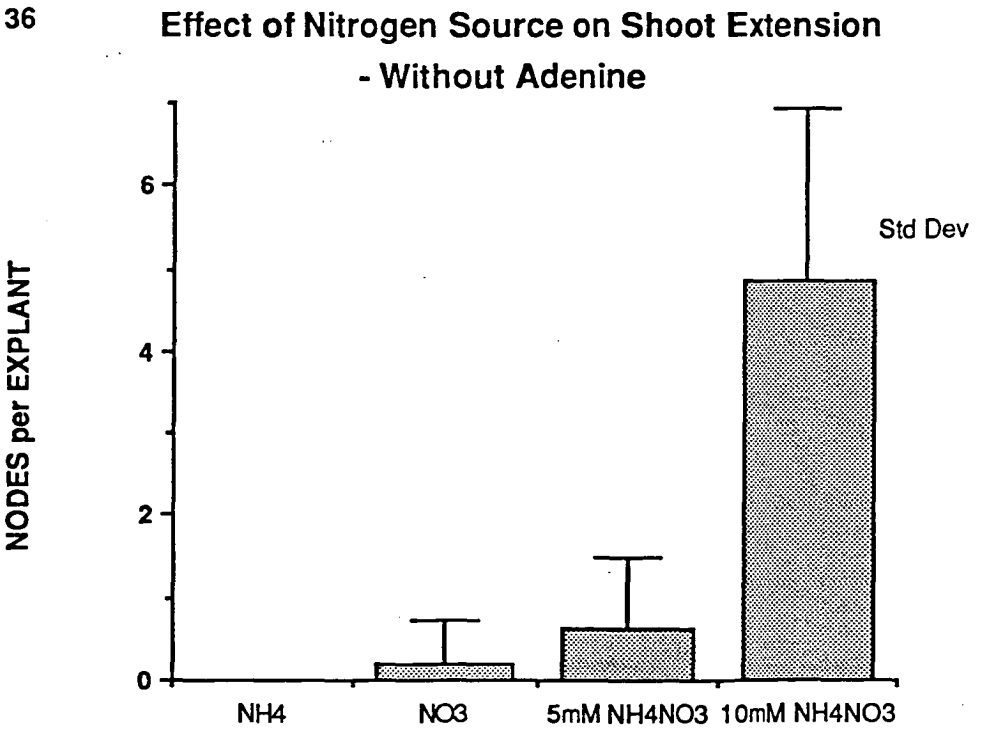

Effect of Nitrogen Source on Shoot Initiation
- Without Adenine
SHOOTS per Explant
60
40
20
0
Std Dev
NH4
NO3
5mM NH4NO3
10mM NH4NO3

Fig 36


Effect of Nitrogen Source on Shoot Extension
- Without Adenine
NODES per EXPLANT
6
4
2
0
Std Dev
NH4
NO3
5mM NH4NO3
10mM NH4NO3

There is a difference in the way in which explants grow on media containing NH4 or NO3 alone. Explants growing on NH4 medium were not observed to undergo any shoot extension, while those growing on NO3 medium did, in some flasks, show some shoot extension. It is also interesting that the addition of adenine, a source of reduced nitrogen, had a greater effect in the NH4 medium than in the NO3 medium, where it might be expected that reduced nitrogen would be more needed. This suggests that whatever the cause of the poor growth on the nitrate medium, it is unlikely to be caused by a shortage of reduced nitrogen. This might suggest that nitrate toxicity may be a factor in this medium, however further evidence is required.

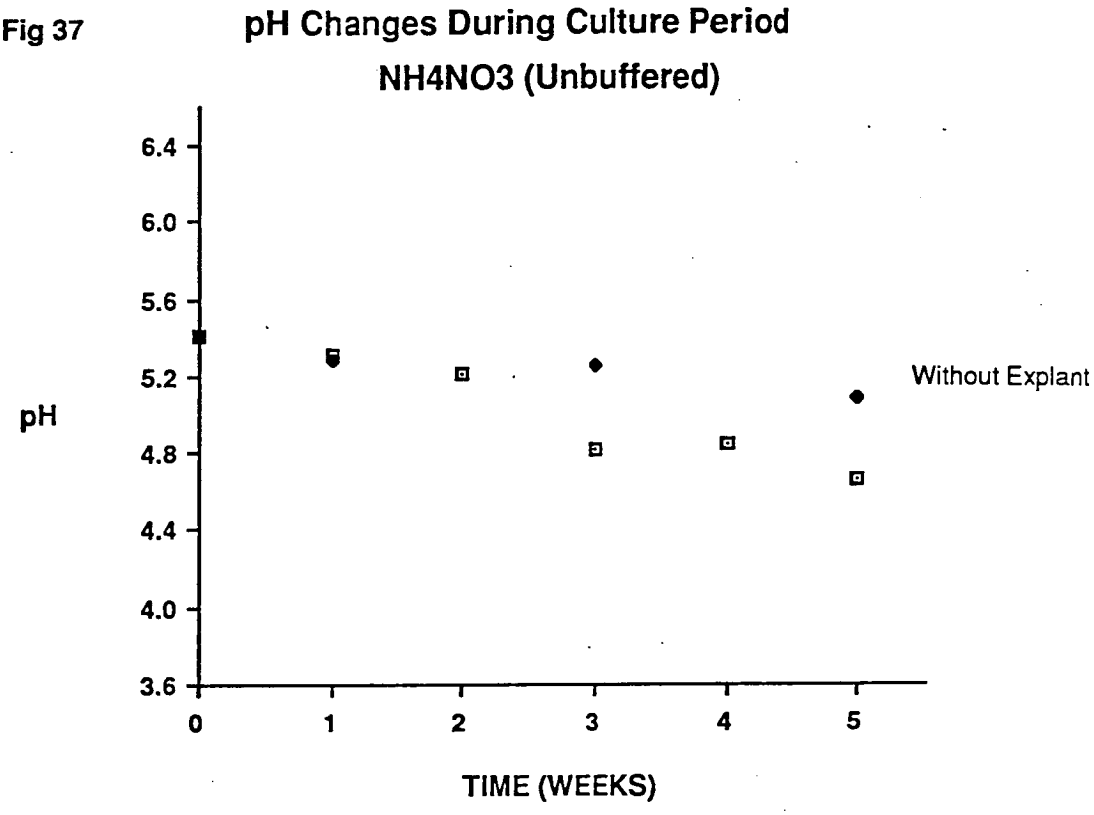
Bufferred Media Trial

pH Changes

The increase in pH observed during autoclaving in this experiment is similar to that observed in the G8 pH Response experiment and is probably related to the rapid cooling of the medium in the laminar flow cabinet as compared with the slower rate in the autoclave, as is normally the case. The effect of this pH change is that the starting pHs of the media are probably slightly higher than the optima for the unbuffered media, although this does not appear to have affected growth significantly.

The graphs of pH for the unbuffered media (figs 37, 38 & 39) show quite clearly the effect on pH that absorption

Fig 37


pH Changes During Culture Period
NH4NO3 (Unbuffered)
pH
6.4
6.0
5.6
5.2
4.8
4.4
4.0
3.6
0
1
2
3
4
5
TIME (WEEKS)
Without Explant

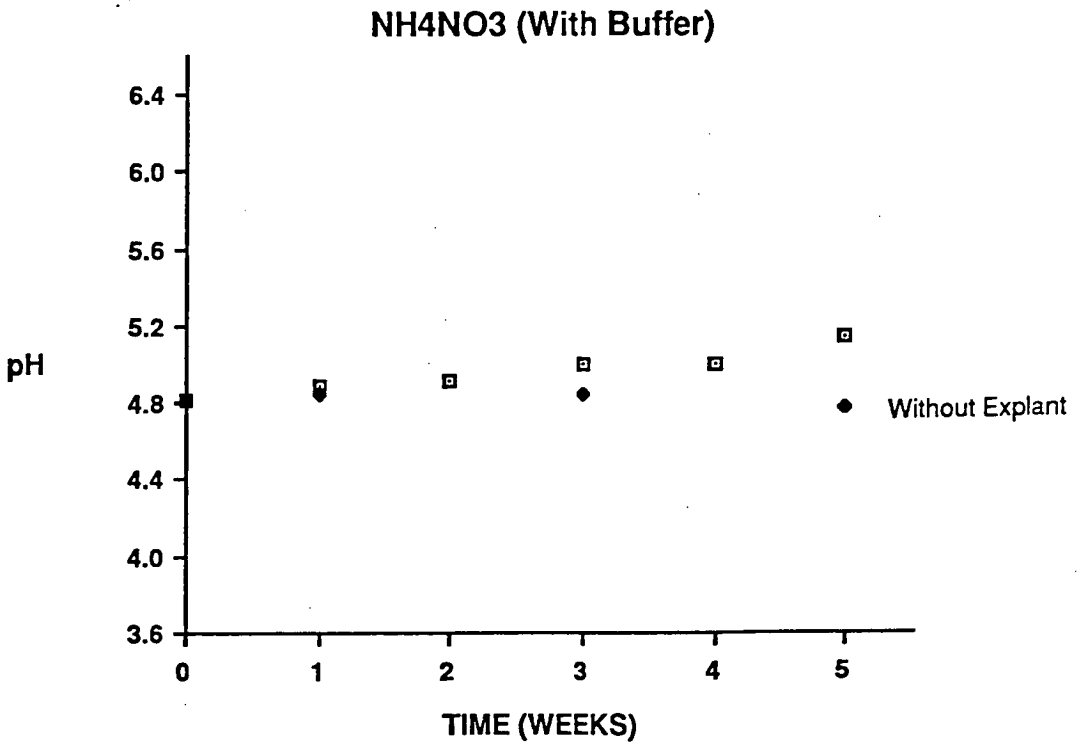
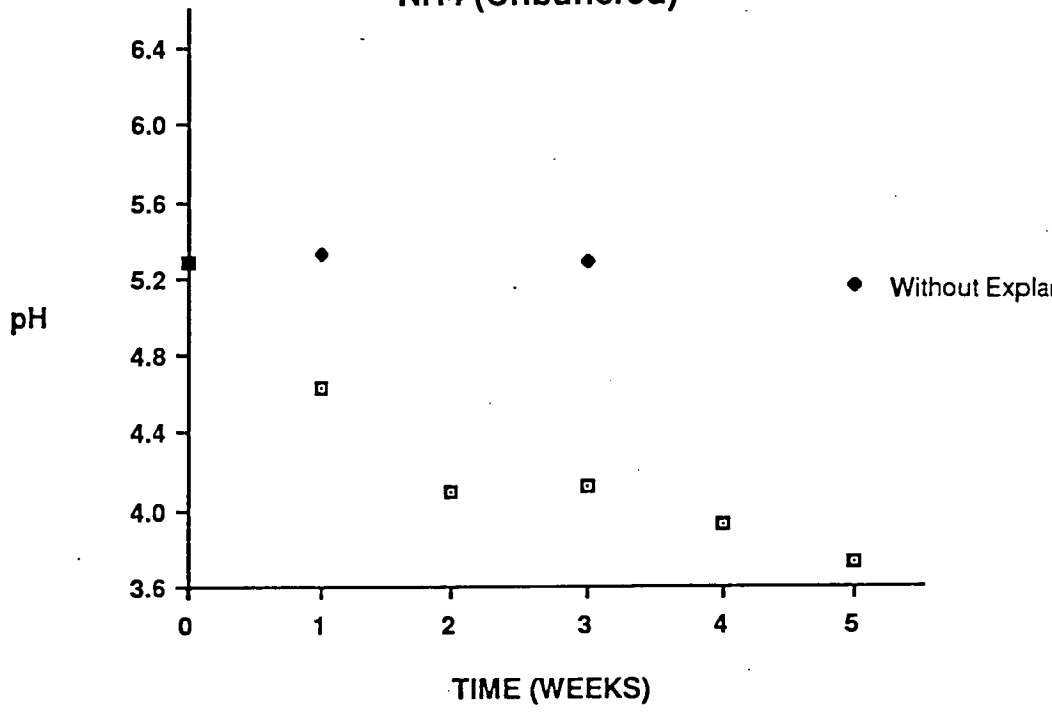

NH4NO3 (With Buffer)
pH
6.4
6.0
5.6
5.2
4.8
4.4
4.0
3.6
0
1
2
3
4
5
TIME (WEEKS)
Without Explant

Fig 38

pH Changes During Culture Period

NH4 (Unbuffered)



NH4 (With Buffer)

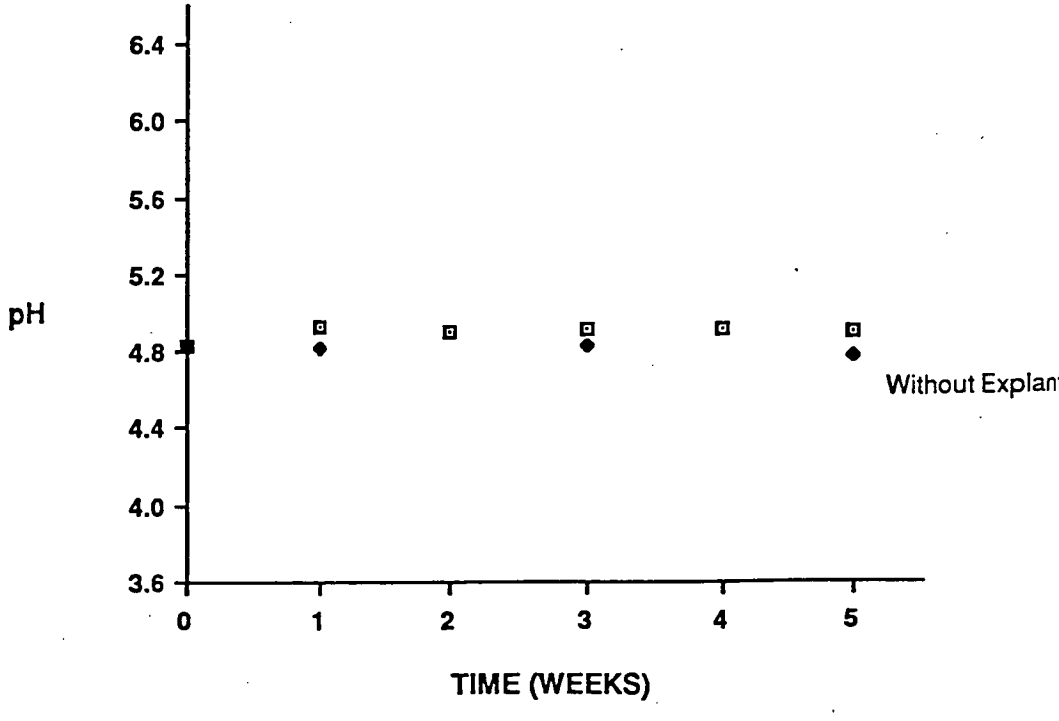
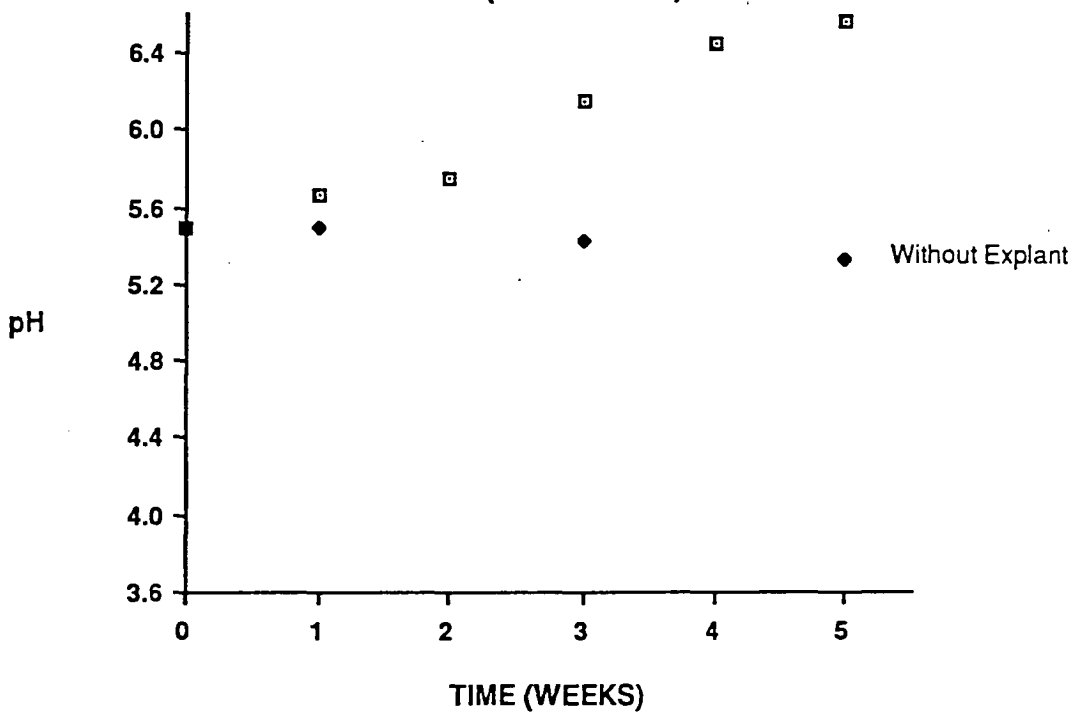


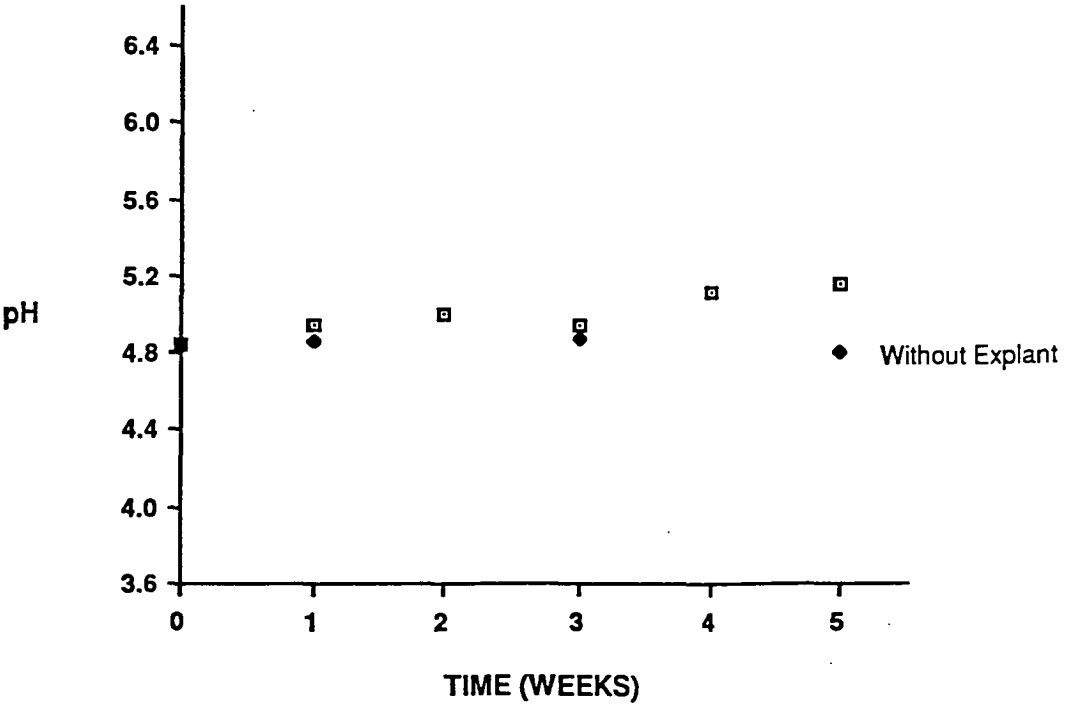
Fig 39

pH Changes During Culture Period

NO3 (Unbuffered)



NO3 (With Buffer)



of NH4 and NO3 and NH4NO3 have on the respective media. It is also clear that the pH changes observed in this experiment are the result of the action of the explants on the medium. There are no significant changes occurring in medium without the presence of plants, as has been observed by Skirvin et al (1986). The addition of 20 mMol succinate buffer was largely successful in holding the pH of the media constant. It is surprising that the pH of the NH4NO3 treatment rose slightly in the presence of buffer, in much the same manner as pH of the NO3 treatment rose. This suggests that in this treatment more nitrate was absorbed than ammonium.

