

1998

The in vitro propagation of seagrasses : halophila ovalis, ruppia megacarpa and posidonia coriacea

Melissa Grace Henry
Edith Cowan University

Follow this and additional works at: https://ro.ecu.edu.au/theses_hons



Part of the [Botany Commons](#), and the [Marine Biology Commons](#)

Recommended Citation

Henry, M. G. (1998). *The in vitro propagation of seagrasses : halophila ovalis, ruppia megacarpa and posidonia coriacea*. https://ro.ecu.edu.au/theses_hons/742

This Thesis is posted at Research Online.
https://ro.ecu.edu.au/theses_hons/742

**THE *IN VITRO* PROPAGATION OF SEAGRASSES:
HALOPHILA OVALIS, *RUPPIA MEGACARPA*
AND *POSIDONIA CORIACEA***

MELISSA GRACE HENRY

THESIS SUBMITTED IN PARTIAL FULFILMENT
OF THE REQUIREMENTS FOR THE AWARD OF
B.SC. (BIOLOGICAL SCIENCE) HONOURS

SCHOOL OF NATURAL SCIENCES
EDITH COWAN UNIVERSITY

JUNE 1998

ABSTRACT

Seagrass communities are of high ecological and economic significance. They provide a nursery area for commercial and recreational juvenile fish and crustacea. Seagrasses also play an important role in influencing the structure and function of many estuarine and nearshore marine environments. Unfortunately, the decline of seagrasses, as a result of human impact, has increased in recent years. This decline has become a major problem throughout the world.

Current methods used to restore degraded seagrass beds are limited, the most promising being transplanting material from healthy donor beds. This approach is expensive because it is labor intensive and damages the donor bed. Consequently, large scale transplanting programmes are not considered to be feasible.

An alternative to using donor material may be found in the propagation of seagrasses. This has been attempted through the production of seedlings in tissue culture. Tissue culture has shown to be successful in the rapid cloning of terrestrial plants and may be applied to develop a protocol which can be utilised to restore seagrass meadows.

Five clones of *Halophila ovalis* Hook F., (initiated from seed) and one clone of *Ruppia megacarpa* Mason (initiated from rhizome) were obtained from stocks at Edith Cowan University, School of Natural Sciences. *Posidonia*

coriacea Cambridge and Kuo was initiated in tissue culture during this study. These trials were undertaken in order to develop suitable tissue culture methods to be applied to the propagation of seagrasses for future revegetation programs.

The addition of sucrose to the medium resulted in increased growth and chlorophyll content of *H. ovalis*. There was no difference between the concentrations applied (30mM, 60mM and 120mM) with regard to growth, but between clones there were observed differences in the chlorophyll content. A comparison of one, two, four and eight week periods between subculture on basal medium showed no effect on the growth of *H. ovalis*, though after two weeks, cultures appeared healthier.

Cultures of *H. ovalis* grown in buffered (10mM MES) medium showed an increase in growth and chlorophyll content between initial pH 6 and 8 compared to those grown on unbuffered medium. These results suggest that medium buffering is important for tissue culture of seagrasses. When cytokinins (5 μ M concentration) were added to the medium, there was no effect on growth or chlorophyll content for three *H. ovalis* clones or one *R. megacarpa* clone.

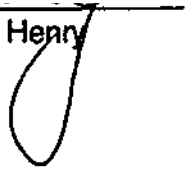
Seeds with the pericarp intact were more successful in initiating *P. coriacea* in tissue culture than those with rhizomes and those without the pericarp. These have continued to grow over seven months, but have not produced rhizome extension as in *H. ovalis* or *R. megacarpa*.

These studies have shown that the requirements for tissue culture of seagrasses may be substantially different from that of terrestrial plants, and have produced a good base line of information for the propagation of seagrasses in tissue culture.

DECLARATION

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

Melissa Grace Henry
12 June 1998



ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr Ian Bennett, not only for his guidance and assistance throughout my Biological Sciences degree and Honours project, but most of all, for his complete faith in my ability.

Acknowledgement must go to the other higher-degree students, for their moral support and optimism throughout my studies, in particular, to Mr Bryan Duff.

Thanks also to Ms Bernadette Gunn, Ms Debra McDavid, and Mr Andrew Woodward for their advice and assistance. Mr Clay Millar, and the technicians in the School of Natural Sciences, Edith Cowan University are also to be commended for their help and worthwhile suggestions throughout the year.

Finally, to my family and friends, for their words of wisdom and encouragement throughout my studies, and to the wonder dogs, Jess and Calypso, who, when all was said and done, saved my sanity.

TABLE OF CONTENTS

Abstract	ii
Declaration	v
Acknowledgements	vi
Table of Contents	viii
List of Tables	xi
List of Figures	xii
CHAPTER 1: INTRODUCTION	1
1.1 The Significance of Seagrasses	4
1.2 Decline of Seagrasses	7
1.3 Seagrass Restoration	11
1.4 Advances in Restoration	13
1.5 Propagation of Seagrasses	16
1.6 Experimental Aims	20
CHAPTER 2: MATERIALS AND METHODS	22
2.1 Plant Material	22
2.2 Sterile Technique	23
2.3 Culture Maintenance	23
2.4 Culture Media	24
2.4.1 Stock Solutions	24
2.4.2 Media Composition	24
2.4.3 Media Components and Preparation	24

2.5 Assessment and Analysis	29
2.5.1 Assessment of Growth	29
2.5.2 Chlorophyll Determination	29
2.5.3 Determining Axenicity	30
2.5.4 Statistical Analysis	30
CHAPTER 3: HALOPHILA OVALIS AND RUPPIA MEGACARPA	31
3.1 Introduction	31
3.2 Materials and Methods	34
3.2.1 Experiment 1. The effect of sucrose concentration on <i>H. ovalis</i> growth.	34
3.2.2 Experiment 2 and 3. The effect of medium pH on <i>H. ovalis</i> growth.	34
3.2.3 Experiment 4. The effect of subculture length on the growth of <i>H. ovalis</i> growth.	35
3.2.4 Experiment 5 and 6. The effect of cytokinins on the growth of <i>H. ovalis</i> and <i>R. megacarpa</i>.	36
3.3 Results	37
3.3.1 Experiment 1. The effect of sucrose concentration on <i>H. ovalis</i> growth.	37
3.3.2 Experiment 2. The effect of medium pH on <i>H. ovalis</i> growth.	40
3.3.3 Experiment 3. The effect of buffered medium pH on <i>H. ovalis</i> growth.	44

3.3.4	Experiment 4. The effect of subculture length on the growth of <i>H. ovalis</i>	48
3.3.5	Experiment 5. The effect of cytokinins on the growth of <i>H. ovalis</i> .	50
3.3.6	Experiment 6. The effect of cytokinins on the growth of <i>H. ovalis</i> and <i>R. megacarpa</i> .	50
3.4	Discussion	56
3.4.1	Sucrose	56
3.4.2	Effect of pH	57
3.4.3	Subculture periods	58
3.4.4	Cytokinins	59
CHAPTER 4:	<i>POSIDONIA CORIACEA</i>	62
4.1	Introduction	62
4.2	Materials and Methods	64
4.2.1	Experiment 1. Surface sterilization of rhizome tissue	64
4.2.2	Experiment 2. Surface sterilization of fruit	65
4.3	Results	66
4.3.1	Experiment 1. Surface sterilization of rhizome tissue	66
4.3.2	Experiment 2. Surface sterilization of fruit	66
4.4	Discussion	70
4.4.1	Surface sterilization of rhizome	70
4.4.2	Surface sterilization of fruit	70

CHAPTER 5: GENERAL DISCUSSION	72
5.1 Concluding Remarks	76
References	77

LIST OF TABLES

TABLE	PAGE
2.1 The contents of the basal medium	26
2.2 Composition of Murashige and Skoog (1962)	27
2.3 Composition of Sea Salts	28
3.1 Unbuffered pH levels recorded before and after autoclaving, and the mean pH recorded at the end of the experiment.	43
3.2 Medium pH before and after autoclaving, and the mean pH recorded at the end of experiment for buffered pH 5 to 8, and unbuffered pH 8.	47
4.1 Medium percentage contamination of <i>P. coriacea</i> rhizomes sterilized using three treatments of 100 replicates each.	68
4.2 Mean percentage contamination of <i>P. coriacea</i> fruits sterilized using two treatments of 40 replicates each.	69

LIST OF FIGURES

FIGURE	PAGE
3.1 Mean number of nodes of <i>H. ovalis</i> clones 3, 6, and 12, after 28 days in 0mM to 120mM sucrose.	38
3.2 Mean chlorophyll content of <i>H. ovalis</i> clones 3, 6, and 12, after 28 days in 0mM to 120mM sucrose.	39
3.3 Mean number of nodes of <i>H. ovalis</i> clones 2, 9, and 12, after 28 days in unbuffered medium pH 5 to 8.	41
3.4 Mean chlorophyll content of <i>H. ovalis</i> clones 2, 9, and 12, after 28 days in unbuffered medium pH 5 to 8.	42
3.5 Mean number of nodes of <i>H. ovalis</i> clones 3, and 12, after 21 days in buffered medium pH 5 to 8, and unbuffered medium pH 8.	45
3.6 Mean chlorophyll content of <i>H. ovalis</i> clones 3, and 12, after 21 days in buffered medium pH 5 to 8, and unbuffered medium pH 8.	46
3.7 Mean number of nodes of <i>H. ovalis</i> clones 6, and 12, subcultured at one, two, four and eight weeks.	49
3.8 Mean number of nodes of <i>H. ovalis</i> clones 3, 6, and 12, after 28 days in no cytokinin and 5 μ M kinetin, 2iP, BAP or zeatin.	52
3.9 Mean chlorophyll content of <i>H. ovalis</i> clones 3, 6, and 12, after 28 days in no cytokinin and 5 μ M kinetin, 2iP, BAP or zeatin.	53

3.10	Mean number of nodes of <i>H. ovalis</i> clones 6, and 12, and one <i>R. megacarpa</i> clone after 14 days in no cytokinin and 5 μ M kinetin, 2iP, BAP or zeatin.	54
3.11	Mean chlorophyll content of <i>H. ovalis</i> clones 6, and 12, and one <i>R. megacarpa</i> clone after 14 days in no cytokinin and 5 μ M kinetin, 2iP, BAP or zeatin.	55

CHAPTER 1. INTRODUCTION

Seagrasses are aquatic, monocotyledonous angiosperms, that grow in saline water. According to Cronquist (1981) there are four recognised families of seagrasses: Hydrocharitaceae, Posidoniaceae, Cymodoceaceae and Zosteraceae. These contain 12 genera and approximately 58 species (Kuo & McComb, 1989). Recognised genera and their families include: *Enhalus*, *Thalassia*, *Halophila* (Hydrocharitaceae), *Posidonia* (Posidoniaceae), *Syringodium*, *Halodule*, *Cymodocea*, *Amphibolis* and *Thalassodendron* (Cymodoceaceae), *Zostera* and *Heterozostera* (Zosteraceae) (King, Adam & Kuo, 1990). Fossil records date back to the Cretaceous (~100 million years ago) and indicate that seagrasses are the only known present day group of higher plants to have returned to a completely submerged marine existence (Larkum & den Hartog, 1989).

Arber (1920, cited by King *et al.* 1990) listed four special traits required by aquatic angiosperms in order for them to be considered seagrasses. These are: a tolerance of saline environments, a capacity to grow whilst fully submerged, the ability to anchor against wave action and tidal currents, and the capacity for hydrophilous pollination.

Other groups that are not conventionally considered to be seagrasses, but are sometimes included, are species of the genera *Ruppia* and *Lepilaena* (King *et al.*, 1990). These groups are often found in estuaries, and coastal

and inland saline lagoons. Certain species of *Ruppia* and *Lepilaena* have been found to tolerate a wide range of salinities, some up to ten times that of seawater (King *et al.*, 1990).

Seagrasses are found associated with all continents except Antarctica. Seven of the 12 genera are considered tropical, while the remaining five are confined to temperate waters (Kuo & McComb, 1989). Though the boundaries are not clearly defined, typically they inhabit shallow coastal waters and estuaries. Here they are found to grow in large numbers to form what is often referred to as seagrass "beds", "meadows" or "communities". These terms are not necessarily restricted to an area comprised of a single species and may contain a mixture of species (Kuo & McComb, 1989).

The greatest seagrass diversity is found around the coastline of Australia, with particularly high species diversity occurring in Western Australia (Larkum & den Hartog, 1989). The coastline of Western Australia has a wide range of environments, extending 12,500 km from the tropical waters of the Timor Sea to the temperate waters of the Southern Ocean. Approximately 20,000 km² of Western Australia's coastline is covered by seagrasses, hence they make up a major component of the nearshore environment (Kirkman & Walker, 1989).