Growth Pattern

The growth on the unbuffered medium confirms the results for the N-source experiment and shows that poor growth on NH4 and NO3 free media is accompanied by substantial and rapid pH changes. It would seem reasonable to assume that the adsorption of NH_4^+ results in a net absorption of OH^- to act as a balancing ion, resulting in an increase in H^+ concentration. For the absorption of NO_3^-, the flow of H^+ and OH^- ions would be reversed.

The growth patterns on media containing succinate buffer are quite unexpected (Figs 37, 38 & 39). Explants on the NH4 medium grew quite well, however the NO3 treatments still grew poorly. These results indicate that the explants are unable to utilise nitrate as a nitrogen source and that holding the pH of the medium constant does not increase the

Fig 40 **Effect of Buffer and Nitrogen Source on Growth**

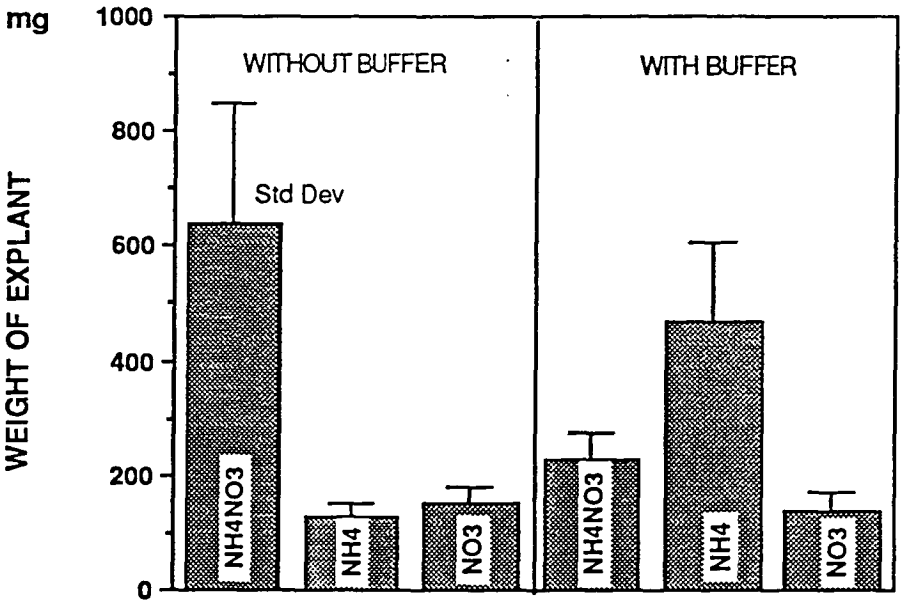


Fig 41

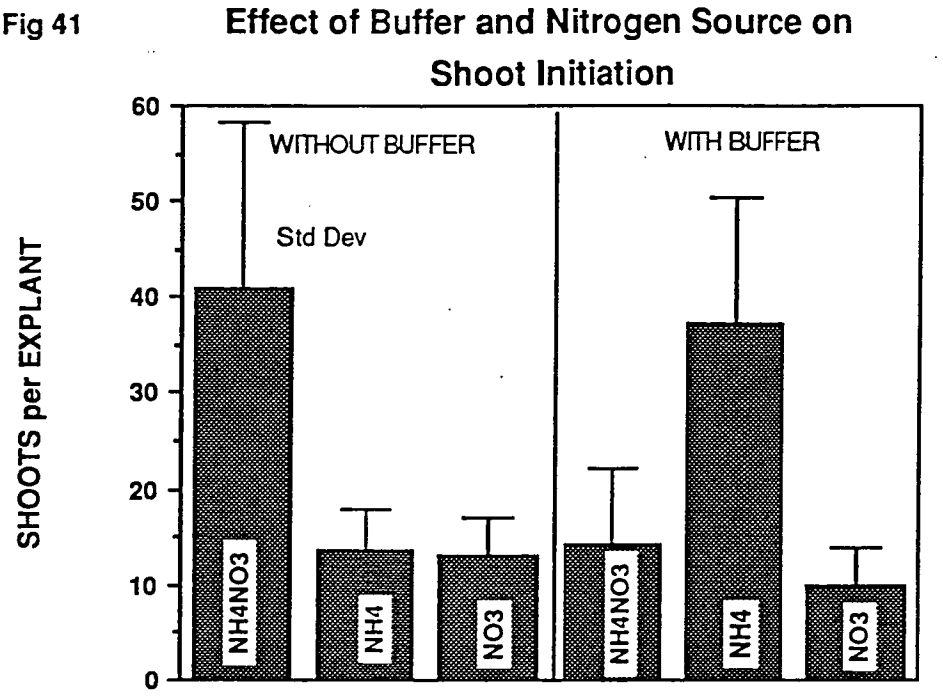
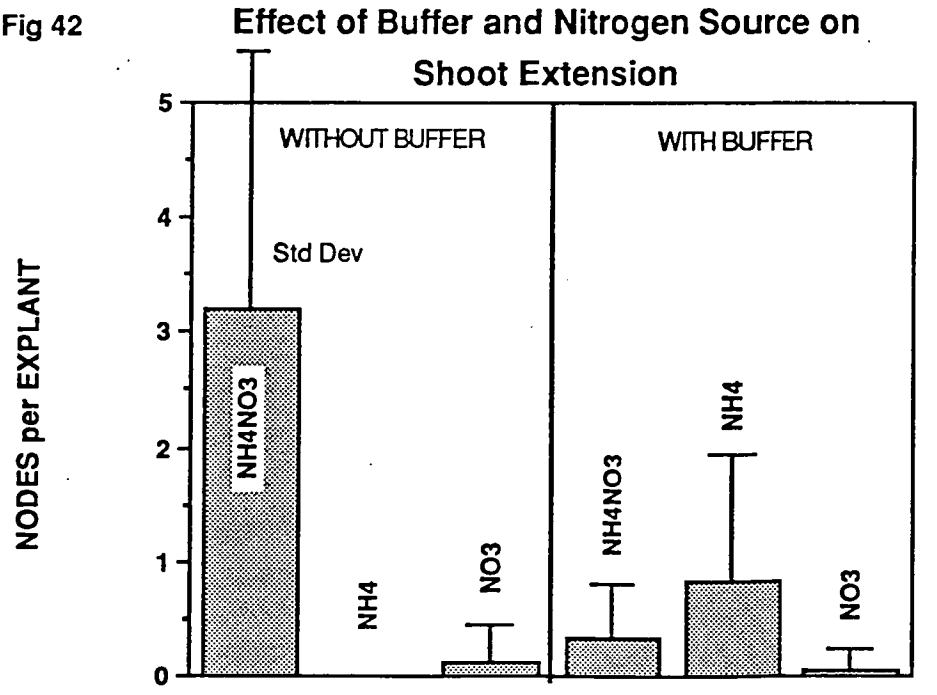

Effect of Buffer and Nitrogen Source on Shoot Initiation
SHOOTS per EXPLANT
60
50
40
30
20
10
0
WITHOUT BUFFER
WITH BUFFER
Std Dev
NH4NO3
NH4
NO3
NH4NO3
NH4
NO3

Fig 42


Effect of Buffer and Nitrogen Source on Shoot Extension
NODES per EXPLANT
5
4
3
2
1
0
WITHOUT BUFFER
WITH BUFFER
Std Dev
NH4NO3
NH4
NO3
NH4NO3
NH4
NO3

ability of the explant to utilise the nitrate in the medium. It can also be concluded that pH change is the main reason why the cultures do not grow well using ammonium as the sole nitrogen source in the absence of buffer. However there must be some other explanation why nitrate is not able to be used as the sole nitrogen source. This is further evidence for nitrate toxicity being a problem in this type of medium.

These results imply that ammonium is the preferred source of nitrogen in boronia cultures and that in normal culture NH4 is absorbed for metabolic purposes. NO3 is most probably only absorbed as a balancing ion once the pH of the medium begins to decline below the optimum for sustained growth, presumably at pH 4.5-4.7.

No explanation can be offered as to why the NH4NO3 treatments failed to grow in the presence of buffer, or why the pH of the medium tended to rise slightly for this treatment. It seems unlikely that the succinate was toxic to the plantlets, since the NH4 treatment grew well. The slight rise in pH in the presence of the buffer is probably important and may indicate that more nitrate was being absorbed than ammonium. If this is the case then it is possible to account for the poor growth through the effects of nitrate toxicity. This still does not explain why more nitrate than ammonium is being absorbed in the presence of succinate when the reverse is clearly the case in the absence of succinate. It could be suggested that the succinate is being absorbed (as an energy source) and that

this results in increased NO3 absorption.

It is probably important to note that the pattern of growth for the buffered NH4 treatment was quite different to normal (NH4NO3) growth. Although the weight and shoot initiation was comparable, there was almost no shoot extension in the ammonium treatment. It can be speculated that perhaps this lack of extension growth is associated with a lower auxin level in the explants. This might result either from decreased absorption of exogenous supplies or changed auxin or giberellic acid metabolism within the explants.

It is not strictly accurate to refer to the NH4 and NO3 media as having these as the sole nitrogen source as all media in this experiment contained 80 mg/L adenine. This, as has been mentioned previously, can act as a nitrogen source in media where nitrogen is deficient. If this nitrogen source and the other nitrogen containing compounds in the medium, (vitamins and glycine plus impurities in the sucrose and agar) are accounted for then the amount of growth that could be attributed to utilisation of nitrate would be very small indeed and may indicate a total inability to utilise nitrate.

Adenine

The results obtained from this experiment make it clear that adenine does exert some effect on the growth of boronia cultures. Growth in G5 media containing adenine was significantly better than those not containing adenine. It would appear that the level of adenine in the pre-treatment medium did not have any substantial effect on subsequent growth of the cultures. The only exception to this is treatment 10, where the absence of adenine in the pretreatment medium conferred some benefit to the culture. In none of the treatments was there any appreciable difference in the pattern of shoot initiation or extension attributable to the presence or absence of adenine. Nor was there any difference in the final pHs of the media that might indicate that adenine was in any way influencing pH.

Lack of homogeneity of variance between the treatments again prevents the direct use of an analysis of variance to interpret the results. In particular, there is an apparent increase in variance in the high nitrogen treatments. This can be overcome by separating the experiment into two sections, that using G5 medium and that using, G10 medium and performing the analysis of variance on each section separately. This greatly improves the reliability of the analysis of variance and LSD tests, although it prevents direct comparison of means between the two analyses. The analysis of variance on shoot extension is still un-reliable

because of the large number of zero values recorded for some treatments. Transformation of this data to Log (Nodes +1) does not improve the data in any material way. See appendix 1

The results from the experiment suggest that adenine does exert some growth promoting effects at low nitrogen levels. However, the fact that the effect is reversed at higher nitrogen levels would suggest that the differences are primarily a result of the nitrogen content of adenine being available to the cultures where there is a nitrogen deficiency. If this is the case, then the higher growth in treatment 10 could be the result of a lower level of nitrogen in the pre-treatment medium being closer to the optimal level for the culture. This would result in a better growth rate in the pre-treatment medium, which would carry through into the treatment medium.

It would be expected that if adenine were acting as a cytokinin or a cytokinin synergist there would be some noticeable differences in the pattern of shoot and node production as well as some effect on growth overall. This was not observed.

The results of this experiment, the discovery that adenine appears to affect growth because of its ability to act as an alternative nitrogen source, should perhaps not be surprising. Nitrogen comprises 34.6% of adenine-hemisulphate and thus is overall approximately 13.6% of the total nitrogen in the G5 medium, but falls to 7.3% in the G10 medium, where nitrogen is near or above optimum levels.

Fig 43

Effect of Adenine on Growth

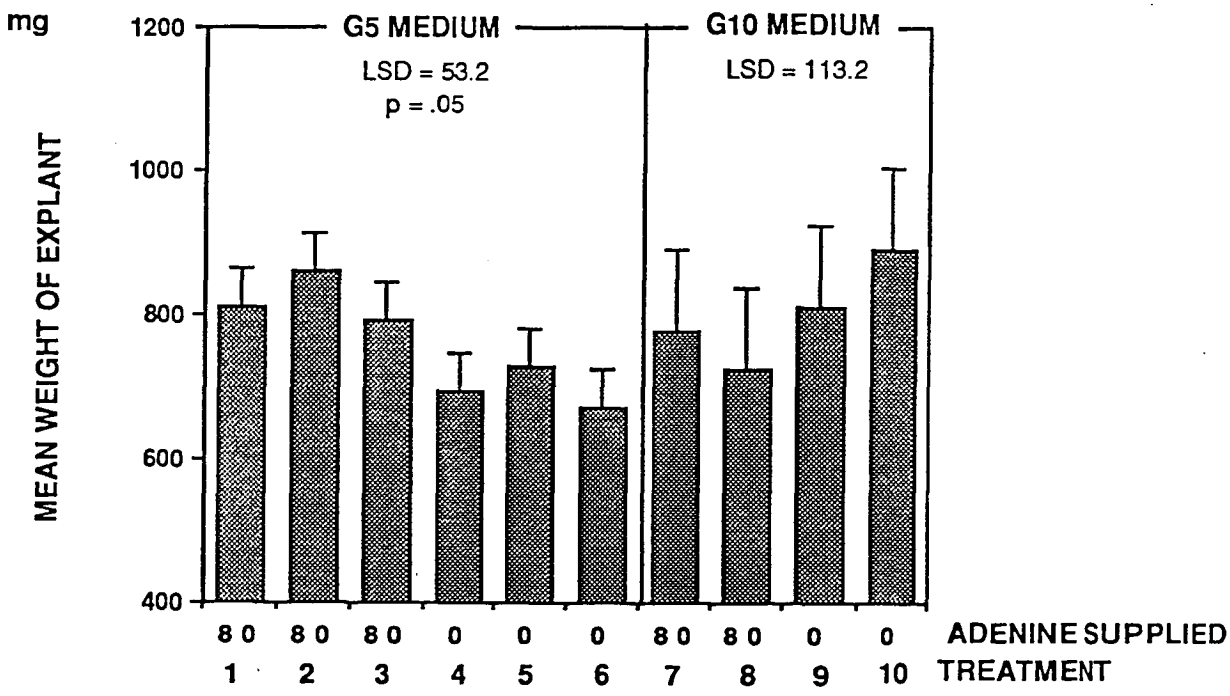


Fig 44 Effect of Adenine on Shoot Initiation

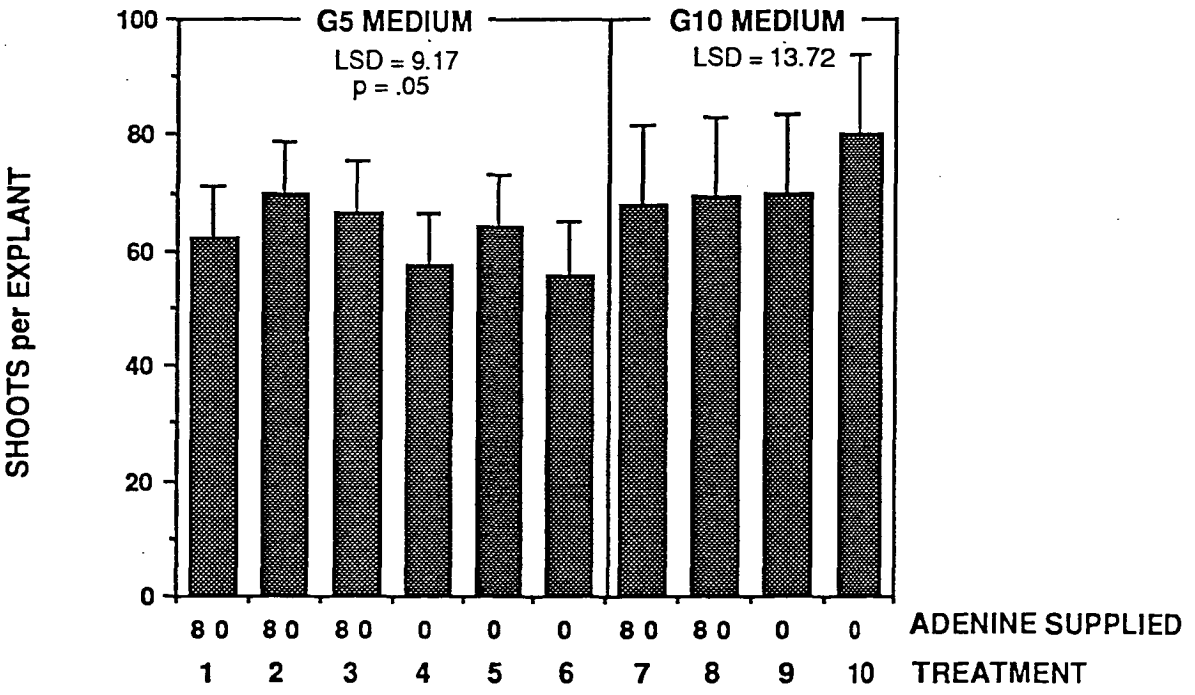
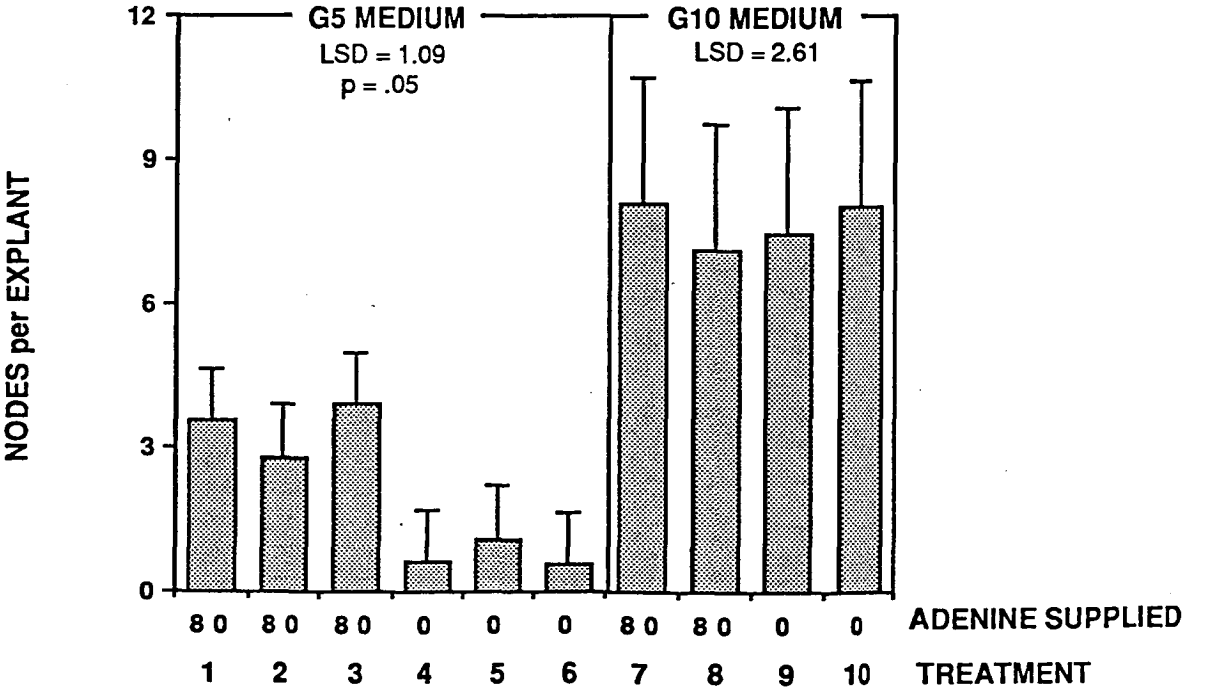


Fig 45 Effect of Adenine on Shoot Extension



In addition, it is in a form which can be readily absorbed and utilised by the plant. Adenine is commonly included in media and it is surprising that there is not more positive evidence of its importance to the cultures on which it is used.