The high diversity of seagrass genera (10) and species (25) along this coastline is unparalleled elsewhere in the world (Kirkman & Walker, 1989).

In terms of coverage, species of the genera *Posidonia* are very important along the coastline of Western Australia. Their colonisation and growth in cleared areas is extremely slow, taking between 60-100 years to cover cleared substratum (Kirkman & Kuo, 1990). This may be attributed to the fact that species of this genus regrow from seedlings only. There is no evidence of any Australian species of *Posidonia* colonising naturally through asexual reproduction (Kirkman & Kuo, 1990).

Despite advances in the study of seagrass biology over the past twenty years, the ecological significance of seagrasses is only just being realised, and is therefore believed to be underestimated. Seagrasses provide a nursery area for commercial and recreational juvenile fish and crustacea (Kirkman, 1989; Lenanton, 1982), and are therefore of high ecological and economic significance.

Seagrass decline has been recorded in many areas. The major cause has been attributed to development associated with high human populations, industrial effluent discharge, and the resultant pollution of the inshore marine environment (Carruthers and Walker, 1997). Detrimental changes associated with the subsequent loss of seagrasses may be irreversible, hence there is demand from governments and environmental managers to prevent continuing destruction. In addition, there is considerable need to develop improved protocols for restoring seagrass beds (Kirkman, 1989).

1.1 The Significance of Seagrasses

There is increasing evidence worldwide to show that seagrasses play a primary function in ecosystem structure of inshore marine environments and estuaries. In many parts of the world it has been shown that seagrass beds are a refuge for assemblages of organisms not present in unvegetated sediment. This has led to the realisation that seagrasses play an important role in influencing many structural and functional aspects of estuarine, coastal and inshore marine environments (Kuo & McComb, 1989).

Most of the productivity of seagrasses is directly transferred to detrital food chains (Walker 1989; Poiner, Walker & Coles 1989), and the total primary productivity of seagrass meadows is greatly enhanced by providing a substrate for other photosynthetic organisms (Orth & Van Montfrans, 1984). Seagrasses produce a large quantity of leaf matter at extremely high rates, which in turn directly supplies the detrital food chain. The epiphytes and bacteria on living seagrass leaves, together with the detritus associated with microbes produced from shed leaves, provides a continuous source of food for detritivores (Cambridge, 1979).

Carruthers and Walker (1997) liken seagrass meadows to terrestrial crop plants, in that the seagrass canopy is a functional unit that impacts greatly in modifying the surrounding environment. Cambridge (1979) compares dense stands of seagrass meadows to a 'pump and filter system', as they

greatly influence current flow, in turn slowing the rate of water flow over the substrate. The reduced velocity of water within the system allows sediment to be trapped, and existing sediments to be confined, thereby stabilising the environment (Walker, 1989). The trapping of sediment within a seagrass bed facilitates the efficient re-shifting of minerals, enabling a highly productive system to operate in what would otherwise be regarded as low nutrient waters (Walker, Sim & Penniford, 1994).

The efficiency with which seagrasses are able to resorb nutrients, such as nitrogen and phosphorous, has been suggested to be an important mechanism to reduce the plants' dependence on external nutrient input in environments that are nutrient poor (Stapel & Hemminga, 1997). The nutrients taken up by seagrasses enable the release (via their roots and leaves) of metabolites such as oxygen and complex organic compounds (Cambridge, 1979).

One of the effects seagrasses have on the surrounding environment is to reduce the turbidity of an area. Studies performed in tropical Queensland found that areas of low turbidity carried a greater abundance and distribution of juvenile fish (Blaber & Blaber, 1980). Hence, the inshore marine environment has been found to provide an alternative nursery area for fish species commonly found in estuaries.

Studies in the south of Western Australia by Lenanton (1982) found that 16 commercially and recreationally important fish species, normally found in

estuarine environments, utilised the inshore marine environment as an alternative nursery area. Rather than reduced turbidity, in this instance it was likely to be the amphipod food source provided by densely packed leaves from "drift" seagrass, which accounted for the high abundance of juvenile fish fauna. "Drift" seagrass is a term used for detached clumps of seagrass leaves which can be found lying in depressions of nearshore marine habitats (Lenanton, 1982).

The enhancement of feeding sources is also supported by studies done in Western Australia. *Posidonia* beds in Cockburn Sound support a diverse array of both epiphytic algae and invertebrates. Eleven fish species have been found to confine their feeding to the seagrass blades, or to organisms found in seagrass beds (Scott, Dybdahl & Wood, 1986). *Posidonia* beds act as a substrate for amphipods, which are considered to be a major food source for juvenile fish. Many species of fish and shell-fish lay their eggs within seagrasses, and may also complete some or all of their stages of growth to maturity within the confines of seagrass beds (Thorhaug, 1986). The distribution of juvenile fish may therefore be attributed to the reduction of turbidity of an area and the supply of food and shelter as a direct result of seagrasses.

Seagrasses also act as a direct food source for large herbivores. This includes the endangered dugong and green turtle, which are largely confined to tropical Australian waters, together with some fish and

crustacea (Klump, Howard & Pollard 1989; Lanyon, Limpus & Marsh 1989).

1.2 Decline of Seagrasses

Seagrass beds may undergo changes that lead to their decline as a result of either natural occurrences or human-related activities. Some natural disturbances may include storms, salinity changes (as a result of droughts or floods), erosion and disturbance from burrowing animals (Thorhaug, 1986). However, the loss of seagrass species as a result of human-activity is becoming far more frequent than that caused by natural perturbations. In most cases, the initial factor responsible for the decline interacts with other independent or secondary factors which may also play a role in a large reduction of seagrass species (Cambridge & McComb, 1984). For example, the initial factor may be heavy pollution or high nutrient influx, which may increase the susceptibility of the seagrass to secondary deleterious factors such as over-grazing.

Increased epiphyte loads have also contributed to seagrass decline in areas found to be highly eutrophic as a result of effluent discharge. The shading of seagrass leaves by epiphytes under such conditions can reduce photosynthesis by as much as 80%. Hence, productivity is sufficiently reduced to explain the level of the decline observed (Silberstein, Chiffings & McComb 1986; Masini, Cary, Simpson & McComb 1990; Hillman, Lukatelich, Bastyan & McComb 1991). Gordon, Chase, Grey and Simpson

(1994) found that not only did the attenuation of light reduce leaf and shoot densities, primary productivity and leaf production per shoot of *Posidonia sinuosa*, but that even after light intensity had returned to normal, the meadow had suffered irreparable damage.

Underground biomass (roots and rhizomes) differs between seagrass species (West & Larkum, 1983). In Port Adelaide, South Australia, losses of *Amphibolis* beds were primarily attributed to effluent flow, and subsequently to the small amount of underground biomass found in species of this genus (West & Larkum, 1983). *Posidonia* has a greater underground biomass than that of *Amphibolis* spp., enabling *Posidonia* to exhibit greater resistance to environmental disturbances than *Amphibolis*.

Their greater underground biomass allows species of *Posidonia* to store products in the rhizome which can supply more energy for respiration (Carruthers & Walker, 1997). The amount of underground biomass is believed to be important in determining whether or not a species of seagrass will survive small perturbations in environmental conditions (Carruthers & Walker 1997; Walker *et al.* 1994).

Disturbance can also lead to increased grazing pressure. Disturbed meadows of *Thalassia* in the United States and *Zostera* in the West Indies have been subjected to heavy grazing by sea urchins (Orth & Van Montfrans, 1984). Polluted areas of *Posidonia* meadows in Cockburn Sound in south-western Australia and areas which have been disturbed in

Botany Bay, New South Wales, were also found to be more susceptible to overgrazing by sea urchins, leading to permanent losses of seagrass (Shepherd, McComb, Bulthuis, Neverauskas, Steffensen & West, 1989).

The common element identified in all observed instances of over-grazing of *Posidonia* by sea urchins was the initial patchiness of the meadow. Dense, healthy meadows did not have outbreaks of overgrazing (Cambridge, Chiffings, Brittan, Moore & McComb, 1986). As a direct result of fragmentation, over-grazing has been found to greatly retard resorption in seagrass leaves, due to the premature loss of leaves and leaf fragments (Stapel & Hemminga, 1997).

Although there appears to be no single factor responsible for the loss of seagrasses, it is clear that human impact has resulted in the initial decline of seagrass meadows, which then allows other factors to disturb the system. More than a decade ago, it was estimated that the meadow area in Cockburn Sound had been reduced from 4 200 to 900 ha since the onset of industrial development in 1955 (Cambridge & McComb, 1984). Although these figures may not be entirely accurate due to the extensive period over which they were taken, and the lack of accurate measuring apparatus available in 1955, they still provide a good indication of the impact industrial development has had in this area.

In Princess Royal and Oyster Harbours, near Albany, Western Australia, similar problems to that experienced in Cockburn Sound have been

observed. The increasing amount of industrial effluent discharged into the waters has included wastewater and effluent contaminated with nitrogen, phosphorous and associated compounds, hydrocarbons, and a range of phenolics (Gordon *et al.* 1994; Cambridge & McComb 1984).

Studies relating to seagrass ecosystems are still at an early stage. However, it has been reported that the loss of seagrass has had significant implications for the coastal and inshore environment. These include a fall in leaf detritus production accompanied by alterations in food chains, a loss of structural diversity which is replaced by bare sand, and alterations in the principal primary producers (from benthic to planktonic). Fish faunas exclusive to seagrass meadows have been found to change with the onset of seagrass degradation, and it is predicted that as a result of alterations to sediment structure, beach morphology will shift. This can lead to the onset of erosion of dune systems (Cambridge & McComb, 1984).

In order to preserve the seagrass resources which remain, it is important that more information is gathered on seagrass biology and ecology. Research should be directed at a greater understanding of the ecological processes involved in seagrass ecosystems, so that appropriate management strategies can be implemented. Such studies are essential in implementing the development of a successful restoration protocol.

1.3 Seagrass Restoration

Natural extension of most seagrass meadows occurs at very slow rates. The successful establishment of seagrass seedlings through sexual reproduction is, in most cases, extremely low (McConchie & Knox, 1989). Therefore, asexual reproduction through seagrass rhizome extension is very important for some species. This not only prevents the erosion of beaches by stabilising sediment, but also extends meadow boundaries. However, asexual reproduction is a slow mechanism of meadow extension. This form of extension has been witnessed in stands of *Posidonia*, *Amphibolis*, *Zostera* and the tropical species *Enhalus acoroides*. But this appears to be very slow, and it has been estimated to have taken decades, or longer for the beds to develop (Kuo & McComb 1989; Kirkman 1989).

Seagrasses have a slow rate of natural recovery. *Zostera marina*, once found along the east coast of the United States and the west coast of Europe, did not recover for many decades following a wasting disease in the 1930's. In South Australia, an area mined for *Posidonia* fibre in 1917 is still visibly identified by the lack of re-growth. Tracks made during World War II in Botany Bay, New South Wales, over *Posidonia australis* beds could still be seen nearly five decades later (Shepherd *et al.*, 1989).

The primary method of restoring damaged or lost beds has typically been transplanting. This is thought to shorten the colonising period of a site by up to ten times (Clarke & Kirkman 1989; Kirkman 1989). However,

success depends on many factors which include growth rate, planting configuration and the species to be restored.

The restoration of degraded seagrass meadows is also a costly process. In discussing the cost involved with seagrass transplants in the United States, Fonseca, Kenworthy and Thayer (1987, cited by Kirkman 1989) claimed it would cost approximately US\$25 000 to plant an acre of seedlings and other planting units. However, this figure was reached on the basis that machinery was not available to plant the units, and that they would have to be planted manually. Information with regard to optimising the efficacy of restoration is scarce. Factors to be considered include the optimum number of planting units, the distance between planting units, the material used to secure the units to the substrate, and the costs involved in obtaining the transplant material.

As the economic importance of seagrasses has not been fully realised, the expense involved in restoring areas which have suffered from seagrass loss may be a mere fraction of the ecological and economic benefits. In 1983 in tropical Australia alone, it was estimated that the prawn fishing industry returned more than \$A161 million in export earnings, which accounted for 41% of Australia's fisheries export revenue (Poiner *et al.*, 1989). Similarly, the seagrass meadows of the west coast of Australia which provide foraging grounds for the western rock lobster (*Panulirus cygnus*), has an annual catch of approximately \$A200 million (Walker & McComb, 1992). If seagrass beds continue to decline, so too will the

nursery areas which are critical to the survival of juvenile fish and crustacea important to the fishing industry.

Transplanting may be successful only if the cause of the decline has been eliminated, and the site modified to allow successful restoration. Some operations have involved transplanting a species other than that which was originally present (Kirkman 1989). However, without adequate knowledge of the biological, physical and chemical characteristics of the area to be planted, this might lead to further unfavourable modifications to the ecosystem.