Vitamins

Table 11

Effect of Different Vitamin Treatments on Growth

Treatment	Mean Weight (mg)	Std Dev
1	632	82.3
2	631	59.5
3	551	79.4
4	620	59.6
5	592	83.3

LSD (5% level) = 41.7

Only treatment three (Millers medium) is significantly different from the zero treatment, treatment 5. It is unexpected to find that treatment 3 has lower growth than the zero. None of the treatments vary much in quantitative terms. It would appear from these results that the vitamin mix of Millers medium is slightly inhibitory to boronia. However, it is difficult to identify how this might be occurring, especially since it only contains vitamin levels that are intermediate to those of other treatments. The growth obtained from the zero vitamin treatment is not significantly different from any of the other treatments.

This suggests that vitamins do not exert much effect on the boronia tissue cultures in these conditions and may not need to be included in routine media. It is, however, probably prudent to continue to include vitamins in the medium, to prevent the possibility of stressed or damaged explants becoming deficient in any of the vitamins.

Sucrose

The major difficulty in interpreting the results of this experiment is that an analysis of variance is again not applicable because of the wide range of standard deviations for individual treatment means. The highly variable nature of the data also has an effect on an attempt to fit regression equations to it.

Despite the difficulties imposed by the lack of an analysis of variance, a number of facts emerge. Sucrose levels quite obviously have a major effect on all measures of growth and development of the cultures. It would appear that there is a distinct optimum level for sucrose at about 30 g/L. At sucrose levels above this, growth and shoot initiation decline quite steadily, while shoot extension is affected more noticeably

The slight differences in growth between the two nitrogen levels need to be treated cautiously as they may not be statistically significant. However, the pattern of growth might suggest that increased nitrogen levels, to some

Fig 46 **Effect of Sucrose Concentration on Growth**

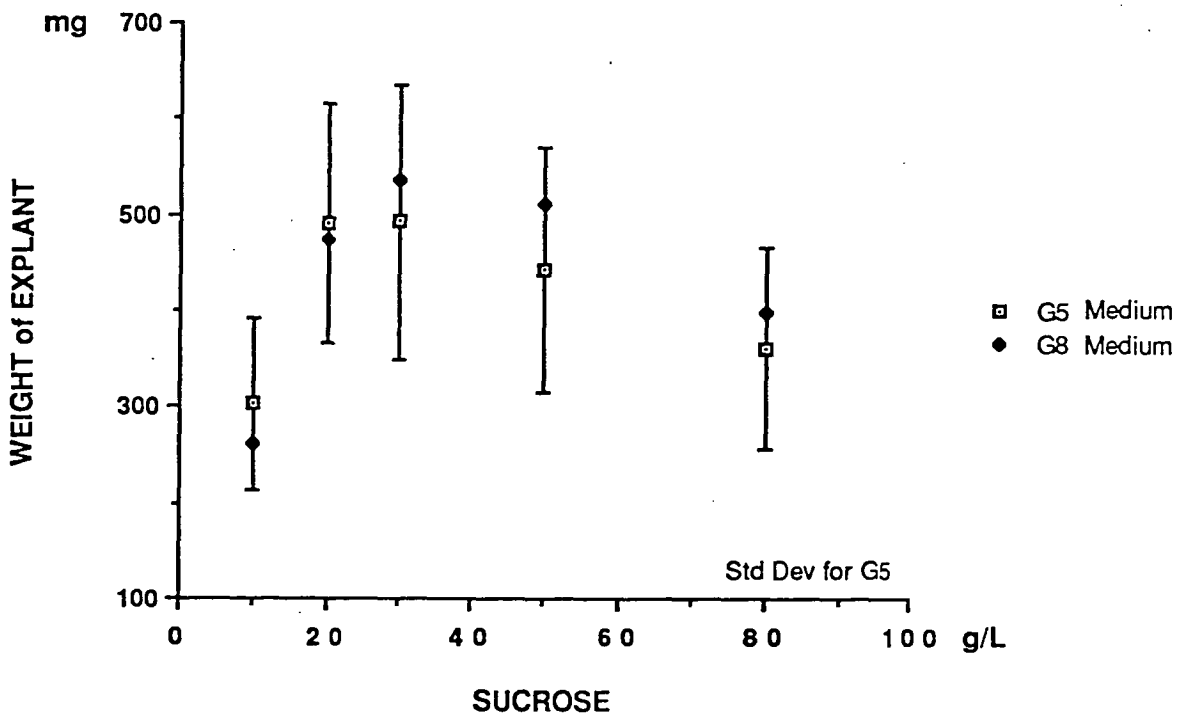


Fig 47

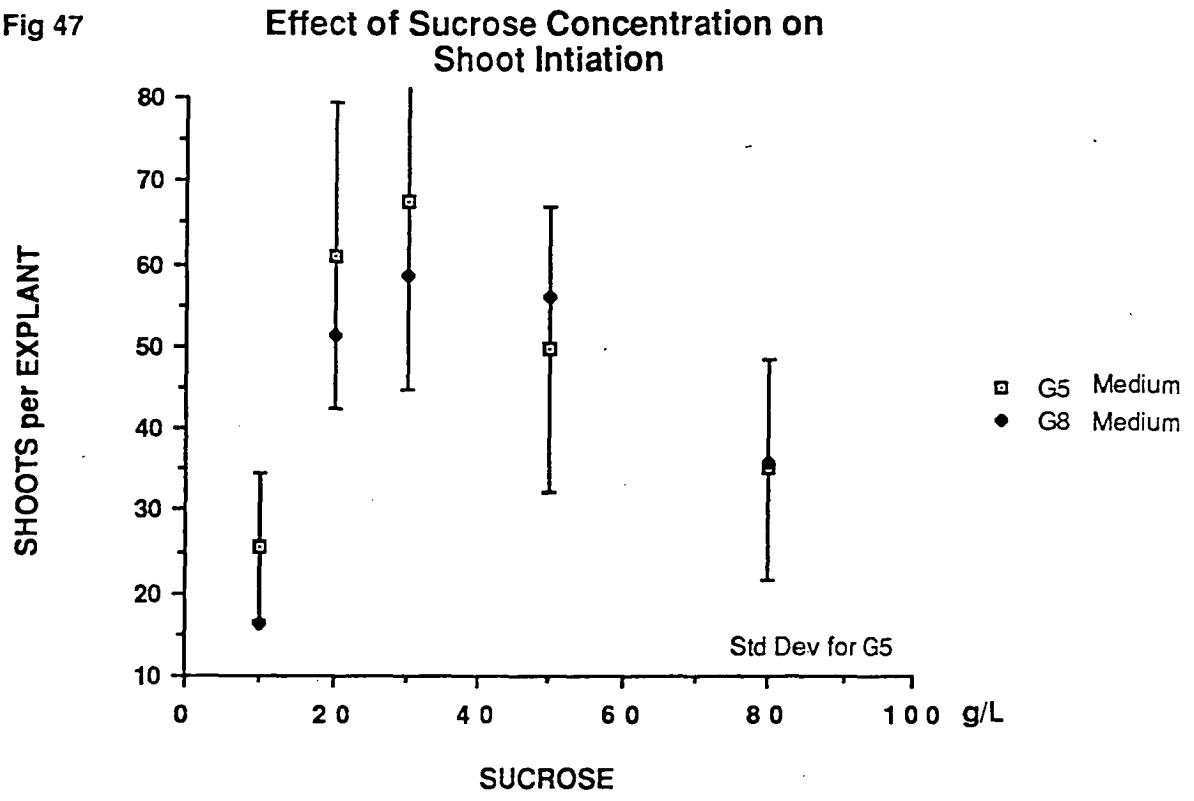
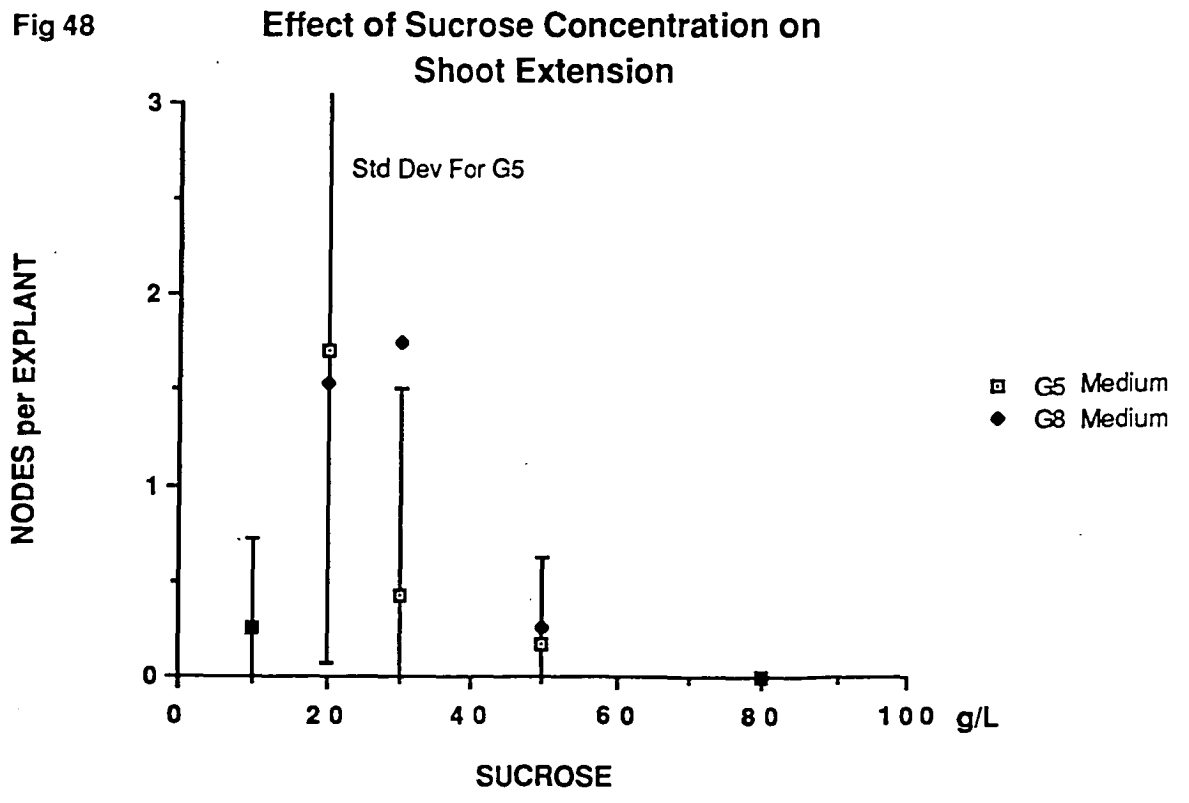

Effect of Sucrose Concentration on Shoot Intiation
SHOOTS per EXPLANT
80
70
60
50
40
30
20
10
Std Dev for G5
0
20
40
60
80
100
g/L
SUCROSE
G5 Medium
G8 Medium

Fig 48


Effect of Sucrose Concentration on Shoot Extension
NODES per EXPLANT
3
2
1
0
Std Dev For G5
0
20
40
60
80
100
g/L
SUCROSE
G5 Medium
G8 Medium

extent, negate the effect of high levels of sucrose. If this were the case, it would imply that part of the adverse effect of high sucrose is through increased osmotic potential of the medium decreasing the adsorption of nutrients.

Root Initiation Experiments

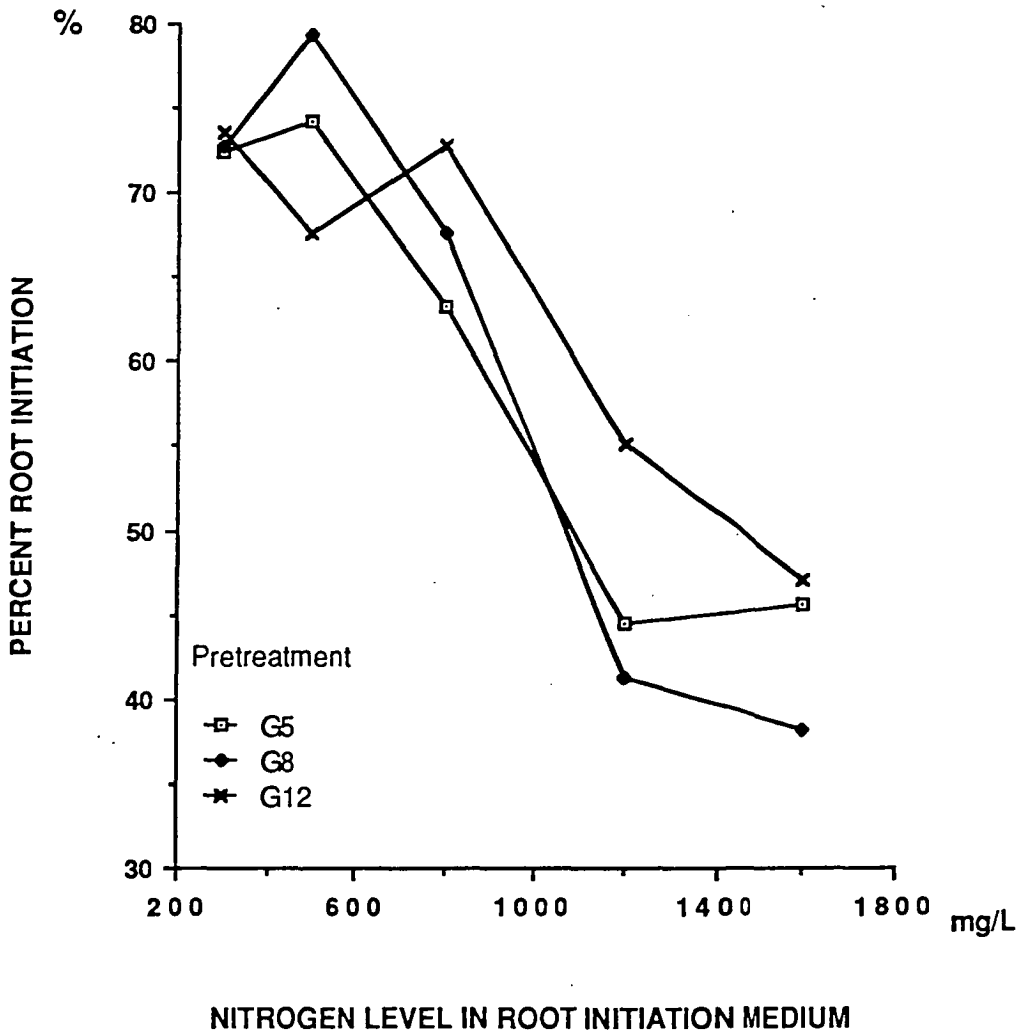
Root Initiation - Nitrogen Effects

Factors Affecting Root Initiation

As can be seen from Figure 49, the level of nitrogen in the pre-treatment medium appears to have had little effect on the rate at which explants subsequently initiate roots. This is somewhat surprising as it had been expected that the level of nitrogen in the shoots at the time of transfer to the root initiation medium would affect the vigour of the shoot and its ability to initiate roots in the flask. It is clear from this graph that the factor most influencing the rate of root initiation is the level of nitrogen in the root initiation medium.

Root initiation is adversely affected by high levels of nitrogen. This results in much lower numbers of explants having roots at the time of transfer to soil. It would appear that the higher level of nitrogen might be interfering with the balance of hormones in some manner, to prevent root initiation. Alternatively, the more active growth of shoots that results from high nitrogen levels might be preventing the diversion of "resources" to the base of the shoots where root initiation is occurring.

Fig 49 Effect of Nitrogen Level on Root Initiation



Factors Affecting Root Development After Transplanting to Soil.

The pre-treatment medium has clearly affected the development of roots after the plantlets have been transferred to soil, especially for those plantlets that have initiated roots prior to the transplantation. This is most readily seen in figure 50 which shows that plantlets which received a low nitrogen level in the pre-treatment medium had much slower root development after the root initiation phase. This effect is most obvious on R12 rooting medium (Fig 50) where, six weeks after the plantlets were planted in soil, 62% of plantlets that had G8 pretreatment were well developed while only 24% of plants from G5 pretreatment were well developed. The effect was also observed on R5 and R8 medium (Fig 51), but was less pronounced. On R3 and R16 medium the effect does not appear (Fig 52). The end result of this effect is that more plantlets are well rooted quicklyusing G8 medium followed by R12.