1.4 Advances in Restoration

The first successful seagrass transplant into the field was claimed by Kelly, Fuss & Hall (1971, cited Kirkman 1989). They attached short shoots of *Thalassia testudinum* that were treated with the artificial auxin naphthalene acetic acid (NAA) to a construction rod. Phillips (1974, cited in Kirkman 1989) successfully transplanted *Zostera marina* on a large scale between Alaska and Puget Sound, Washington in 1974. However, since these initial achievements, little progress has been made with the large scale establishment of seagrasses (Kirkman, 1989).

The primary planting units used in the restoration of seagrass have been "plugs", "sprigs", seeds, and sprouting stems. Plugs are planting units which consist of the rhizome, leaves and roots with sediment intact. These

have been regarded as the most successful of the planting units employed in restoring seagrass beds. However, they also have the greatest impact on the donor bed (Bird, Jewett-Smith & Fonseca 1994; Kirkman 1989) because of the large quantity of plant material taken and the creation of patchy areas. Sprigs are virtually the same as a plug, but they are devoid of sediment. In order for the successful re-establishment of both plugs and sprigs, the rhizomes must contain apical meristematic tissue. This may involve an excessive amount of time in obtaining suitable material, and may also prove wasteful to the donor bed (Kirkman, 1989). Methods are still being developed to overcome problems associated with securing the planting units, together with the most cost effective and efficient means of planting.

Currently under investigation is the development of a protocol to produce artificial, plastic planting units. The use of these has been suggested to restore areas which may not be successful for transplanting methods (Kirkman, 1989). However, using a plastic substitute for seagrass is far from being a suitable replacement, and may be more problematic than beneficial. Seagrass meadows form a complex ecosystem; such artificial systems would be devoid of many of the biological and physical functions of living seagrass beds.

Bird *et al.* (1994) examined whether *in vitro*¹ propagated *Ruppia maritima* would survive transfer back into the field. The first experiment compared

¹ For the purposes of this review, the terms "in vitro" or "tissue culture" refer to the axenic growth of seagrass within closed aseptic vessels. The term "culture" refers to the non-axenic growth of seagrass in aquaria.

two different planting methods. One method looked at the planting of *in vitro* propagated material which was attached to metal staples. The material was directly planted into the field at four different sites. However, within one month, almost all of these transplants disappeared from the four planting sites. This was attributed to the morphology of *R. maritima*, in that the thin rhizomes were not able to be held in place by the staples. In the second method, *in vitro* propagated material was transferred to peat pots and grown in a flowing seawater system for six weeks before transplanting into the field. After 11 months, these transplants were still growing in three of the four sites, with an observed 20-80% survival rate (Bird *et al.*, 1994).

The second experiment showed the greatest transplant success, in which *in vitro* propagated *R. maritima* was directly rooted *ex vitro* in peat pots and grown in a flowing seawater system for six weeks. After 12 months, two of the three planted sites showed a significant percent cover; one had a cover of approximately 99%. The increase in shoot numbers and cover of *R. maritima* suggested that plants propagated *in vitro* could be used successfully for habitat restoration (Bird *et al.*, 1994). However, after 23 months *R. maritima* showed a marked decrease in percent cover, which was attributed to the natural competition of *Zostera marina* (Bird *et al.*, 1994).

Although there has been some success with current transplant methods, Fonseca, Kenworthy & Thayer (1986, cited by Kirkman 1989) believe that there is still a net loss of habitat involved with any restoration project. The

collection of any planting unit from a donor bed will inflict damage to that bed. The larger the quantity of planting units cleared from a donor bed, the harder it is for a meadow to successfully regenerate. With evidence that overgrazing can occur in patchy areas of a seagrass meadow, leading to the loss of the meadow as a whole (Cambridge *et al.*, 1986), removing donor material for rehabilitation purposes should be reviewed. An alternative to using plant material from donor beds is to use propagated seagrass material. For this reason, developing a reliable technique for the propagation of seagrass may play an important role in providing material for restoration projects in the future.

1.5 Propagation of Seagrasses

Following recommendations in the Report of the Steering Committee of the Seagrass Ecosystem Study held at the University of Alaska in 1973, there has been a reasonable amount of effort invested into the culture of seagrass. Most of the studies have been undertaken at the University of Texas, since 1973. However, a reliable protocol for the propagation of seagrasses is still unavailable (McMillan, 1980).

The culturing of seagrasses commenced in a bid to develop an improved understanding of seagrass biology (McMillan, 1980). With the increasing decline of seagrasses and the realisation of their substantial contribution to the marine environment, the need to develop more efficient methods of propagation for restoration has become more immediate.

Most success has been found with the non-axenic cultivation of seagrasses in tanks. Many of the studies have involved developing methods suitable to maintain the various species in culture. Other studies include monitoring the tolerance of various plant species to fluctuations in temperature, salinity and light, along with photoperiodic responses (McMillan 1980; Meinesz, Caye, Loques & Molenaar 1991). The successful culture of nine of the twelve genera, including *Thalassia*, *Halodule*, *Halophila*, *Posidonia*, *Zostera*, *Cymodocea*, *Syringodium*, *Enhalus*, and *Thalassodendron*, has been achieved. However, most of these plants were not found to survive more than two years in culture, some dying after only several months (McMillan, 1980).

The non-axenic germination, flowering and seed production in cultures of *Halophila engelmannii* was the inspiration behind the first successful attempts at tissue culture of seagrass (McMillan 1987; McMillan 1988; Jewett-Smith & McMillan 1990).

Tissue culture methods have been successfully developed for many terrestrial plants, providing a rapid alternative to more conventional means of propagation. However, the techniques developed for terrestrial plants have not been successfully applied to the tissue culture of seagrasses, primarily because of heavy microbial associations and the subsequent deleterious surface sterilization of plant and seed material, and the incapacity to maintain tissues *in vitro* for extended periods (Moffler & Durako, 1984).

There is enormous potential offered in developing tissue culture techniques for the rapid production of seagrass. In addition, the ability to screen vast numbers of explants for genotypes able to survive in reduced light, or carrying improved resistance to the effects of many pollutants such as heavy metals and hydrocarbons may prove beneficial for the restoration of seagrass meadows.

As mentioned, sterilization techniques frequently used for terrestrial plant material have been inadequate when applied to seagrass, due to the presence of high amounts of both surface and endophytic bacteria and fungi. The lack of a well-developed cuticle in seagrass, together with the large variety of associated microorganisms, makes effective, non-destructive surface sterilization a difficult task (Koch & Durako, 1991).