The results discussed above imply that the proliferation treatment used prior to root initiation affected how the plants developed, even though this pre-treatment had ceased 6 weeks before the plantlets were transferred to soil. This occurred even though the nitrogen level of the pre-treatment medium had no discernible effect on the rate of root initiation. In the best treatment (Fig 50), it can be seen that 100% of plants that initiated roots in culture had good root

Fig 50

Effect of Pre Treatment on Root Development
R12 Medium - Plantlets with Pre-Initiated Roots

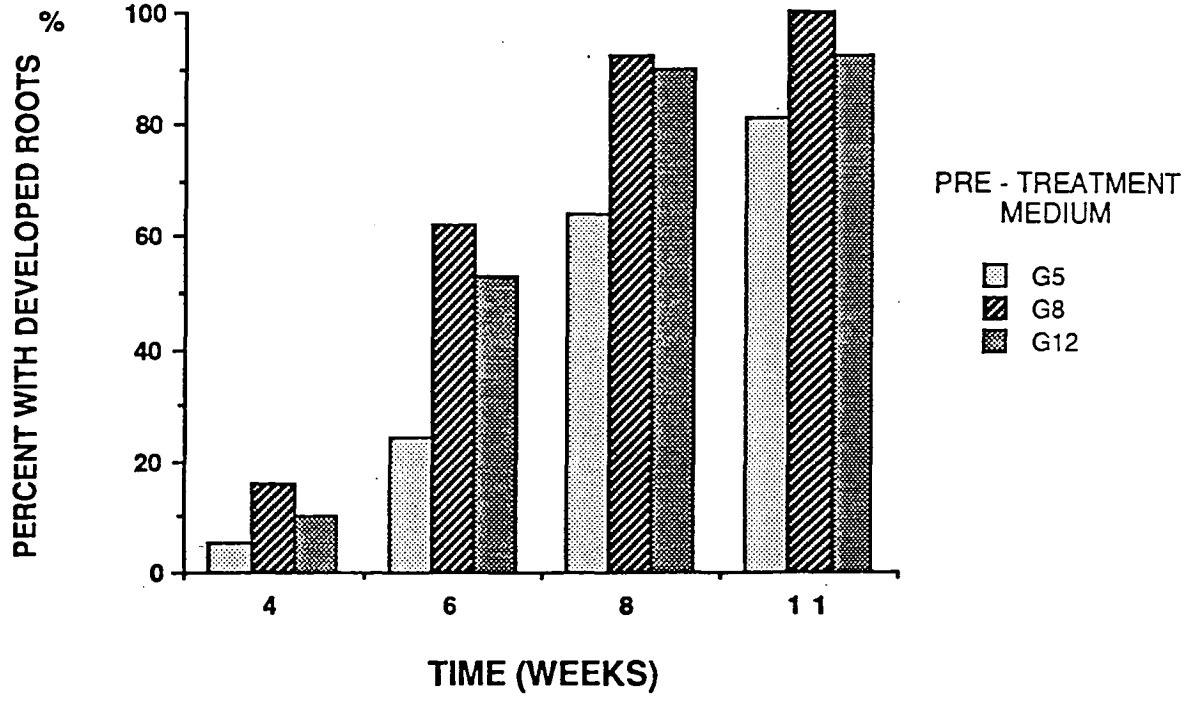
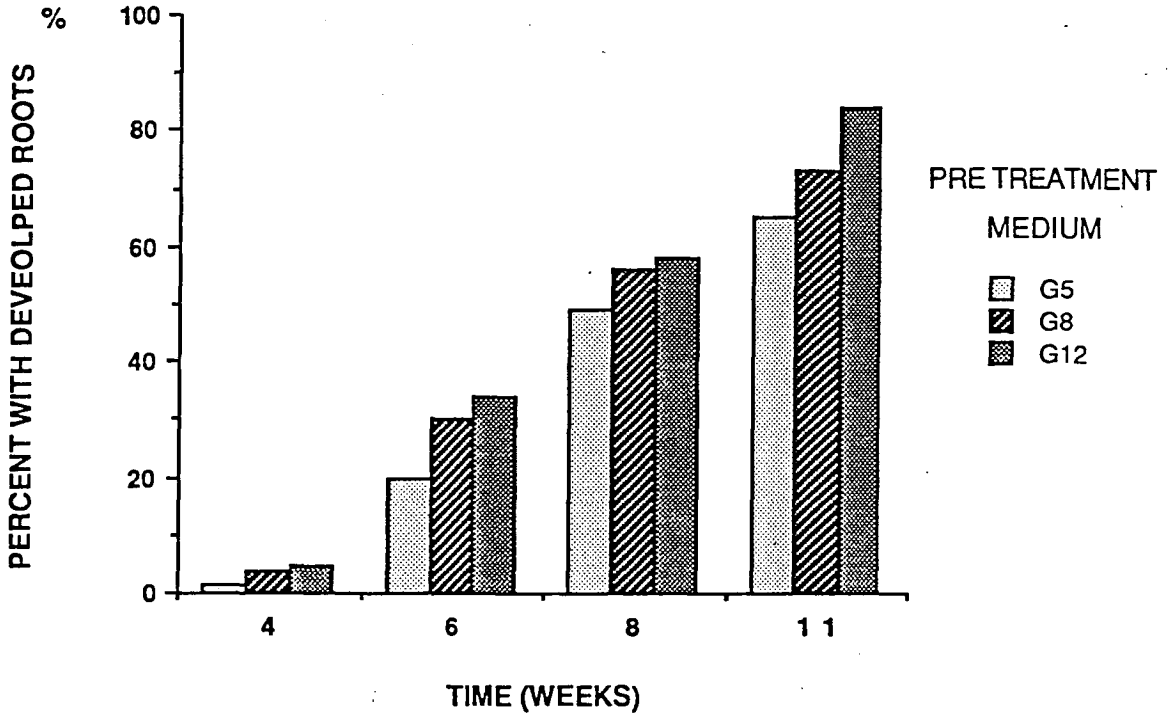


Fig 51

The Effect of Pre-Treatments on Root Development
R5 Medium - Plantlets With Pre-Initiated Roots



R8 Medium - Plantlets With Pre-Initiated Roots

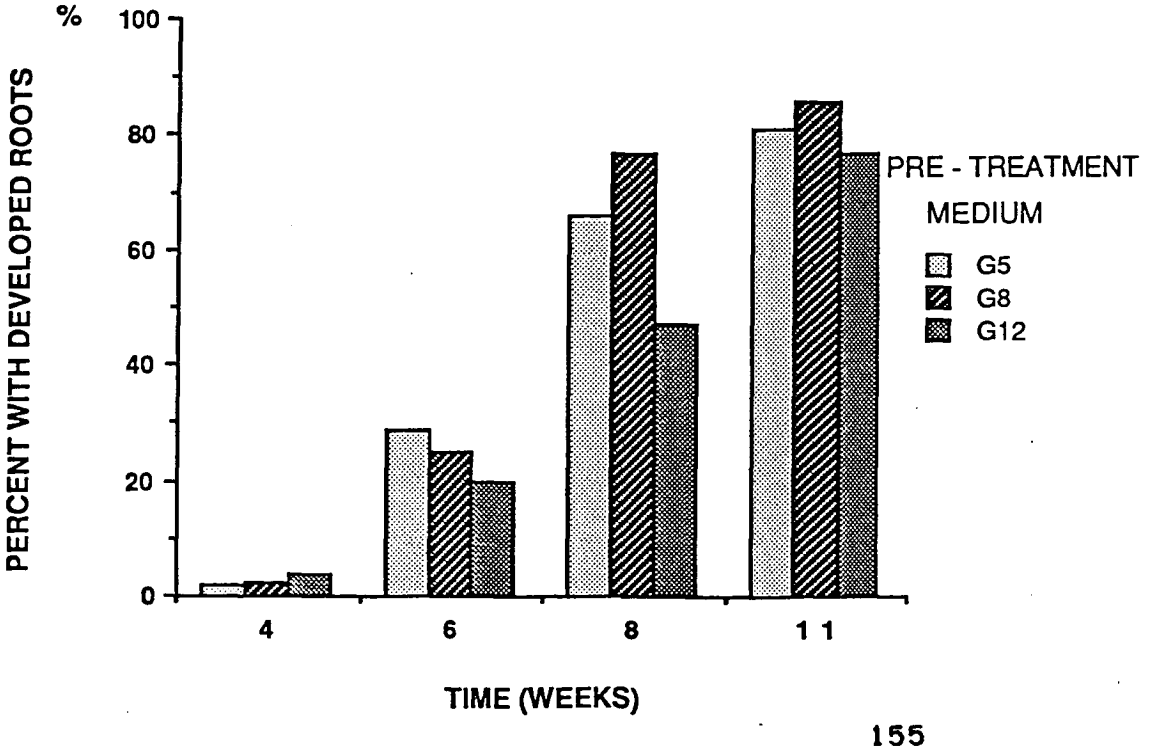
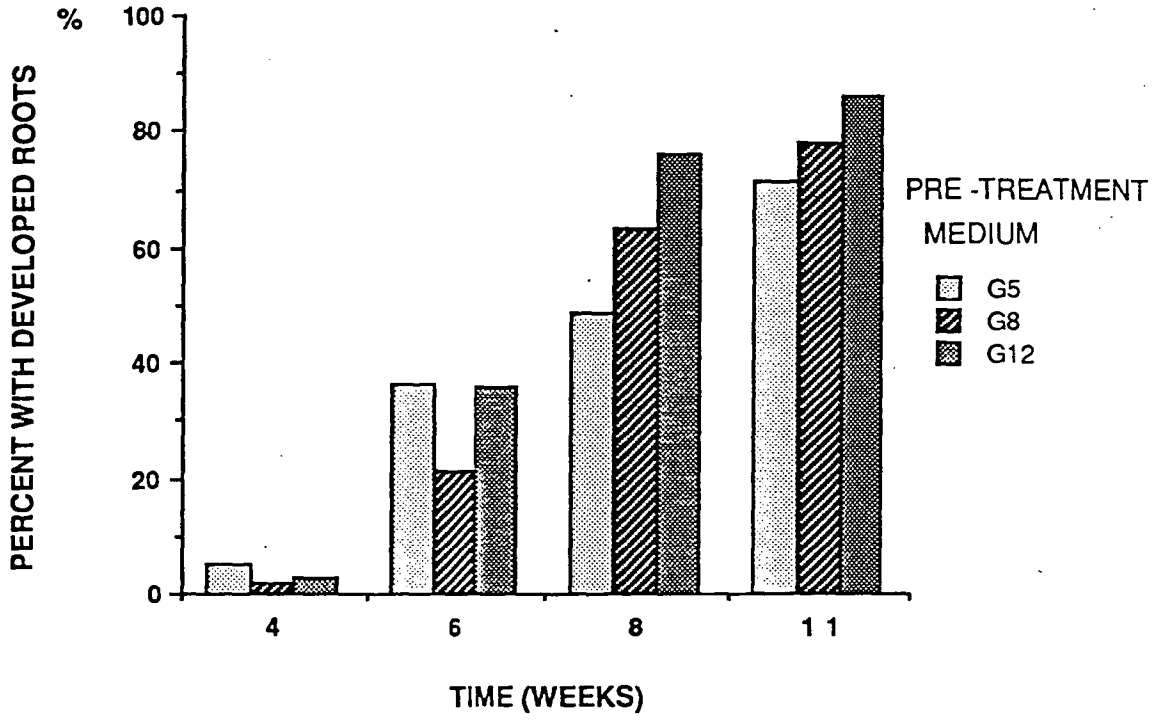


Fig 52

Effects of Pre Treatment on Root Development
R3 Medium - Plantlets With Pre-Initiated Roots



R16 Medium - Plantlets With Pre-Initiated Roots

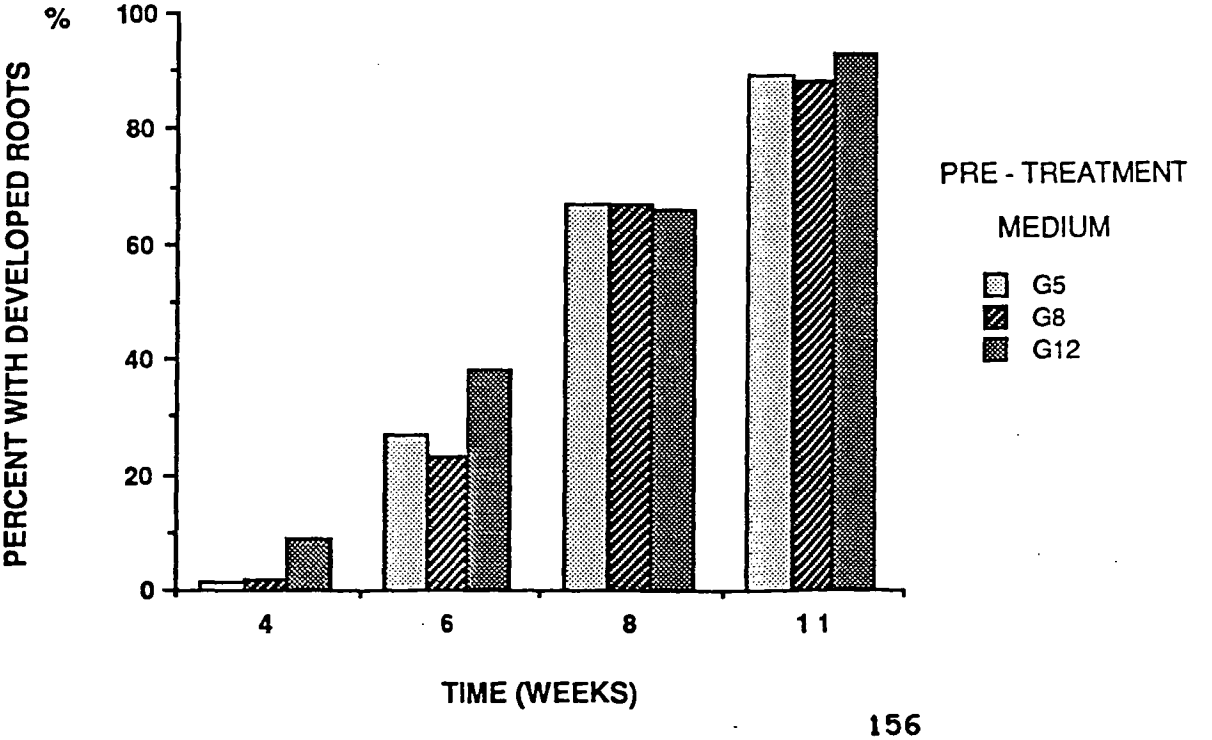
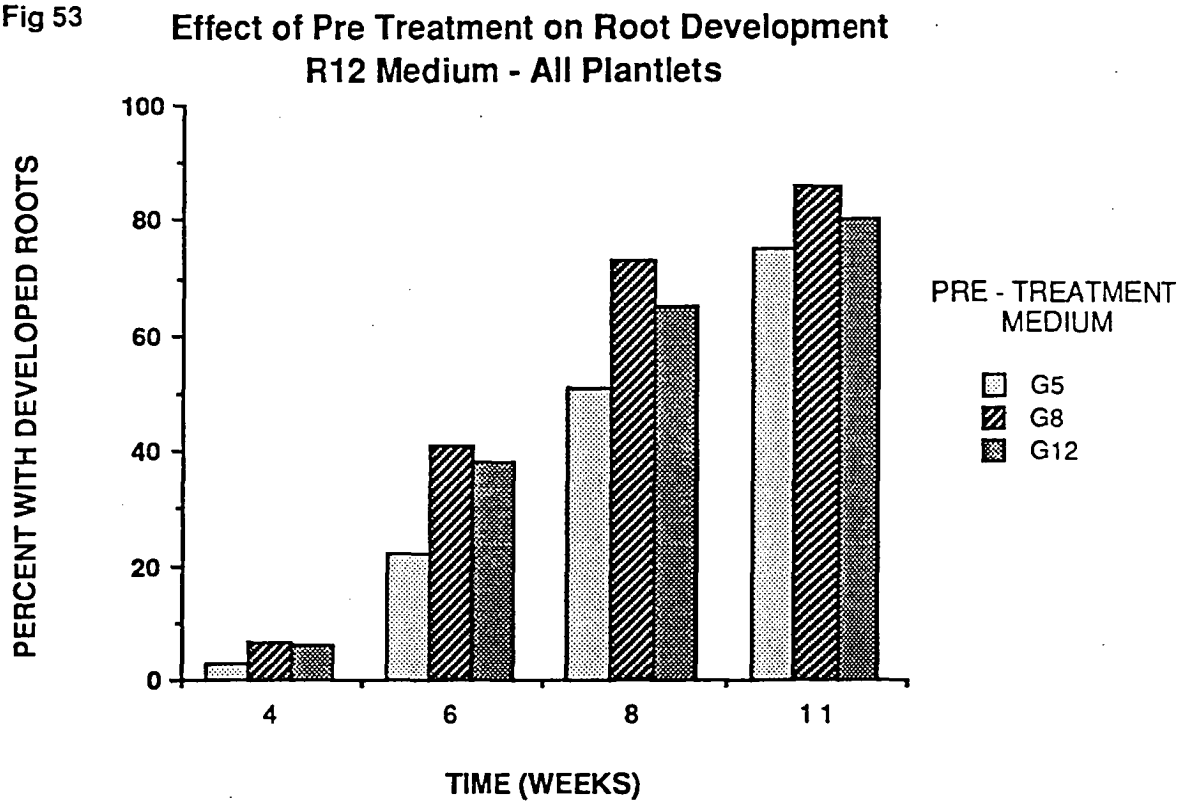


Fig 53


Effect of Pre Treatment on Root Development
R12 Medium - All Plantlets
PERCENT WITH DEVELOPED ROOTS
100
80
60
40
20
0
4
6
8
11
TIME (WEEKS)
PRE - TREATMENT MEDIUM
G5
G8
G12

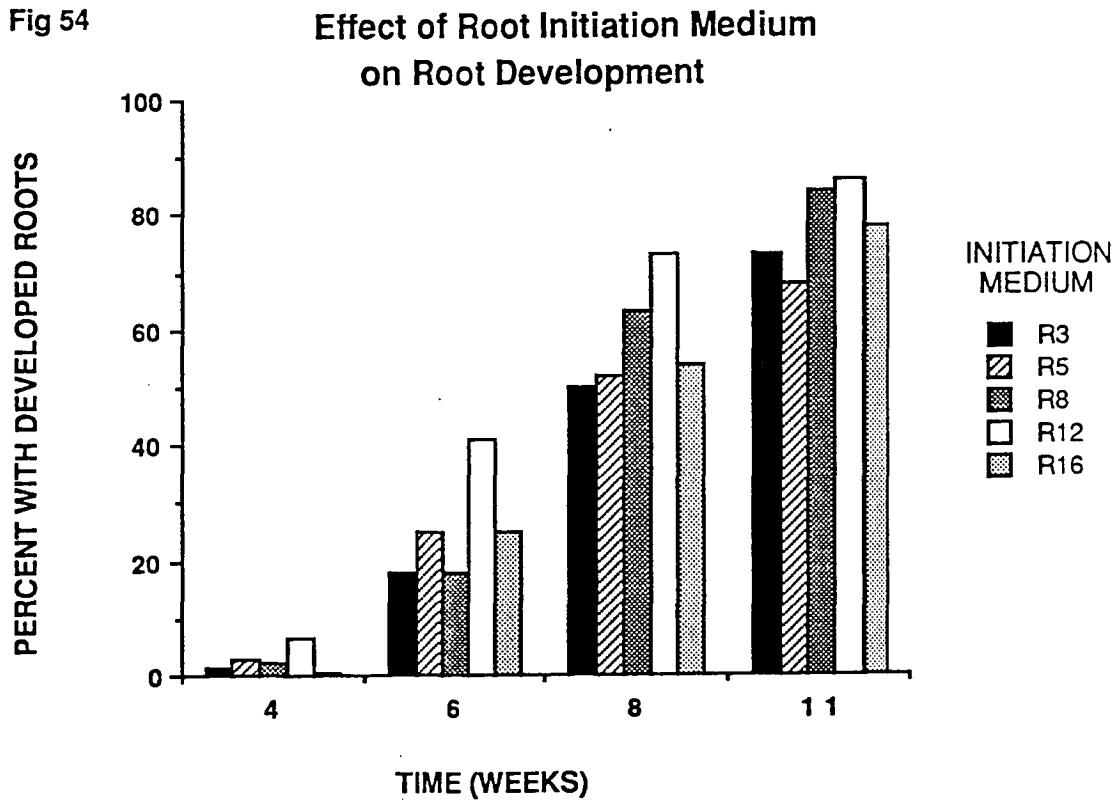
development after 11 weeks and 86% of all plants in that treatment (Fig 53).

The root initiation medium also appears to be exerting an effect on the subsequent development of the plants. It is perhaps less surprising that those plants on rooting media with high levels of nitrogen should develop faster than those plants that were on low nitrogen root initiation media. The latter would carry lower nitrogen reserves onto the mistbed. What is more significant is the fact that the low percentage of root initiation observed in the high nitrogen treatments did not prevent those plants from developing rapidly once transferred to the mistbed. The effect of this was such that, even though R12 and R16 media produced very much lower root initiation rates, they still produced more plants ready for transplantation at each time period (Fig 54). This means that lowering the nitrogen level in the root initiation medium to achieve a higher rate of root initiation will result in an overall slower production of plants because their roots are much slower to get established.

Drawbacks in the Design of This Experiment

During the progress of this experiment a couple of major problems were encountered with its design and execution. Probably most significant of these was the absence of replication during the post culture measurement. This is a result of the size of the Celpak trays, which

Fig 54


Effect of Root Initiation Medium
on Root Development
PERCENT WITH DEVELOPED ROOTS
100
80
60
40
20
0
4
6
8
11
TIME (WEEKS)
INITIATION
MEDIUM
R3
R5
R8
R12
R16

contain 200 plantlets each. To use whole trays as replicates for each of the 15 treatments would have required more plantlets than can be handled in a single experiment. A more reasonable approach would have been to use a split plot design.

A second major fault with the experiment was the failure to measure the height of plantlets resulting from the different treatments. Nitrogen levels are known to affect the rate of shoot extension and so the differences in height between nitrogen levels were quite expected. Regular measurement of plantlet height would have provided a further estimate of the rate of growth and development of plantlets.

This experiment suffered from a further difficulty in that the Celpak trays that were used to assess the extent and speed of root development were almost certainly responsible for obscuring some of the treatment effects. This occurred because the soil volume available to each plantlet was quite restricted, resulting in the larger plants running out of room very quickly. This gave the appearance that the growth of the plantlets from the low nitrogen treatment was catching up. The measure still has a great deal of use because, for practical purposes, the factor that is most important is the time taken for plants to reach a transplantable size, suitable for field planting.

Root Initiation Time

Table 12

Effect of Time of Culture on Root Initiation and Survival

	STARTING HEIGHT (mm)	HEIGHT AT WEEK 15 (mm)	% SURVIVAL
WEEK 4	16.1	29.1	92.3
SD	0.47	10.7	
WEEK 6	20.9	26.0	95.2
SD	0.55	10.0	
WEEK 8	27.7	18.7	85.1
SD	0.79	8.27	

NOTE - Measurement at week 15 means that explants planted at week 4 were measured 78 days after planting, week 6 plants 64 days after planting and week 8 plants 50 days after planting. This means that week 4 and week 6 plantlets were left under mistbed conditions significantly longer than would normally be the case, to give continuity of growth conditions and thus allow for better comparison with week 8 plants.

As might be expected, the plantlets continued to grow in the medium to week 8. However, increased size of plantlets at the time of planting in the soil did not result in larger plants after the plants were established in soil, nor did it improve the survival rate of plantlets. The decrease in size of week 8 plants at week 15 reflects the considerable susceptibility of these plants to damage due to desiccation of shoots. In some cases, this results in a considerably smaller plant. In other instances it appears

that the damage allows entry of pathogens and the plantlet dies. This desiccation effect was observed, to some extent, in week 6 plants but at the time of measurement the plants had recovered sufficiently for it to be no longer noticeable. The damage due to desiccation did not appear to be severe enough to affect the percentage survival of week 6 plantlets.

The differences in survival rate appear to suggest that the week 8 plants were too large and that the roots were not able to cope with the moisture stress experienced after removal from the flasks. The differences between survival rate for week 4 and week 6 plants are slight. Those differences may reflect the fact that some of the week 4 plants were still quite small and soft and were less able to withstand damage incurred during handling.

An important factor that is not reflected in this data is the differences in speed of handling of material of different sizes. It was found that the week 8 plantlets tended to have longer roots as well as longer shoots. This meant that the plants tended to be difficult to remove from the agar and were easily tangled and damaged when being planted into the Celpak trays.

Root Initiation Conditions

The significantly lower root initiation percentage measured for the treatments receiving some temperature variation was an unexpected side effect of the treatments.

This may reflect the effect that an increased transpiration load on the plant has on the ability of the plant to initiate roots. The differences in plantlet height, while significant, are slight. They are probably a reflection of the 30% less time those explants spent at higher temperatures that are more favourable to growth.

Table 13

Effect of Culture Conditions on Root Initiation and Survival.

	Treatments			
	T1	T2	T3	T4
% Root Initiation LSD = 7.21	58.5	49.4	49.7	44.6
Mean Height LSD = 3.12	23.5	18.5	19.6	20.7
% Losses at week 4	1.0	1.0	10.4	10.2
% Losses at week 6	23.9	22.0	65.0	50.0

The losses due to 'damping off' were very much higher than normally experienced and may reflect inadequate humidity control or generally unsatisfactory growing conditions. Such very high losses are well above acceptable levels and need to be regarded with some caution as they cannot be taken as an indication of the normal effects of these treatments. The fact that the two "pulse" treatments both gave lower survival rates than the constant temperature treatment would suggest that the effects of temperature are not solely related to humidity changes in the flasks. There

is no obvious reason why these two treatments should give such obviously poor survival rates.

Adenine in Rooting Media

The slight differences in the percentage root initiation and height for the two treatments is not statistically significant, indicating that adenine does not have any measurable effects on the growth or development of boronia on this rooting medium.

Table 14

Effects of Adenine on Root Initiation

	No. Measured	% Root Initiation	Mean Height of Plants
With Adenine	271	58.3	32
No Adenine	277	64.6	32

Sucrose Effects on Root Initiation.

The effects of increasing sucrose levels are quite obviously deleterious both on the rate of root initiation and on the size of the rooted plantlet. (Figs 55 & 56) The effect on shoot extension might reasonably have been expected given that increased sucrose levels had a similar effect in proliferation media. No conclusive evidence can be presented on the cause of the lower growth but the appearance of the explants might indicate that there was reduced nitrogen uptake associated with high sucrose levels. The explants on the high sucrose treatments were mostly quite yellow, with a tendency to develop purple on leaf and

Fig 55 Effect of Sucrose Concentration on Root Initiation

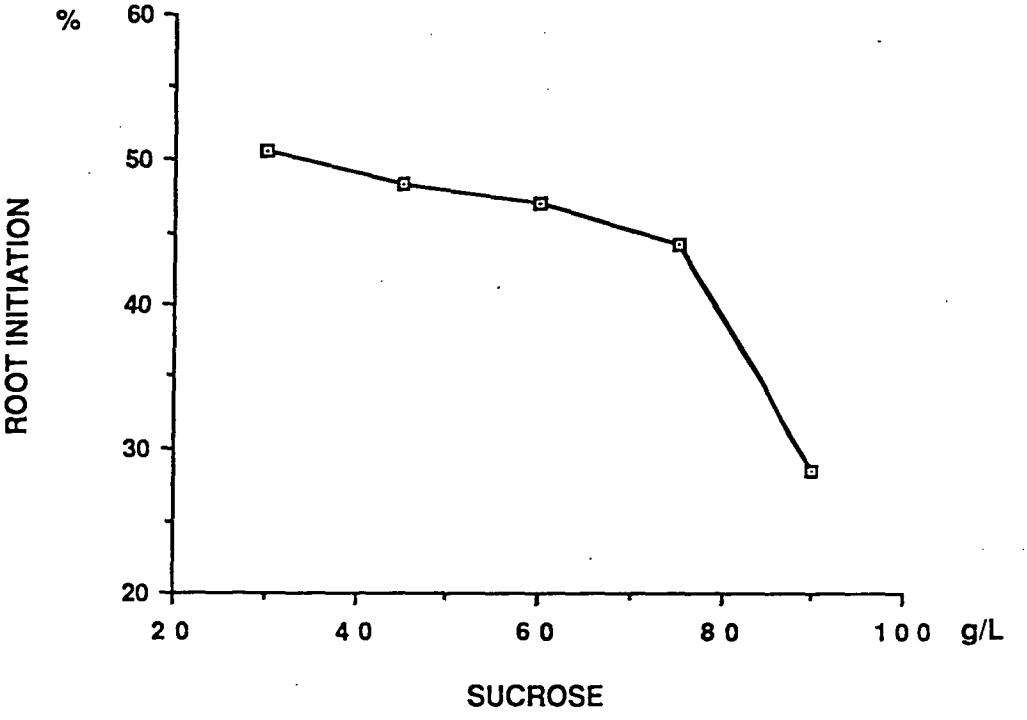
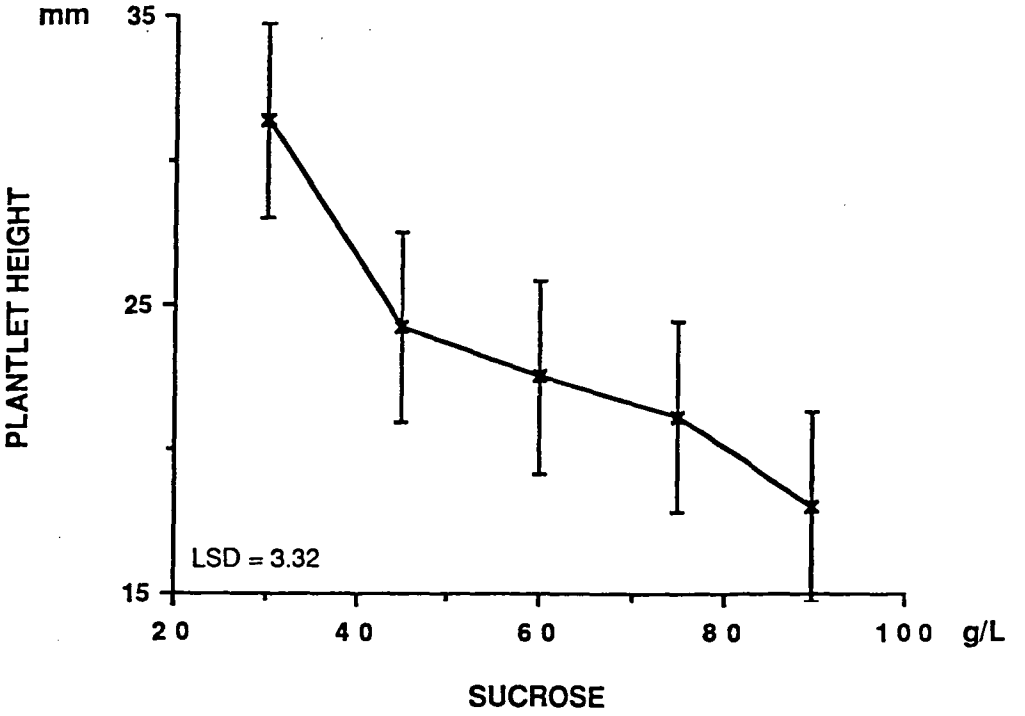


Fig 56 Effect of Sucrose Concentration on Plantlet Height



shoot tips. These are characteristics which have been observed in the past on explants grown on low nitrogen media.

Given the substantially lower growth caused by high sucrose levels it is probably not surprising that the rate of root initiation was also decreased. It had been noted, in previous work, that there was a tendency for proliferation cultures to form occasional roots on media with higher than normal, above 50 g/L, levels of sucrose (unpublished data). These results clearly indicate that this is unlikely to occur on root initiation media.

It should be noted that, in this experiment, there was a tendency to understate the height differences between treatments. This occurred when the explants were planted in the Celpak trays. There was a slight tendency to place tall plantlets into the soil a little deeper to ensure that they were stable in the soil and would not fall over. This means that when the height above soil was measured after planting, the height of such explants was understated slightly. It is quite likely that this effect occurred in other root initiation experiments.

V GENERAL DISCUSSION

Growth conditions and Measurement

The results of most of the experiments reported in this thesis have been obscured to a greater or lesser extent by the difficulties arising from large variations within treatments. This has been combined with the fact that, in many experiments, this variation is apparently distributed in association with treatments and not randomly distributed. Thus, the application of standard tests of the significance of the differences between means becomes very much more difficult. Particular emphasis has been directed towards attempting to identify and quantify the sources of this variation in the hope that it could be isolated from treatment effects. The results obtained have allowed some sources of variation to be eliminated from experiments and to increase the accuracy of measurements. The two areas of investigation have been: a) the extent of pH changes during media preparation, the causes of this change and its implications for growth of cultures and b) accurate preparation and measurement of growth in explants.

pH Variation

It is apparent that there are two types of pH effects occurring that need to be considered. First, there is the

pH change that occurs as a result of autoclaving. This may have a totally different effect on growth from the effect of the pH drop that results from using a high initial pH. The pH change that results from autoclaving signifies that some substantial, but as yet unidentified, chemical changes are occurring in the medium. In some cases, this change appears to have been ascribed to agar degradation (Harris and Stevenson 1982). This is quite understandable, as the most noticeable effect of the pH change is the decrease in the gel strength and darker appearance of the agar. This might be assumed to be the result of heat related breakdown of the agar. However, it has been shown that agar is not responsible for this pH change through the results of the 'Source of pH Variation' experiment. Consideration must therefore be given to the possible chemical reactions that might result in such a pH change and the consequence of those reactions for growth of the culture.

However, it can be assumed that the effects of these chemical reactions, if any, are not the only effects of the pH change, since the G8 pH-Response experiment showed some effect from different pHs, even though the pH changes associated with autoclaving are slight and mostly in the other direction. This last point also highlights the complexity of the reactions which must be occurring, since autoclaving under one set of conditions results in pH rising and autoclaving under very similar conditions but with a slower cooling phase results in a pH decline. Detailed

chemical analysis is needed to identify exactly what chemical reactions are taking place in the medium during autoclaving and the effects they have on the availability of nutrients in the medium.

The 'Buffer Trial' and 'G8 pH Response Trial' both showed that the pH of media does not necessarily have to decline during autoclaving. They would tend to indicate that the autoclaving temperature, in itself, is not the sole controlling factor affecting how the pH changes in the medium. A combination of the degree of heating and the duration of the period during which the media is heated would be more important. This suggests that the slow cooling cycle of large autoclaves is much more likely to result in large pH changes. The variations measured in the pH changes indicate that this cooling pattern is probably quite uneven. As it has been observed that autoclaves are still warm after 16 hours cooling during the summer, even factors such as the time of year the experiment is conducted could contribute to the pH changes.

The importance of specifying the pH of the medium after autoclaving is emphasised. If the results for the two pH experiments can be used as a guide to the effects of medium pH on the growth of explants, then this may be an explanation for the wide fluctuation in growth within individual treatments. It should be noted that the range of pHs encountered in the autoclave test would, on the basis of the pH effect in G8 media, result in approximately 50 %

difference in growth between the lowest and highest pHs encountered in the type 2 autoclave (range of pH 4.8 to 5.4). Such an extreme variation, applied randomly over a series of treatments, would be sufficient to obscure all but the most extreme treatment effects.

Consideration of possible mechanisms for the effect of pH on growth needs to take into account the fact that the pH of the medium does not stay at the same level for very long. The results of the buffer experiment (see Fig 37) showed that the pH in normal media drops quite quickly to the optimum level. By week 3 the pH has dropped quite substantially and so it must be assumed that much of the growth occurs after the pH of the medium has neared the optimum. So whatever effect media pH has on plant growth patterns is most probably occurring in the first couple of weeks of the culture cycle. Further work needs to be undertaken to try to identify exactly why the pH can have such an effect on the growth of cultures and to quantify this effect. It seems that use of buffers to hold the pH at a particular level is unlikely to offer information on this point because of the effects the buffers have on other factors affecting growth. One technique which may add to the information would be to grow the explants on a much larger aliquot of medium. This would mean that changes due to absorption of nutrients would have a much smaller effect on the pH of the medium and would allow it to stay near constant for a longer period.

Other Factors Increasing Within Treatment Variation

It is obvious that pH variation is not the only cause of variation. The error variance for the 'G8 pH Response Trial' was still large in an experiment where the pH variation within treatments was known to be very small. Physical factors, such as orientation, trimming, as it affects uniformity of initial explants, and pH, as it affects agar hardness, are other factors that may add to variation. Random effects, such as the cracking pattern of agar, would also make a contribution. This may be related to medium pH, since agar is significantly more gel like at pHs above 5.0. and much more prone to crack under pressure from growth and desiccation. Thus, contact between the explant and the agar may be decreased, especially in the later stages of growth, when the agar has begun to dry out. It is not clear how the mobility of media constituents might be affected by medium pH, although Aitken-Christie and Jones (1987) claim that agar does not interfere with diffusion of nutrients through media.

Accurate Growth Measurement

Another substantial difficulty encountered relates to the accurate measurement of growth. In most of the experiments, growth is measured purely with respect to mean explant weight at the conclusion of the experiment. No reference is made to the initial weight of the explant even though this has, in most cases, been measured. It has been

found that Relative Growth does not apply to the system under study because there is an inverse relationship between initial weight and Relative Growth. That is, explants with a low initial weight will tend to have a higher relative growth than explants of higher initial weight. The effect of this is that it tends to increase the variation within treatments. This increases the error variance and may cause treatment differences to be declared to be non-significant when, in fact, they are significant. Linear growth parameters such as % weight increase, are clearly not appropriate since they would have the effect of making the differences between the growth of small explants and large explants appear even greater.

It is possible to construct an Adjusted Relative Growth value which takes into account the non-linearity of the Relative Growth (R) measurement. By using the regression co-efficient from the regression of R against Ln initial weight (Ln W-0) it is possible to arrive at an adjusted Relative Growth, R(adj), such that the regression of R(adj) has a regression co-efficient of zero. That is, R(adj) is constant for all values of Ln W-0. In this case, such a regression, using the combined data of the G8 and G5 media, yields an equation of :

$$R(adj) = Ln\ (W-1) - 0..366\ Ln\ (W-0).$$

The difficulty with applying such an adjusted measure is that it is difficult to be certain under what conditions the relation will remain valid. If, as seems reasonable,

the surface area to volume ratio affects the rate at which the explant is able to absorb nutrients, then any treatment which changes the rate of absorption or availability of growth limiting nutrients may affect the relationship between initial size and the growth rate.

All three ways of measuring growth described above have a degree of inaccuracy or uncertainty associated with them. Weight has been used and continues to be used because it is the easiest to apply. Both Relative Growth and Adjusted Relative Growth are less readily associated with how the plant is actually growing, due to their having time as their only dimension.