Loques *et al.* (1990) applied many disinfection techniques to various tissues of *Posidonia oceanica*, in order to initiate them into axenic culture. However, most explants developed heavy bacterial and fungal infection. The greatest success was with macromeristematic tissue. But this was short-lived, as the explants only survived four months.

As with many terrestrial plants, the sterilization of seeds has been very useful. This has proved to be more successful than the use of plant segments with species of *Halophila* and *Thalassia* (Moffler & Durako 1984; Bird & Jewett-Smith 1994). *Thalassia testudinum* was germinated under axenic culture, however, the plants died prior to subculturing (Bird &

Jewett-Smith, 1994). Balestri, Piazza and Cinelli (1998) recently initiated *P. oceanica* in tissue culture by the surface sterilization of seed but the cultures died after 10 months.

The disadvantage in relying on seed alone, is that it is not always easily found or available. For example, *P. sinuosa*, a dominant species of seagrass found along the southern end of the south-west of Western Australia, seeds once a year for a couple of months, and in some years will not produce seed at all (Kirkman, 1989). However, once cultures are established there will not be a need to collect every year.

The first report of successful *in vitro* culture of a seagrass was for *Ruppia maritima*, in which a large amount of rhizomal division was generated (Koch & Durako, 1991). The report examined the effects of various plant growth regulators; five cytokinins and one auxin. Root suppression was observed in the media containing auxin, and the various cytokinins were observed to stimulate a three to four-fold increase in the growth of rhizomes and shoots. 2-iso-pentyladenine was the only cytokinin found to stimulate dose-dependent growth and development. Koch and Durako (1991) attributed this to the fact that this compound is found to occur naturally in ocean sediment.

Bird, Brown, Henderson, O'Hara and Robbie, (1996) claimed to have developed a routine culture medium adequate to support the axenic growth of *R. maritima* clones for six years. They have contrasted tissue culture

media containing inorganic carbon (as bicarbonate), organic carbon (as sucrose), and cytokinin addition to carbon-based media. In a sucrose-based medium, *R. maritima* showed a growth response to 6[γ,γ -dimethylallylamino]-purine (2iP) twice that of the growth response to the cytokinin 6-benzylaminopurine (BAP). This response was not linearly dose-dependent. Instead, once a concentration of 14.8 μ M 2iP was applied, growth did not improve. Growth in the bicarbonate-based medium with 2iP was less than that in the sucrose-based medium (Bird *et al.*, 1996).

1.6 Experimental Aims

Following on from the work of Bird *et al.* (1996), this project aimed to: determine the optimum culture medium for the maintenance and growth of *H. ovalis* and *R. megacarpa*, provide a sterilization protocol for the establishment of *P. coriacea* in tissue culture and compare the culture requirements of seagrasses with those for terrestrial plants. These species will be used in this project as they are local to Western Australia.

Data gathered with respect to optimum growth of *H. ovalis*, and *R. megacarpa* in tissue culture and sterilization of *P. coriacea* would provide useful information to develop a reliable protocol for the propagation of seagrasses. This would have implications for the restoration of seagrasses.

The project examined, under *in vitro* conditions, the growth responses of *H. ovalis*, to a range of sucrose concentrations, pH levels, pH levels buffered with 2-(N-morpholino)ethanesulfonic acid (MES), and length of time between subculture. *H. ovalis* and *R. megacarpa* were also exposed to several cytokinins including 6-furfurylamino-purine (kinetin), 6-benzylaminopurine (BAP), 6[γ,γ -dimethylallylamino]-purine (2iP), and 6-(4-hydroxy-3-methyl-but-2-enyl-amino)-purine (zeatin).

Sterilization procedures for initiation of *Posidonia coriacea* in tissue culture was also examined. Three treatments were applied to sterilize rhizome tissue but only one sterilizing treatment was applied to the fruits, with and without the pericarp present.

CHAPTER 2. MATERIALS AND METHODS

2.1 Plant Material

Shoots including rhizomal material of *Posidonia coriacea* were collected at a depth of 10m using Self Contained Underwater Breathing Apparatus (SCUBA) off the coast of Fremantle, Western Australia on the 25th September 1997. Shoots bearing fruits of *P. coriacea* were collected in November 1997 using SCUBA, from the shoreline at Cockburn Sound, Western Australia. *P. coriacea* shoots with fruits attached were transported back to the laboratory in seawater.

The rhizome material and fruits of *P. coriacea* were washed under running tap water and then stored in aerated beakers of artificial seawater for no more than 24 hours after collection prior to sterilizing.

Five clones of *Halophila ovalis* (initiated from seed) and one clone of *Ruppia megacarpa* (initiated from rhizome) were obtained from stocks that had been established one year previously by the School of Natural Sciences, Edith Cowan University. Material of *H. ovalis* was used in all experiments except for those pertaining to sterilization. *R. megacarpa* responses to cytokinin were examined.

2.2 Sterile Technique

Tissue culture involves growing plants in a sterile environment and under a defined set of conditions. Any materials involved with handling, growing and storing cultured plant tissues must be sterilized first. Any materials used with the plants or media containers (eg. media, media containers, instruments, plastic cutting plates, ethanol, artificial seawater), were sterilized in an autoclave at 121°C for 20 minutes.

Plant material was aseptically handled in a laminar flow cabinet which had been exposed to ultra-violet radiation for 15 minutes prior to use, and then wiped with 70% ethanol. Instruments (eg. forceps and blades) were routinely re-sterilised during culturing with a BactiCinerator sterilising unit (Sigma-Aldrich, Castle Hill NSW).

2.3 Culture Maintenance

Cultures were grown in 250mL screw top polycarbonate containers containing 30 mL of liquid medium. All material was subcultured onto basal medium (described in Table 2.1) every two weeks, except where mentioned for cultures in experiment. Cultures were maintained at 25°C \pm 4°C in a photoperiod 16h light/8h dark. Light was provided by cool white fluorescent tubes.

2.4 Culture Media

2.4.1 Stock Solutions

Stock solutions of the plant growth substances kinetin, 2iP, BAP and zeatin were used in media preparation. These were prepared by dissolving powdered cytokinins (Sigma-Aldrich, Castle Hill NSW) in analytical grade 1M NaOH, and made up to the required volume with milli Q water (ion-exchange filtered to 15 m Ω electrical resistance). Solutions were stored in the dark at 4°C.