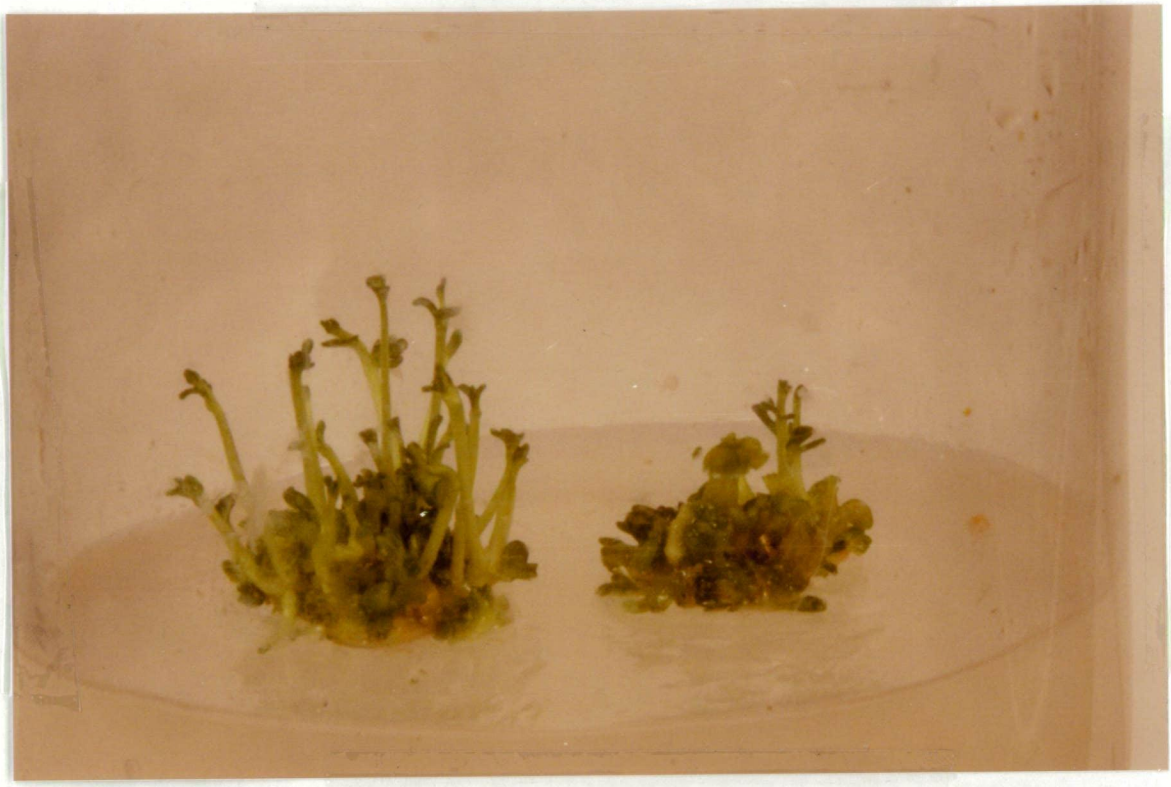
An alternative, to overcome the difficulty of initial size affecting growth rate, is to keep initial weights to as narrow a size range as is practically possible. The weights should also be kept as large as possible, since the effect of size is most noticeable at small sizes and decreases on a logarithmic scale. However, large explants do not grow as quickly and have the potential to have a much greater pre-treatment effect through such factors as the carry-over of nutrients. Experimental material was arbitrarily selected in the range 60 - 100 mg for most experiments, with the aim of giving a good balance between the competing needs. Further restricting the size range of initial explants would be desirable but presents practical difficulties. It has proved impossible to provide explants in sufficient numbers within a range smaller than 60 - 100 mg while relying on

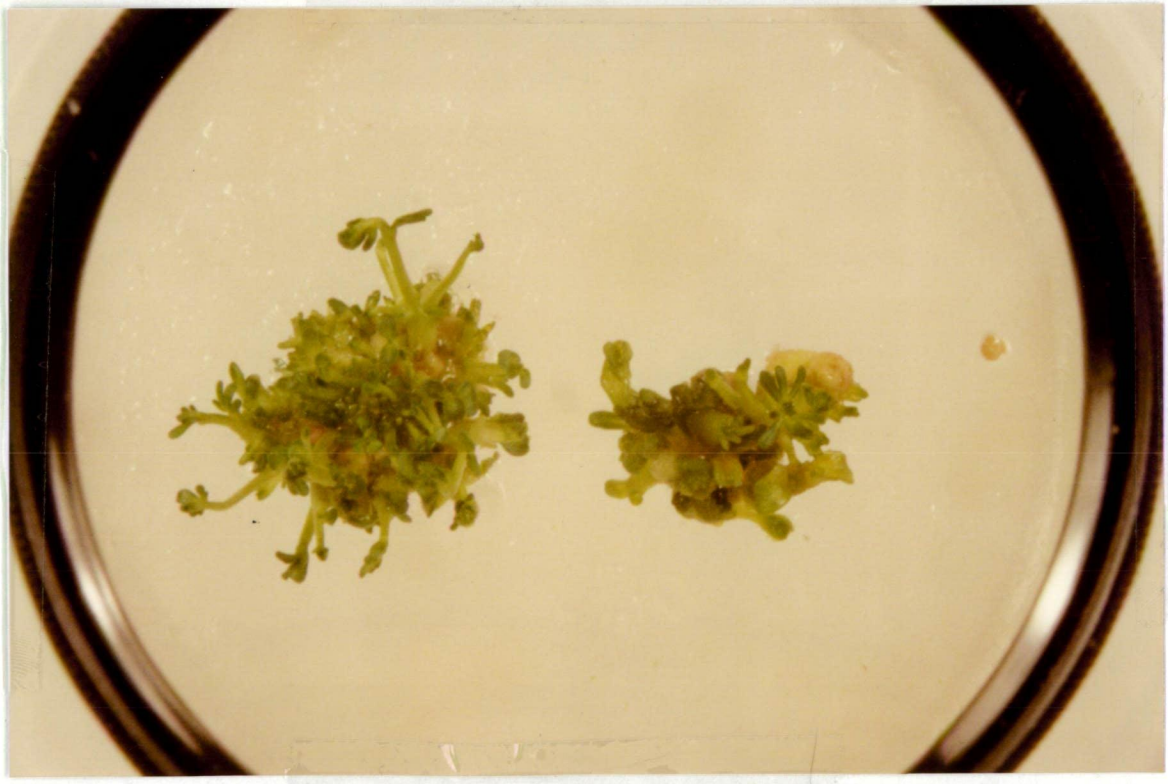
visual assessment to guide preparation of explants. Attempts to obtain a narrower range of initial weights tends to result in excessive mechanical and desiccation damage to explants. It also greatly extends the time needed to cut and weigh each explant.

In summary, using Relative Growth as a measure is technically incorrect in this case. However, not taking account of variation in starting weight is also obviously inaccurate.

Accurate measurement of growth is important to allow accurate measurement of the effects of different treatments. The problems caused by heterogeneous variance prevent the use of analysis of variance and multiple comparisons of means. One of the basic assumptions upon which the analysis of variance rests, that the experimental error will be homogeneous among the treatments, is not fulfilled. It may prove to be impossible to correct this problem until all the major factors causing the variation are identified and can be controlled or at least accounted for. Unfortunately, this may prove extremely difficult to achieve. Figure 57 shows the growth of two explants that were grown for five weeks in the same flask. Both explants were cut from a single proliferation, had the same initial weight, and were selected for their uniform appearance. Reasons why one should grow well, while the other becomes stunted and vitrified are difficult to discover and may prove impossible

Fig 57 Variation in growth under uniform conditions.
(Side and top view)





to isolate and eliminate. While such wide variation in growth rate can occur under identical conditions, it will be difficult to measure treatment effects with any greater accuracy than at present.

The measures selected to describe the growth and development of cultures have not proved entirely satisfactory. Shoot initiation is obviously an important selection criteria in developing commercial micropropagation media. However, in practical terms it has proved difficult to apply. Although intended to be used as objective measure of growth, the difficulty in providing a consistent definition of what constitutes a shoot leads to some difficulties. As the definition is to some extent subjective, there is the possibility of biassed measurement. If the experiment is measured blind, that is the experimenter does not know what treatment the individual explant belongs to, this possibility is negated to some extent. The other difficulty involved in this measure is the time required to make each measurement. This restricts the size of the experiment and the amount of replication that can be handled in one experiment. In many of the experiments there was little difference between the growth, as measured by weight, and the growth, as measured by shoot initiation. However, there were substantial differences in some experiments, most noticeably those involving different nitrogen levels. It is difficult to conceive of a way of

deciding which experiments are likely to require this time consuming measurement without actually counting the shoots.

Selection of Measurement Parameters

The ability to accurately determine the effect of media changes on resultant growth and development is of critical importance. Many of the changes that need to be studied are of a subtle nature but could be of some significance in determining the mode of action and the extent of interaction between different media constituents and conditions. Until the subtle changes in growth can be distinguished and quantified, it may not be possible to separate interactions from the main effects. This may give a misleading impression of the control of growth and morphogenesis in vitro.

The substantial changes in pH observed during autoclaving are an arguement in favour of eliminating from the medium ingredients, such as vitamins and adenine, for which there is no clearly established need. While the concentration of these compounds is low, and their direct contribution to changing the pH is unlikely to be great, it is certain that the contribution would be less if they were not included in the medium at all. This would not stop the pH changes from occurring but it would almost certainly make isolation of the key chemical changes a little easier.

Consideration should probably also be given to the role that myo-inositol is playing in proliferation and root initiation media. It was originally included in media developed for callus and suspension cultures. The role of all of these chemicals in cultures that have a greater resemblance to whole plants is by no means certain. On the other hand, if some other changes in media or growth conditions occurs then the absence of these compounds could easily result in much more stress being imposed on the plant than is presently the case.

Nutrition

Of the factors investigated, the one having the greatest effect on the growth and development of boronia in tissue culture has been found to be nitrogen. It has been found that the growth of cultures is influenced by both the level and the source of nitrogen. The effects of other factors have been found to be very slight, in the case of adenine and vitamins and closely linked with nitrogen nutrition, in the case of adenine and sucrose.

Despite the results of the experiments already discussed, there is still quite a lot of uncertainty about the extent to which factors such as media pH affect the results. Some of the experiments were conducted before the effects of pH on growth and development had been discovered. Thus, there is no information about the pH of the media in

these experiments, apart from the fact that the pH was set to 5.8 before autoclaving. Unfortunately, this is a measure which has been shown to be of limited use. The uncertainty raised by this problem apply particularly to the 'Nitrogen Trial', the 'Clone Trial' and the 'Sucrose Trial'.

Effects of Nitrogen on Growth

The results of the 'Nitrogen Trial' have some important implications for the design of a culture system. It is important that the optimal balance between proliferation and shoot extension is obtained for mass propagation. In micropropagation it is not just the proliferation rate that is important. The ease of handling shoots and the speed at which individual shoots can be dissected out to give a single stem plant after root initiation can have a substantial effect on efficiency. The fact that boronia changes its development pattern so markedly over a range of nitrogen levels, gives another potential method for obtaining the preferred growth pattern rather than adjusting the hormone levels, which may produce undesired effects. Instances where this type of influence over explant development in vitro have been found in other plants do not appear to have been reported in the past.

There are two possible ways in which this growth pattern might be occurring. The changes in level of available nitrogen in the medium may be influencing the rate at which other nutrients are being absorbed or may influence

the rate of absorption of exogenous hormones. The pattern of development obtained would suggest, that if this is the case, either the rate of absorption of IAA has been increased, causing less shoot proliferation and greater shoot extension, or the rate of absorption of BAP is decreased, to give the same effect. Alternatively, the changed pattern of growth may be the result of general increases in metabolic activity as the nitrogen level increases. The improved level of nitrogen within the plant tissues may be closer to that found in normal plants, allowing a growth and development pattern that is more like that encountered in vivo. Changes in the pattern of development of boronia associated with higher levels of nitogen nutrition were noted by Reddy (1987). It would probably be useful to look at the rate of absorption of exogenous hormones, to see what factors influence their absorption and the rate of metabolism within the plant.

Effect of Nitrogen on Different Clones

The apparently large differences in growth patterns observed between the 'Nitrogen Trial' and 'Clone Trial' (Figs 58, 59, 60) for clone HC 4 are difficult to explain. The fact that both experiments were carried out before the importance of pH measurement was known offers one possible explanation. In both experiments, the pH of the media were set at 5.8 before autoclaving. The nitrogen experiment was autoclaved in the 'Type 1' autoclave, while the clone trial

Fig 58 **Comparison of Growth in Nitrogen Trial and Clone Trial Weight**

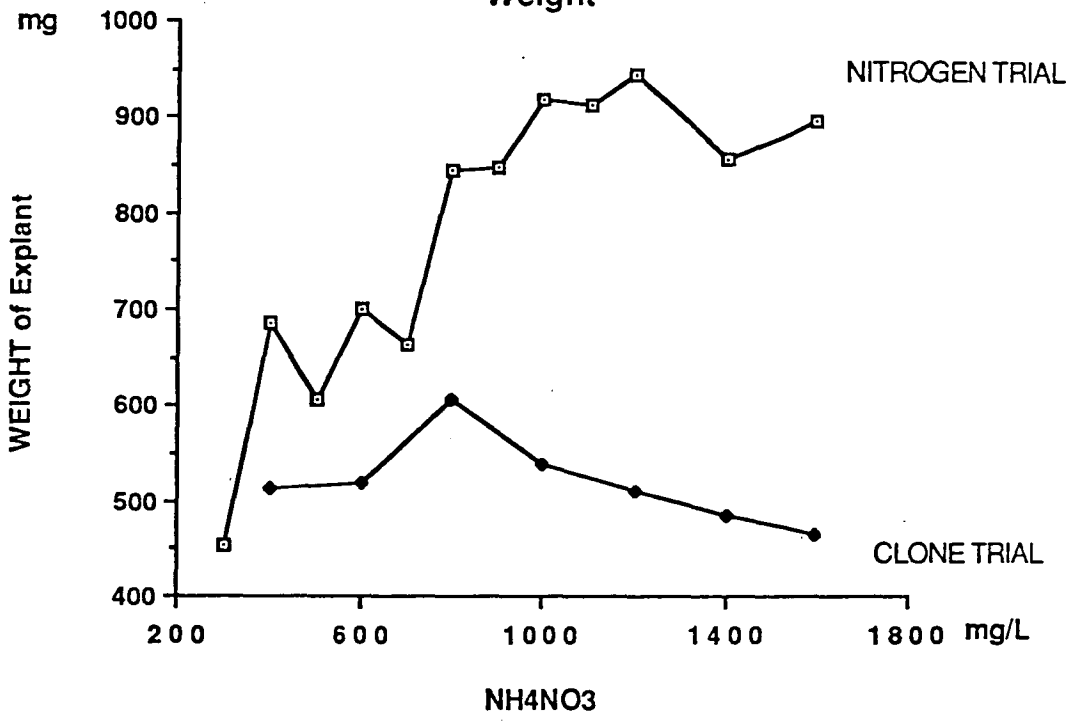


Fig 59

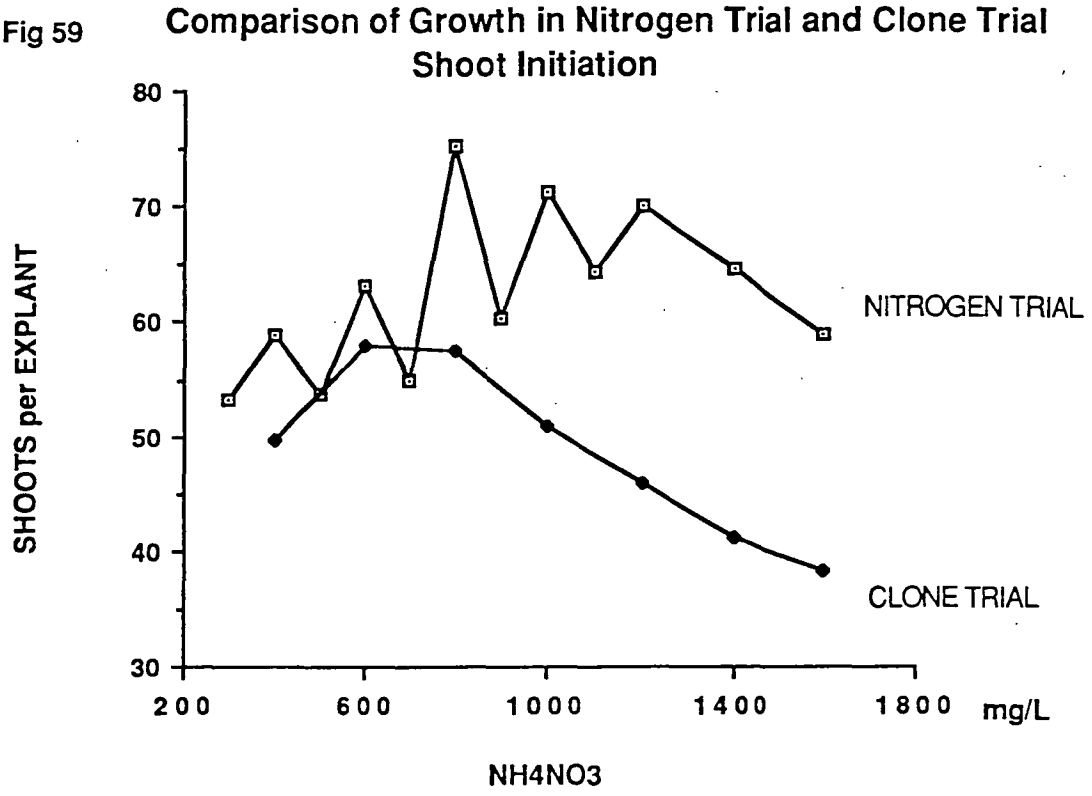
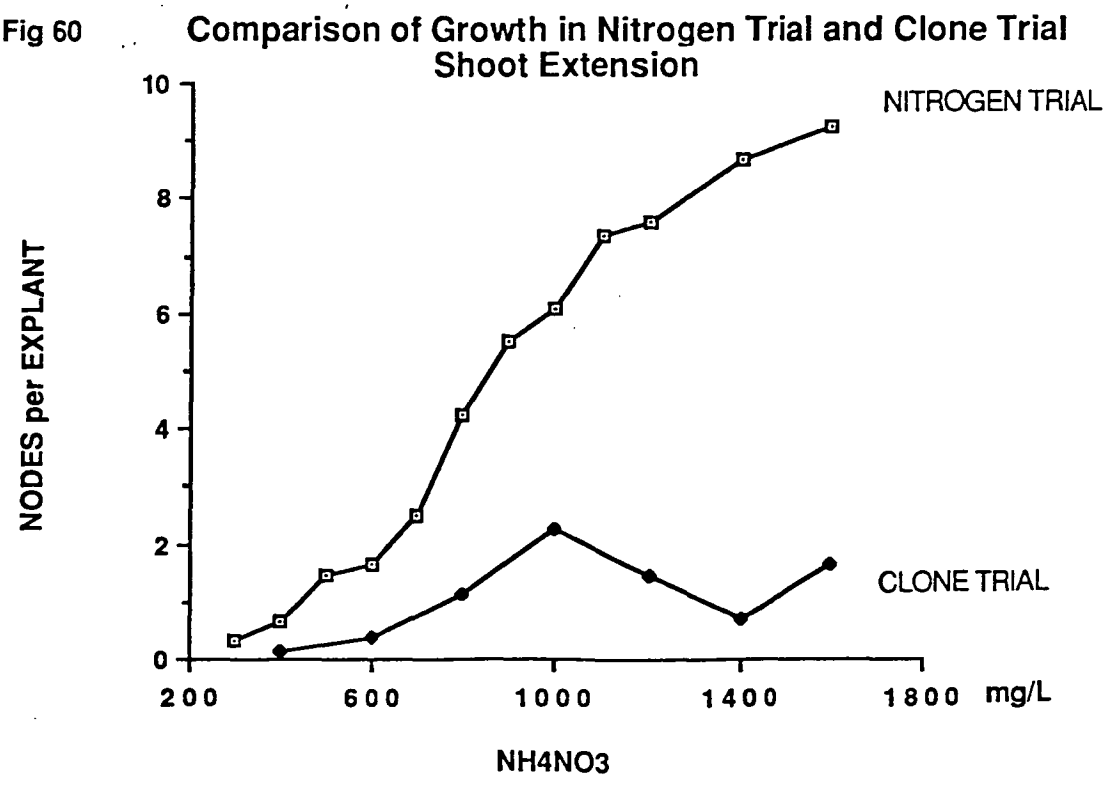

Comparison of Growth in Nitrogen Trial and Clone Trial
Shoot Initiation
SHOOTS per EXPLANT
80
70
60
50
40
30
NITROGEN TRIAL
CLONE TRIAL
200
600
1000
1400
1800
mg/L
NH4NO3

Fig 60


Comparison of Growth in Nitrogen Trial and Clone Trial
Shoot Extension
NODES per EXPLANT
10
8
6
4
2
0
NITROGEN TRIAL
CLONE TRIAL
200
600
1000
1400
1800
mg/L
NH4NO3

was autoclaved in the 'Type 2' autoclave. Without the benefit of post-autoclaving pH measurements it is impossible to be certain what range of initial pHs were presented to the explants in the two different experiments. If there were substantial differences in the two autoclave runs, there could easily be significant pH differences, which might account for the observed changes. Otherwise, it is very difficult to explain. This is unfortunate because it places some doubt over the extent to which the results for the other clones can be relied upon. Obtaining more data on the effect of media pH on growth at different nitrogen levels would clarify whether this is the cause of the discrepancy.

Nitrogen Sources and the Influence of Succinate Buffer

The response of the cultures to different initial pHs cannot be satisfactorily explained by the results of the 'Buffer Trial'. In fact, these results appear to be contradictory. It is known that when ammonium is the sole nitrogen source the pH drops sharply and that the pH rises when nitrate is the sole nitrogen source. It therefore seems reasonable to assume that a decline in the pH of the medium when both ammonium and nitrate are available indicates that more ammonium is being absorbed than nitrate. From the buffer experiment, it is clear that ammonium is readily used by the plant and that nitrate is used less readily, if at all. It would be expected from these

statements that a high initial pH of the medium, falling to the presumed optimum during the culture period, would facilitate the absorption of more ammonium than nitrate. This should lead to at least equal, if not faster, growth from those cultures with a high initial pH. This is the opposite of what has been measured. Further investigation is needed to reconcile this major contradiction. Possible mechanisms for the effect of initial pH have been discussed previously.

The other piece of contradictory information relates to the failure of the ammonium nitrate treatment to grow in the presence of buffer. It is difficult to suggest a reason why this has occurred since results of the ammonium plus buffer treatment show that succinate is not toxic to the plant. A possible explanation could be that the inclusion of succinate, for some reason, causes an increased uptake of nitrate, resulting in excess levels of nitrate in tissues during the early phase of growth. The trend of the pH for the NH4NO3 treatment was upwards, suggesting that this may have occurred. The growth and appearance of the explants was quite similar to that for the nitrate treatments, again suggesting that nitrate toxicity could be a factor. A reason for succinate having such an effect on the plant is much less readily apparent. There needs to be much more work done to look at the effect of succinate and other buffers on the growth of boronia and on the utilisation of ammonium and nitrate. Confirmation, or otherwise, of the

ability of the cultures to use nitrate under different circumstances is needed.

If, as appears probable, the boronia cultures do not use nitrate at all then this would allow for some very useful changes to be made to the standard methods of growing and measuring growth in plant suspension cultures. If nitrate is not being used then it would be reasonable to develop a liquid culture medium that omits this form of nitrogen. The difficulty of pH change that would arise can be quite readily be overcome by the addition of NH4OH, under the control of a pH stat system, as described by Martin, Rose and Hui (1977). This would allow the long term growth of cultures with ammonium as the sole nitrogen source, without the complication of adding buffers or other chemicals to counteract pH changes. The rate of growth in this system can be readily estimated from the amount of NH4OH added to the system to keep the pH of the medium constant. This eliminates any possible ill-effects resulting from nitrate or buffers being included in the medium. Unfortunately, this technique is less readily applicable to proliferation culture studies because of the difficulties of growing proliferations in liquid media, where vitrification can be a problem.

Interactions between Sucrose and Nitrogen Concentrations

The effects of sucrose on the growth and development of explants, in both proliferation media and root initiation media, suggest that the level of sucrose may be influencing growth rates by altering the availability of other nutrients in the medium. This would explain the apparent slight interaction between sucrose levels and nitrogen levels in this experiment. This would be entirely consistent with the idea that much of the effects related to sucrose levels in tissue culture media is related to the place of sucrose as the largest factor in controlling the osmotic potential of the medium. The difficulty in interpreting this experiment, caused by the high level of variation within treatments, makes confident prediction of the extent of this effect difficult. More information might be obtained from a similar experiment using a higher level of nitrogen for the second nitrogen treatment.

The results of both the adenine and vitamin experiments show the difficulties of adopting, without question, the media developed for other culture systems. It seems certain that vitamins are not fulfilling any major nutritional role in this culture system. However, the possibility that longer term culture might uncover some slight decrease in growth cannot be discounted.

In the case of adenine, it seems certain that the role claimed for adenine in other culture systems does not apply to this culture system. There is no reason to believe that

adenine is able to influence growth and development of the boronia cultures, apart from the effects it has as a source of nitrogen. As such, it does have the capacity to change slightly the balance of shoot initiation and extension.

In the cases of both vitamins and adenine, there are a large number of references indicating they have been included in media without evidence of having any beneficial effects. This is apparently done on the basis that they have been found to be useful in some other, completely unrelated, culture system, which happens to use the same medium.

Definition of Culture Media

One of the difficulties in studying plant tissue culture media, especially a medium low in salts, such as this one, is that the medium should not be thought of as having a constant composition. Over the duration of the growth cycle, substantial changes occur in the medium as large amounts of nutrients are absorbed by the plant cells. The concentration of the remaining nutrients is altered substantially by desiccation of the medium. The concentration of hormones in the medium is changed to an unknown extent by the combination of absorption by the explant and breakdown in the medium. All of these factors would go together to produce a medium which, at the end of the five week growth period, is substantially different from that to which the explants will be transferred to at the

start of the next culture cycle.

The explant is not being exposed to a medium with a constant concentration of nutrients, rather it is receiving a series of pulses of high nutrient and hormones, followed by periods of gradual decline in their concentration. The extent of the decline in nutrient concentration will partly depend on a number of factors. The ratio of explant to media volume, for example, will affect nutrient concentration, since a small explant on a large volume of media will absorb much less of the available nutrients. Other factors that will influence the nutrient concentration are the extent of desiccation that occurs in the growth cycle and the duration of the culture cycle. With these factors in mind, it is misleading to think of a culture medium purely in terms of the initial concentrations, although clearly these are important.

Depletion of exogenous hormones from media has been found to be a significant factor affecting development in other culture systems (Smulders, et al 1988). However, little research seems to have been conducted to see what effect this depletion of phytohormones has on the progress of growth and development of the culture during its growth phase. It would seem quite possible that maintenance of hormone concentrations at a constant level might result in a different growth pattern, particularly if the rate of utilisation or degradation of one hormone is greater than the other.

Desiccation of media is an area that has received very little attention but could be assumed to be having an opposite effect to the above factors. Experiments in this thesis have shown that increasing sucrose concentration has a substantial effect on growth and development. The extent of media desiccation was not measured in any of the experiments but could be expected to be substantial and probably sufficient to cause the sucrose concentration to rise during the culture period. Thus, any changes to growth conditions that affect the rate of desiccation of media (temperature, day length etc.) may result in growth changes due to a sucrose effect as well as the direct effects of the growth conditions.

In order to take account of all these potential interactions it is important to consider in vitro culture in terms of a whole system, including media, growth conditions and pH. When the effects of changes to one factor in the system are being contemplated the effects that the changes may have had on other growth determining factors need to be considered.

Root Initiation and Post Culture Survival

The primary objectives in attempting to initiate roots are to achieve the highest possible number of rooted plantlets in the shortest possible time and at the cheapest possible rate. This must be achieved whilst keeping in mind

the requirements for a healthy single stemmed plantlet. One of the objectives of this thesis was to attempt to identify the conditions that would improve the evenness of growth, provide single stemmed plantlets and improve the post culture survival. The evenness of growth is necessary to cut down the number of times plantlets need to be handled during transplantation and sorting and to minimise the amount of time the plantlets need to be kept under misting conditions.

Effects of Adenine

If adenine were exerting some effect as a source of cytokinin activity, it might have been expected that the influence would be observed in the root initiation medium, where explants are exposed to a much lower level of cytokinin. The lack of significant differences in root initiation rates suggest that there is no such effect and that adenine can be safely omitted from the culture medium without affecting growth in any material way.

Effects of Sucrose and its Interaction with Nitrogen

The effects of sucrose level on both the root initiation rate and on the height of explants at the time of transplantation suggest that there is some potential for improving both these factors through manipulation of the sucrose level. In particular, it would seem likely that there is justification for further investigation of the

effect of lower sucrose levels on the height of explants. Taller explants are easier to handle and root initiation rates may be improved as well. However, if the trend to increased height were to continue too much then post culture survival might be affected, through the plantlets becoming too tall and soft.

Further investigation of the effect of changed sucrose levels and nitrogen would be of benefit. It is not clear, from the results obtained so far, whether the effects observed for changes in sucrose level are the result of the effects of sucrose on the metabolism of the plant or as a result of changes to the osmotic potential of the medium. If the latter is the case, then it may prove possible to use changes in sucrose level to alter the effect of nitrogen levels. The high level of root initiation in R5 medium for example, might be able to be combined with increased plantlet height and vigour by lowering the sucrose level in this medium.

Effects of Nitrogen

The data obtained on the effect of nitrogen levels on root initiation and subsequent development is, to some extent, contradictory and confusing. There is no doubt that root initiation is antagonised by increased levels of nitrogen in the root initiation medium. This can probably be explained by the effects of nitrogen level on the growth and vigour of the plantlets and the effect this might have

on endogenous phytohormone levels and transport within the plant. However, if this is the case, it would also be expected that the root initiation rate would be affected by the level of nitrogen in the pre-treatment medium. This did not happen, which leads to the conclusion that the rate of absorption of exogenous phytohormones may be affected by the level of salts in the medium.

It is important to note that the low level of root initiation in those root initiation media with high nitrogen levels did not prevent those plants from developing roots rapidly after transplanting to soil. This probably reflects the fact that the plantlets had developed the capability to initiate roots while on the initiation medium, even though those roots did not become visible until after planting out. This could be because the conditions required to cause root initiation do not necessarily coincide with the conditions that allow development of the roots in culture. Provided the plantlets are sufficiently vigorous to survive well during the early stages after planting out, there may not be any disadvantage in having plantlets not initiate roots in culture. If, as happens with boronia, there is less than 100% root initiation, there can be extra sorting and handling required to manage plantlets with roots and those without. Rooted plantlets are generally harder to handle without damaging the roots. Any damage to the roots can result in the growth of the plantlets being checked substantially, adding further to the unevenness of growth of

the plants. If few of the plantlets have developed roots at the time of planting out, but have been exposed to initiation conditions sufficient to cause rooting in the soil, then it may prove simpler, and in the long run, more cost effective.

Effects of Culture Conditions

Some improvement in growth and health of cultures, due to the inclusion of a temperature differential in the growth cabinets, had been noted. Much of this improvement was attributed to a lessening of the appearance of shoot tip necrosis in large actively growing proliferations (unpublished data). The theories proposed by Debergh (1983) and others, suggest that lowering the humidity in the flask results in better development of the plant vascular system and epidermis. It is suggested that this will allow for better survival during post culture handling. It was expected that a temperature differential would affect plantlet survival by decreasing in-flask humidity. A short period of cool temperature should give a similar reduction in humidity and result in a similar survival rate. Similarly, it was expected that two periods of cool temperature would result in a longer period of low humidity and so give even better survival.

The failure of the 'Rooting Conditions' experiment to provide any improvement in the overall post culture survival of the plantlets is surprising. The low survival attained

in this experiment probably reflects the importance that post-culture conditions have for plantlet viability. If the conditions of the mistbed are not suitable for the growth of the plantlets, then no amount of pre-conditioning of the explants will prevent some losses from occurring. It is very difficult to propose a reason why the two treatments receiving short pulses of cool temperatures had such a poor survival. It would appear that something about this treatment made the plants more prone to fungal attack.

The failure to identify any positive benefits from temperature changes in this experiment should not necessarily lead to discarding the proposal that culture conditions have a significant effect on post culture survival. Clearly, some types of culture conditions have a very marked effect on survival. The reasons for this negative effect need to be identified, as the factors may be occurring, to a lesser extent, under normal treatments. If culture conditions do have the potential to alter humidity within the flask then they also have the ability to alter the pattern of absorption of those parts of the culture medium that are normally absorbed passively. This would occur by altering the rate at which water is absorbed by the plantlets.

VI. CONCLUSIONS

The results of the experiments in this thesis are in many cases inconclusive. This does not prevent those results from providing a useful basis for the successful micropropagation of boronia. In addition they will allow future research to be directed at particular problems with a greater degree of confidence in the techniques being employed.

In particular, they have established some of the techniques and growth parameters that can be used to accurately measure the effects of changes to media and growth conditions. They provide a basis upon which research can be undertaken to apply some of the more difficult and potentially beneficial in vitro culture techniques for the benefit of boronia production, disease control and plant improvement. An understanding has been gained of the factors which have the most influence over the success, or otherwise, of cultures. This will be of particular use in the establishment of suspension cultures and the regeneration of plants from these cell suspensions.

Results from a number of experiments have led to the possibility that the rate of exogenous phytohormone absorption is being affected by other media factors. This has been proposed to explain :-

a) The changes to proliferation rates caused by increased levels of nitrogen and sucrose.

b) Changes in growth pattern as a result of initial media pH.

c) The effect of both nitrogen and sucrose levels on root initiation.

In all three areas, the hypothesis is entirely speculative. However, the use of sensitive assays to measure the rate of removal of hormones from the medium and the fate of these compounds in the explant may go some way towards explaining the observed effects. It would also provide a greater understanding of the ways in which plant tissue cultures are controlled.

There is still room for improvement on the rate of micropropagation. Particular emphasis needs to be placed on increasing the rate of root initiation and survival of tissue culture produced plants, whilst decreasing the amount of labour required to produce the plants. The results of the experiments conducted so far have provided important information about the techniques that can be employed to make this a commercially viable method of plant propagation.

The task of gaining an understanding of the growth and development of boronia in tissue culture is by no means complete. However, the results obtained so far will provide a sound starting point for future research and points to a number of areas where future research may be of particular use.

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APPENDIX

Summary of Data for Experiments

Data Summary - pH Response

- G5 Medium

	WT-0 MEAN	WT-0 STD DEV	WT-1 MEAN	WT-1 STD DEV	SHOOT MEAN	SHOOT STD DEV	NODES MEAN	NODES STD DEV	PH START MEAN	PH START STD DEV
1	51.000	11.192	721.00	193.44	69.950	25.607	2.5000	2.3731	4.4600	0.0001
2	53.500	12.258	731.50	132.72	64.200	15.956	3.2500	1.8602	4.6500	0.0014
3	53.500	9.881	780.00	106.67	67.800	21.267	4.4000	4.0833	4.7100	0.0001
4	58.000	13.611	665.50	133.59	51.250	16.444	4.6000	3.2347	4.9000	0.0014
5	53.500	11.821	661.50	148.23	58.350	17.878	3.5500	1.9595	4.9200	0.0010
6	54.000	12.732	690.50	156.62	61.450	29.765	4.1500	2.6413	5.2600	0.0010
7	53.000	11.743	581.00	211.46	52.150	26.988	5.3500	4.7270	5.7500	0.0000
ALL	53.786	11.842	690.14	165.68	60.736	23.090	3.9714	3.2028	4.9500	0.4027

- G8 Medium

	pH MEAN	W-0 MEAN	W-0 STD DEV	W-1 MEAN	W-1 STD DEV	SHOOTS MEAN	SHOOTS STD DEV	NODES MEAN	NODES STD DEV	pH-final MEAN	pH-final STD DEV
1	4.1800	75.909	11.816	795.45	205.56	58.000	20.573	2.6364	2.1279	4.4777	0.2127
2	4.4700	80.455	13.620	875.45	169.19	61.409	12.250	4.2273	1.6015	4.4055	0.0884
3	4.6400	77.273	11.205	802.27	203.35	51.136	17.294	3.0909	2.5431	4.4423	0.0990
4	4.8800	75.455	11.434	843.18	155.91	53.273	15.166	4.0000	3.2805	4.4555	0.0875
5	5.0500	80.455	13.965	594.74	147.81	42.842	11.102	3.5789	2.3645	4.4368	0.1783
6	5.1700	78.182	10.970	798.18	173.94	48.636	12.625	4.1364	2.3563	4.5814	0.2018
7	5.5400	76.364	12.927	597.27	151.73	44.318	15.047	4.0909	3.2354	4.4427	0.0986
ALL	4.8471	77.727	12.235	761.32	200.42	51.543	16.201	3.6821	2.5778	4.4636	0.1535

Analysis of Variance - pH Response G5 Medium

ANALYSIS OF VARIANCE ON WT-1

SOURCE	DF	SS	MS	F	p
TRMT	6	481537	80256	3.20	0.006
ERROR	133	3334060	25068		
TOTAL	139	3815597			

LEVEL	N	MEAN	STDEV
1	20	721.0	193.4
2	20	731.5	132.7
3	20	780.0	106.7
4	20	665.5	133.6
5	20	661.5	148.2
6	20	690.5	156.6
7	20	581.0	211.5

POOLED STDEV = 158.3

ANALYSIS OF VARIANCE ON SHOOT

SOURCE	DF	SS	MS	F	p
TRMT	6	6334	1056	2.07	0.061
ERROR	133	67771	510		
TOTAL	139	74105			

LEVEL	N	MEAN	STDEV
1	20	69.95	25.61
2	20	64.20	15.96
3	20	67.80	21.27
4	20	51.25	16.44
5	20	58.35	17.88
6	20	61.45	29.76
7	20	52.15	26.99

POOLED STDEV = 22.57

ANALYSIS OF VARIANCE ON NODES

SOURCE	DF	SS	MS	F	p
TRMT	6	107.49	17.91	1.81	0.102
ERROR	133	1318.40	9.91		
TOTAL	139	1425.89			

LEVEL	N	MEAN	STDEV
1	20	2.500	2.373
2	20	3.250	1.860
3	20	4.400	4.083
4	20	4.600	3.235
5	20	3.550	1.959
6	20	4.150	2.641
7	20	5.350	4.727

POOLED STDEV = 3.148

Analysis of Variance - pH Response G8 Medium

ANALYSIS OF VARIANCE ON W-1

SOURCE	DF	SS	MS	F	p
TREAT	6	1645743	274290	9.02	0.000
ERROR	144	4379391	30412		
TOTAL	150	6025135			

LEVEL	N	MEAN	STDEV
1	22	795.5	205.6
2	22	875.5	169.2
3	22	802.3	203.4
4	22	843.2	155.9
5	19	594.7	147.8
6	22	798.2	173.9
7	22	597.3	151.7

POOLED STDEV = 174.4

ANALYSIS OF VARIANCE ON SH OTS.