2.4.2 Media Composition

Culture media were prepared using half strength Murashige and Skoog (M&S) Basal Medium (Sigma Aldrich, Castle Hill NSW; Product number M5519), containing macro- and micronutrients and vitamins as detailed in Table 2.2. Artificial sea salts (Sigma Aldrich, Castle Hill NSW; Product number S9883) as detailed in Table 2.3, cytokinins (Sigma Aldrich, Castle Hill NSW) and sucrose (CSR Ltd, North Sydney, NSW) were also added.

2.4.3 Media Components and Preparation

Analytical grade reagents and milli Q water were used in media preparation. All glassware and culture vessels were washed in Pyroneg

detergent and rinsed twice in tap water, then in de-ionised water twice, before being oven dried at 60°C.

Media contents (eg. sucrose, M&S, artificial sea salts) were weighed then dissolved in milli Q water. Stock solutions of cytokinins were added to a solution which was made up to the final volume, and pH adjusted to 8.0 with 1M and 0.1M KOH. Media was dispensed into culture containers, then autoclaved. Media were stored in the dark at 4°C until used.

TABLE 2.1: The contents of the basal medium used for *H. ovalis*, *R. megacarpa*, and *P. coriacea*.

Component	Concentration
	(mg.L ⁻¹)
Murashige and Skoog (1962)	2 215
Sucrose	20 000
Sea Salts	20 000
	(μ M)
Kinetin	5.0

TABLE 2.2: Composition of Murashige and Skoog (1962) (Sigma Aldrich; Product number M5519).

Component	Concentration (mg.L ⁻¹)
<u>Macronutrients:</u>	
NH ₄ NO ₃	1 650.0
KNO ₃	1 900.0
CaCl ₂ .2H ₂ O	440.0
MgSO ₄ .7H ₂ O	370.0
KH ₂ PO ₄	170.0
EDTA-Iron (III) Sodium Salt.H ₂ O	36.7
<u>Micronutrients:</u>	
H ₃ BO ₃	6.2
MgSO ₄ .4H ₂ O	22.3
ZnSO ₄ .7H ₂ O	8.6
KI	0.830
Na ₂ MoO ₄ .2H ₂ O	0.250
CuSO ₄ .5H ₂ O	0.0250
CoCl ₂ .6H ₂ O	0.0250
<u>Vitamins:</u>	
Nicotinic Acid (free acid)	0.50
Thiamine HCl	0.10
Pyridoxine HCl	0.50
Glycine (free base)	2.0

TABLE 2.3: Composition of Sea Salts (Sigma Aldrich; Product number S9883).

Component	Concentration (% of total weight)
Chloride	46.943
Sodium	26.047
Sulfate	6.44
Magnesium	3.16
Calcium	0.996
Potassium	0.927
Bicarbonate	0.362
Borate	0.072
Strontium	0.016
Phosphate	—
Solids Total	84.963
Water	14.987
TOTAL	99.95

2.5 Assessment and Analysis

2.5.1 Assessment of Growth

At the start of each experiment, each explant had a known number of nodes. The relative amount of plant growth was determined by counting the number of new nodes at the end of experiment.

2.5.2 Chlorophyll Determination

The total chlorophyll was determined by measuring the amount of chlorophyll a and b for each replicate using the non-maceration method of Moran and Porath (1980). This involved measuring the fresh weight of explants that includes nodes and leaves for each replicate, and then leaving these in 5mL of N,N-dimethyl formamide (DMF) overnight. The absorbance of the extract was then read in a spectrophotometer at wavelengths of 647 and 664 nm. Micrograms of chlorophyll per gram of fresh weight were calculated by using the fresh weight and chlorophyll values as per the following calculation formulae:

$$\text{Chlorophyll a } (\mu\text{g/ml}) = (\text{ABS}_{664} \times 12.64) - (\text{ABS}_{647} \times 2.99)$$

$$\text{Chlorophyll b } (\mu\text{g/ml}) = (\text{ABS}_{647} \times 23.26) - (\text{ABS}_{664} \times 5.6)$$

$$\text{TOTAL CHLOROPHYLL} = (\text{ABS}_{664} \times 7.04) + (\text{ABS}_{647} \times 20.27)$$

2.5.3 Determining Axenicity

The cultures were visually screened for apparent bacterial and fungal contaminants. If there were any signs of cloudiness, discolouration, fungal spores or bacterial colonies, the cultures were autoclaved. The cultures which were not observed to be contaminated were assumed to be operationally axenic.

2.5.4 Statistical Analysis

Statistical analysis was performed using SPSS for Windows Version 7.0. All data were tested for normality, and where necessary, transformed using natural logarithms. Analysis of variance (ANOVA) was used to test the validity of replication within data sets. Two-way ANOVA was performed where experiments of two factors were tested, and where significant ($P \leq 0.05$), a one-way ANOVA was used to test for significant differences between treatments; Scheffe's post hoc analysis was performed when significant differences were found using one-way ANOVA. The χ^2 test was applied to experiments relating to sterilization of *P. coriacea* material.

CHAPTER 3. HALOPHILA OVALIS AND RUPPIA MEGACARPA

3.1 INTRODUCTION

In vitro culture of terrestrial plants has been successfully applied to propagation, the investigation of plant biology, and plant-microorganism associations. However, little work has been done with aquatic, marine plants.

Since the environmental conditions for marine plants are very different to those of terrestrial plants, factors established for the *in vitro* culture of terrestrial plants need re-examination for application to aquatic marine plants such as seagrasses. Hence, it was important to determine some basic conditions necessary to establish media and a protocol for the growth of *H. ovalis* and *R. megacarpa*.

Bird *et al.* (1996) found *in vitro* growth of *R. maritima* in a 29.21 mM sucrose-based medium with 2iP was three times greater to that in a bicarbonate-based medium with 2iP. However, as various levels of sucrose were not examined experimentally, the large increase in growth may have been attributed to any number of medium contents.

Loques, Caye and Meinesz (1990) found the greatest chlorophyll level and survival time of *P. oceanica* macromeristems in medium containing a sucrose concentration of 263 mM, however the cultures were short-lived,

dying after 4 months. The sucrose concentration, although high, was thought to play both a nutritive and osmotic role. However, the concentration of sucrose used for successful *R. maritima* cultures has been as low as 29.21 mM (Bird *et al.* 1994; Bird *et al.* 1996). The level of sucrose applied to *P. oceanica* may have been too high, resulting in the cultures going into osmotic shock.