SOURCE	DF	SS	MS	F	p
TREAT	6	5901	983	4.23	0.001
ERROR	144	33471	232		
TOTAL	150	39371			

LEVEL	N	MEAN	STDEV
1	22	58.00	20.57
2	22	61.41	12.25
3	22	51.14	17.29
4	22	53.27	15.17
5	19	42.84	11.10
6	22	48.64	12.62
7	22	44.32	15.05

POOLED STDEV = 15.25

ANALYSIS OF VARIANCE ON NODES

SOURCE	DF	SS	MS	F	p
TREAT	6	48.93	8.15	1.24	0.290
ERROR	144	947.81	6.58		
TOTAL	150	996.74			

LEVEL	N	MEAN	STDEV
1	22	2.636	2.128
2	22	4.227	1.602
3	22	3.091	2.543
4	22	4.000	3.281
5	19	3.579	2.364
6	22	4.136	2.356
7	22	4.091	3.235

POOLED STDEV = 2.566

Data Summary - Trimming Experiment

	N-LEV MEAN	TRIM MEAN	W1 MEAN	W1 STD DEV	W2 MEAN	W2 STD DEV	SH N MEAN	SH N STD DEV	NN MEAN	NN STD DEV
1	1.0000	1.0000	74.000	12.732	559.00	58.03	54.500	14.540	2.950	1.669
2	1.0000	2.0000	62.000	12.397	526.00	92.70	48.400	15.916	1.600	1.429
3	2.0000	1.0000	58.500	16.311	672.00	120.33	69.650	21.787	3.950	2.235
4	2.0000	2.0000	56.000	12.312	657.50	114.24	57.750	15.687	1.750	1.333
5	3.0000	1.0000	45.500	9.987	517.00	153.56	60.250	19.947	16.450	6.924
6	3.0000	2.0000	57.000	13.018	564.00	229.01	58.850	30.555	5.350	4.004
ALL	2.0000	1.5000	58.833	15.239	582.58	148.81	58.233	21.051	5.342	6.214

Data Summary - Growth Period Experiment

	FR-WT MEAN	FR-WT STD DEV	DR-WT MEAN	DR-WT STD DEV	SHOOTS MEAN	SHOOTS STD DEV	NODES MEAN	NODES STD DEV
0	220.4	56.1	25.29	6.96	22.33	6.66	1.292	1.334
1	271.2	46.4	30.12	6.05	29.87	10.82	1.917	1.283
2	405.4	63.0	40.58	7.34	40.29	10.06	4.167	2.316
3	590.8	160.1	56.83	11.73	50.00	19.87	11.792	3.575
4	696.2	145.8	69.12	13.13	68.33	22.89	15.417	6.691
5	945.0	194.7	96.25	19.74	101.92	25.81	27.417	11.470
6	1035.8	268.7	112.75	24.89	114.21	26.18	35.542	11.190
7	1052.9	258.6	120.58	24.93	114.17	39.67	37.708	13.235
8	1093.7	254.7	123.96	22.61	126.96	46.85	36.875	16.477
9	1051.7	177.0	132.04	18.85	125.83	44.09	40.875	12.909
ALL	736.3	373.7	80.75	42.67	79.39	48.55	21.300	18.002

Data Summary - Trimming Experiment

	N-LEV MEAN	TRIM MEAN	W1 MEAN	W1 STD DEV	W2 MEAN	W2 STD DEV	SH N MEAN	SH N STD DEV	NN MEAN	NN STD DEV
1	1.0000	1.0000	74.000	12.732	559.00	58.03	54.500	14.540	2.950	1.669
2	1.0000	2.0000	62.000	12.397	526.00	92.70	48.400	15.916	1.600	1.429
3	2.0000	1.0000	58.500	16.311	672.00	120.33	69.650	21.787	3.950	2.235
4	2.0000	2.0000	56.000	12.312	657.50	114.24	57.750	15.687	1.750	1.333
5	3.0000	1.0000	45.500	9.987	517.00	153.56	60.250	19.947	16.450	6.924
6	3.0000	2.0000	57.000	13.018	564.00	229.01	58.850	30.555	5.350	4.004
ALL	2.0000	1.5000	58.833	15.239	582.58	148.81	58.233	21.051	5.342	6.214

Data Summary - Growth Period Experiment

	FR-WT MEAN	FR-WT STD DEV	DR-WT MEAN	DR-WT STD DEV	SHOOTS MEAN	SHOOTS STD DEV	NODES MEAN	NODES STD DEV
0	220.4	56.1	25.29	6.96	22.33	6.66	1.292	1.334
1	271.2	46.4	30.12	6.05	29.87	10.82	1.917	1.283
2	405.4	63.0	40.58	7.34	40.29	10.06	4.167	2.316
3	590.8	160.1	56.83	11.73	50.00	19.87	11.792	3.575
4	696.2	145.8	69.12	13.13	68.33	22.89	15.417	6.691
5	945.0	194.7	96.25	19.74	101.92	25.81	27.417	11.470
6	1035.8	268.7	112.75	24.89	114.21	26.18	35.542	11.190
7	1052.9	258.6	120.58	24.93	114.17	39.67	37.708	13.235
8	1093.7	254.7	123.96	22.61	126.96	46.85	36.875	16.477
9	1051.7	177.0	132.04	18.85	125.83	44.09	40.875	12.909
ALL	736.3	373.7	80.75	42.67	79.39	48.55	21.300	18.002

Data Summary - Nitrogen Trial Week 5

	W5 - W0 MEAN	W5 - W0 STD DEV	W5 - W5 MEAN	W5 - W5 STD DEV	W5 - SH MEAN	W5 - SH STD DEV	W5 - NN MEAN	W5 - NN STD DEV
300	87.000	12.183	454.0	68.8	53.200	18.718	0.3500	0.6708
400	86.000	15.009	686.5	109.6	58.900	19.555	0.6500	0.9881
500	84.000	14.654	607.0	136.8	53.700	22.683	1.4500	1.4318
600	87.000	12.183	701.5	102.0	63.200	20.847	1.6500	1.1367
700	82.000	13.219	662.0	111.5	55.050	18.147	2.5000	1.9331
800	86.500	14.244	843.0	93.9	75.300	19.312	4.2500	2.6926
900	85.000	17.014	846.0	199.3	60.300	26.811	5.5000	2.6258
1000	85.000	15.728	917.5	168.0	71.300	24.251	6.1000	3.1772
1100	81.500	15.652	913.5	212.8	64.350	23.965	7.3500	4.0429
1200	86.000	14.290	943.5	316.6	70.000	23.434	7.6000	5.0928
1400	85.000	11.002	856.0	352.8	64.700	34.050	8.7000	5.2626
1600	87.000	11.286	896.5	259.8	58.900	19.555	9.2500	5.3398
ALL	85.167	13.782	777.2	242.9	62.408	23.492	4.6125	4.4773

Nitrogen Trial Week 10

	W10- W5 MEAN	W10- W5 STD DEV	W10- W10 MEAN	W10- W10 STD DEV	W10 - SH MEAN	W10 - SH STD DEV	W10 - NN MEAN	W10 - NN STD DEV
300	75.000	13.572	474.50	45.82	53.30	15.54	0.250	0.444
400	84.000	16.351	522.50	45.87	62.55	17.45	0.900	1.021
500	68.000	7.678	610.50	32.68	76.65	16.46	2.050	1.099
600	72.000	11.050	672.00	48.19	63.90	16.05	1.550	1.317
700	81.000	12.524	718.00	183.18	54.05	18.52	3.400	1.875
800	86.000	15.009	857.00	100.48	68.85	23.91	4.650	2.231
900	79.000	10.208	865.00	104.00	64.90	16.90	8.500	4.785
1000	76.000	10.954	920.00	87.84	84.35	15.75	9.200	4.514
1100	90.000	12.978	984.00	67.07	107.40	35.76	3.950	3.284
1200	86.500	12.258	1028.00	114.92	114.50	28.45	9.100	6.357
1400	86.000	12.732	827.00	227.48	79.05	25.73	21.900	10.568
1600	89.500	12.344	844.50	219.56	77.85	24.83	24.750	12.451
ALL	81.083	13.981	776.92	209.42	75.61	28.41	7.517	9.442

Data summary - Clone Trial Clone HC 4

	W-0 MEAN	W-0 STD DEV	W-1 MEAN	W-1 STD DEV	SHOOTS MEAN	SHOOTS STD DEV	NODES MEAN	NODES STD DEV
1	76.667	17.182	514.00	85.34	49.667	14.690	0.1333	0.3519
2	76.667	17.995	578.00	99.87	58.000	19.332	0.4000	0.8281
3	78.667	14.573	608.67	145.94	57.600	16.194	1.1333	1.3558
4	77.333	15.337	538.00	147.42	50.933	20.869	2.2667	2.3135
5	72.667	17.915	511.33	206.70	45.867	19.935	1.4667	1.5055
6	69.333	11.629	484.00	95.00	41.133	11.313	0.7333	1.2799
7	68.000	18.974	466.00	197.15	38.333	16.680	1.6667	2.2887
ALL	74.190	16.395	528.57	150.10	48.790	18.197	1.1143	1.6717

Clone HC 15

	W-0 MEAN	W-0 STD DEV	W-1 MEAN	W-1 STD DEV	SHOOTS MEAN	SHOOTS STD DEV	NODES MEAN	NODES STD DEV
1	66.000	14.041	294.00	91.56	32.267	16.837	2.6667	2.1269
2	69.333	16.242	268.00	68.47	28.867	11.363	2.9333	2.0862
3	71.333	14.075	280.67	130.25	28.267	13.962	1.6667	1.6762
4	75.333	13.558	284.00	90.62	30.533	12.755	2.6000	2.2928
5	67.333	15.337	241.33	71.40	24.267	9.153	3.3333	2.3805
6	72.000	18.974	289.33	81.02	27.133	8.079	3.0000	1.8516
7	69.333	18.696	296.00	129.55	30.533	12.552	1.5333	1.3020
ALL	70.095	15.781	279.05	96.40	28.838	12.287	2.5333	2.0337

Data Summary - Clone Trial Clone HC 17

	W-0 MEAN	W-0 STD DEV	W-1 MEAN	W-1 STD DEV	SHOOTS MEAN	SHOOTS STD DEV	NODES MEAN	NODES STD DEV
1	65.333	16.417	420.00	52.37	43.333	15.564	0.26667	0.45774
2	69.333	17.099	441.33	130.65	37.133	17.586	0.33333	0.72375
3	72.667	17.512	467.33	128.03	50.867	14.071	0.46667	0.63994
4	68.000	14.243	440.67	136.09	38.400	15.833	0.06667	0.25820
5	72.667	15.337	354.67	155.47	36.733	16.399	0.06667	0.25820
6	74.667	18.074	489.33	98.81	35.133	9.731	0.20000	0.77460
7	60.000	17.321	361.33	90.23	30.000	9.173	0.00000	0.00000
ALL	68.952	16.809	424.95	123.54	38.800	15.265	0.20000	0.52623

Clone HC 129

	W-0 MEAN	W-0 STD DEV	W-1 MEAN	W-1 STD DEV	SHOOTS MEAN	SHOOTS STD DEV	NODES MEAN	NODES STD DEV
1	64.667	18.848	330.00	126.83	56.800	23.665	1.1333	1.4075
2	59.333	13.345	382.00	133.21	56.800	18.675	0.6667	1.5430
3	60.000	11.952	490.00	209.69	69.600	21.885	1.2000	2.4260
4	67.333	14.376	536.00	210.57	91.600	36.237	0.4667	0.9155
5	62.000	11.464	462.67	170.06	63.933	25.650	1.0000	2.3299
6	70.000	19.272	450.67	220.44	58.333	25.933	0.7333	1.3345
7	64.667	22.636	381.33	153.15	57.800	20.267	0.3333	0.8165
ALL	64.000	16.385	433.24	185.73	64.981	27.139	0.7905	1.6273

Data Summary - Buffer Experiment

	W-0 MEAN	W-0 STD DEV	W-1 MEAN	W-1 STD DEV	SHOOTS MEAN	SHOOTS STD DEV	NODES MEAN	NODES STD DEV	pH MEAN	pH STD DEV
1	59.167	12.482	642.08	206.10	40.875	17.479	3.2083	2.4491	4.6604	0.3336
2	55.417	11.413	129.17	25.18	13.625	4.412	0.0000	0.0000	3.7379	0.2322
3	62.083	12.504	151.25	30.97	13.083	4.106	0.1250	0.3378	6.5612	0.4701
4	60.000	12.158	227.08	50.69	14.333	7.872	0.3333	0.4815	5.1446	0.1174
5	57.917	11.025	470.83	138.25	37.292	13.107	0.8333	1.1293	4.9037	0.1902
6	62.500	13.593	138.75	32.21	9.875	3.993	0.0417	0.2041	5.1583	0.2103
ALL	59.514	12.252	293.19	221.59	21.514	15.925	0.7569	1.5877	5.0277	0.8840

Data summary - Sucrose Trial

	SUCR	NITR	WT-1 MEAN	WT-1 STD DEV	WT-2 MEAN	WT-2 STD DEV	SHOOTS MEAN	SHOOTS STD DEV	NODES MEAN	NODES STD DEV
1	10.000	500.00	62.500	8.969	303.75	91.02	25.542	7.419	0.2500	0.4423
2	20.000	500.00	57.083	11.602	491.25	143.48	60.792	19.845	1.7083	1.8528
3	30.000	500.00	63.333	13.406	492.50	95.38	67.292	19.850	0.4167	0.5836
4	50.000	500.00	62.500	10.734	442.08	106.16	49.500	15.094	0.1667	0.4815
5	80.000	500.00	64.167	13.486	360.42	88.39	35.000	11.557	0.0000	0.0000
6	10.000	800.00	61.250	12.959	262.50	84.40	16.417	8.240	0.2500	0.5316
7	20.000	800.00	60.833	12.825	475.00	106.73	51.375	16.447	1.5417	1.4440
8	30.000	800.00	66.250	13.772	536.25	178.98	58.667	25.004	1.7500	2.1315
9	50.000	800.00	60.000	11.421	510.42	139.33	55.917	18.911	0.2500	0.4423
10	80.000	800.00	62.917	11.602	397.50	117.74	35.583	15.059	0.0000	0.0000
ALL			62.083	12.163	427.17	146.04	45.608	22.700	0.6333	1.2505

Data Summary - Adenine Experiment

	WT-1 MEAN	WT-1 STD DEV	WT-2 MEAN	WT-2 STD DEV	SHOOTS MEAN	SHOOTS STD DEV	NODES MEAN	NODES STD DEV
1	80.000	12.034	810.00	130.83	62.100	19.352	3.5667	3.1038
2	86.333	12.726	860.00	89.21	69.733	17.473	2.8000	1.8270
3	81.000	12.690	791.00	138.72	66.433	18.091	3.9000	3.3461
4	81.000	12.690	693.33	85.03	57.400	17.240	0.6333	0.9994
5	81.667	12.617	727.00	77.56	64.033	20.121	1.1000	1.1250
6	84.667	11.958	672.00	86.32	55.733	14.950	0.5667	0.8976
7	80.000	12.865	778.67	221.14	67.733	24.361	8.1000	5.9327
8	76.333	14.735	723.00	177.81	69.267	22.885	7.1333	4.6292
9	85.333	11.958	810.67	252.01	69.900	23.765	7.4667	4.9878
10	80.667	14.126	889.67	228.07	80.333	34.680	8.0333	4.7233
ALL	81.700	12.987	775.53	172.85	66.267	22.627	4.3300	4.6303

Analysis of Variance - Adenine Experiment G5 Medium

ANALYSIS OF VARIANCE ON WT-2

SOURCE	DF	SS	MS	F	p
TREAT	5	801931	160386	14.80	0.000
ERROR	174	1885446	10836		
TOTAL	179	2687377			

LEVEL	N	MEAN	STDEV
1	30	810.0	130.8
2	30	860.0	89.2
3	30	791.0	138.7
4	30	693.3	85.0
5	30	727.0	77.6
6	30	672.0	86.3

POOLED STDEV = 104.1

ANALYSIS OF VARIANCE ON SHOOTS

SOURCE	DF	SS	MS	F	p
TREAT	5	4262	852	2.65	0.025
ERROR	174	56048	322		
TOTAL	179	60310			

LEVEL	N	MEAN	STDEV
1	30	62.10	19.35
2	30	69.73	17.47
3	30	66.43	18.09
4	30	57.40	17.24
5	30	64.03	20.12
6	30	55.73	14.95

POOLED STDEV = 17.95

ANALYSIS OF VARIANCE ON NODES

SOURCE	DF	SS	MS	F	p
TREAT	5	341.49	68.30	15.04	0.000
ERROR	174	789.90	4.54		
TOTAL	179	1131.39			

LEVEL	N	MEAN	STDEV
1	30	3.567	3.104
2	30	2.800	1.827
3	30	3.900	3.346
4	30	0.633	0.999
5	30	1.100	1.125
6	30	0.567	0.898

POOLED STDEV = 2.131

Analysis of Variance - Adenine Experiment G10 Medium

ANALYSIS OF VARIANCE ON WT-2

SOURCE	DF	SS	MS	F	p
TREAT	3	436110	145370	2.97	0.035
ERROR	116	5685260	49011		
TOTAL	119	6121370			

LEVEL	N	MEAN	STDEV
7	30	778.7	221.1
8	30	723.0	177.8
9	30	810.7	252.0
10	30	889.7	228.1

POOLED STDEV = 221.4

ANALYSIS OF VARIANCE ON SHOOTS

SOURCE	DF	SS	MS	F	p
TREAT	3	2981	994	1.38	0.253
ERROR	116	83655	721		
TOTAL	119	86637			

LEVEL	N	MEAN	STDEV
7	30	67.73	24.36
8	30	69.27	22.88
9	30	69.90	23.77
10	30	80.33	34.68

POOLED STDEV = 26.85

ANALYSIS OF VARIANCE ON NODES

SOURCE	DF	SS	MS	F	p
TREAT	3	19.4	6.5	0.25	0.862
ERROR	116	3010.6	26.0		
TOTAL	119	3030.0			

LEVEL	N	MEAN	STDEV
7	30	8.100	5.933
8	30	7.133	4.629
9	30	7.467	4.988
10	30	8.033	4.723

POOLED STDEV = 5.094

Appendix 2

Trt No.	NH_4NO_3 level	Adenine level	Adenine Pretreatment
1	500 mg/L	80 mg/L	Standard
2	500 "	80 "	With held 5 weeks
3	500 "	80 "	With held 25 weeks
4	500 "	0	Standard
5	500 "	0	With held 5 weeks
6	500 "	0	With held 25 weeks
7	1000 "	80 "	Standard
8	1000 "	80	With held 5 weeks
9	1000 "	0	Standard
10	1000 "	0	With held 5 weeks