The effects of pH on photosynthesis for three seagrass species was examined in the field and under non-axenic conditions in the laboratory (Invers, Romero & Perez, 1997). The pH range of 8 to 8.8 was examined for *Posidonia oceanica* and *Cymodocea nodosa* and the range of 8 to 9 was examined for *Zostera noltii*. Photosynthetic rates were greatest for *Posidonia oceanica* and *Cymodocea nodosa* at pH 8.2 and changed to 25-80% at pH 8.8. *Zostera noltii* was less sensitive to pH and maintained high photosynthetic rates up to pH 8.8 (Invers *et al.*, 1997). The preferred pH values under laboratory conditions were similar to those found in the field.

In vitro cultures of *Thalassia testudinum* were successfully establishment and maintained on medium at pH 8 (Moffler & Durako, 1984) whereas *H. engelmannii* was found to grow best at medium pH 5 (Bird & Jewett-Smith, 1994). *R. maritima* were maintained *in vitro* at pH 5.6 (Koch & Durako, 1991), however more recent studies found pH of 7 and 7.5 to be adequate (Bird *et al.* 1994; Bird *et al.* 1996). It must be noted that the complex environmental conditions and medium, made it difficult to determine

whether it was the level of pH affecting growth, or the contents of the medium.

One of the factors which is important, but is absent from published data, is the frequency of subculture. This would play a significant role in the vigour of cultures as components of the medium (eg. carbohydrate source, nutrients, vitamins etc.) would support the plant for a limited amount of time. Likewise, a build-up of toxins in the medium released by the plant would eventually kill the plant.

Plant growth regulators, in particular cytokinins, have shown a marked effect on growth rates of seagrasses *in vitro*, however, the type of cytokinin appears to be species specific. *R. maritima* has observed the greatest amount of growth on 2iP, whereas *H. engelmannii* showed the best growth response to BAP (Bird *et al.* 1996). The greatest longevity of *P. oceanica* macromeristem cultures were observed on medium with ratios of the auxin, indole-3-acetic acid, and the cytokinin, kinetin of 0.2:2 mg L⁻¹ and 5.0:5.0 mg L⁻¹ (Loques *et al.* 1990).

The current experiments examined growth responses of *H. ovalis* to a range of sucrose concentrations, length of subculture period, pH and plant growth regulators.

3.2 MATERIALS AND METHODS

3.2.1 Experiment 1. The effect of sucrose concentration on *H. ovalis* growth.

The effect of different concentrations of sucrose on the mean number of new nodes produced and chlorophyll content of *H. ovalis* was investigated. The basal medium (Chapter 2.) was supplied with different amounts of sucrose 0mM, 30mM, 60mM and 120mM. Three clones were used: 3, 6 and 12. Each treatment contained ten replicates, distributed into five culture vessels. Each explant had two nodes with two leaves per node.

Shoots were grown for a period of 14 days, then subcultured into fresh experimental media. At the end of 28 days the number of nodes was measured (Chapter 2).

3.2.2 Experiment 2 and 3. The effect of medium pH on *H. ovalis* growth.

The effect of pH and the buffer 2-(N-morpholino)ethanesulfonic acid (MES) on the growth of *H. ovalis* was investigated. Initially, the basal medium (Chapter 2.) was adjusted using 1M and 0.1M KOH or 1M and 0.1M HCl to pH 5, 6, 7, and 8 prior to autoclaving; these media were not buffered. Subsequently, these same media were used, but buffered with 10 mM

MES. An additional medium at pH 8 and unbuffered was also included. The pH was measured after autoclaving and at the end of the experiment.

In the unbuffered media three clones were used (2, 9, and 12) and in the experiment using buffered media, two clones were used (3 and 12). Each treatment contained six replicates, distributed into three culture vessels. Each explant had two nodes with two leaves per node.

In the unbuffered experiment, shoots were grown for a period of 14 days, then subcultured into fresh experimental media for another 14 days. The shoots in the buffered media remained in the experimental media for 21 days. At the end of both experiments, the mean number of new nodes and chlorophyll content was determined.

3.2.3 Experiment 4. The effect of subculture length on the growth of *H. ovalis*

The effect of subculture length on mean number of new nodes of *H. ovalis* was investigated. Four periods of time between subculture on basal medium (Chapter 2) were tested. The treatments were one, two, four and eight week intervals between subculture. Treatment one (1 week) was subcultured every week for four weeks and treatment two (2 weeks) was subcultured every two weeks for four weeks. Treatments three (4 weeks) and four (8 weeks) remained in the initial media until the end of the experiment. Two clones were used (6 and 12) and the experimental

material contained six replicates, distributed into three culture vessels, each with two explants bearing two nodes, but not leaves.

The final data recorded included the number of new nodes. It was not possible to measure chlorophyll because of a shortage of plant material.

3.2.4 Experiment 5 and 6. The effect of cytokinins on the growth of *H. ovalis* and *R. megacarpa*

The effect of cytokinins on the growth was investigated for *H. ovalis* and *R. megacarpa*. The basal medium (Chapter 2.) was supplied with 5 μ M of different cytokinin: no cytokinin (control), kinetin, 2iP, BAP or zeatin. Initially, two clones of *H. ovalis* were used (3 and 12) and in the follow up experiment two clones of *H. ovalis* were used (6 and 12) as well as one clone of *R. megacarpa*. Each treatment contained six replicates, distributed into three culture vessels. In the first experiment, each explant had two nodes without leaves. In the second experiment each explant of *H. ovalis* had two nodes with two leaves per node, whereas each explant of *R. megacarpa* had six nodes with leaves.

In the initial experiment, shoots were grown for a period of 14 days, then subcultured into fresh experimental media for another 14 days. The shoots in the second experiment were not subcultured and were scored after 14 days. At the end of both experiments, the mean number of new nodes and chlorophyll content was determined.

3.3 RESULTS

3.3.1 Experiment 1. The effect of sucrose concentration on *H. ovalis* growth.

Growth and chlorophyll levels were both significantly affected by the four treatments of sucrose (0mM, 30mM, 60mM and 120mM). There was no difference in the responses of the different clones with regard to multiplication, with the mean number of new nodes ranging from 5.7 to 7.7 when sucrose was included in the medium. For all three clones, a greater amount of growth ($P=0.000$) was obtained when sucrose was included in the medium but sucrose concentration did not affect the growth (Fig. 3.1. Scheffe's test).

A significant difference for mean chlorophyll content was observed between clones ($P=0.000$) and treatments ($P=0.000$). For clone 12 all treatments containing sucrose had the same level of chlorophyll and only the medium minus sucrose had a significant reduction in chlorophyll (Fig. 3.2C Scheffe's test). Clone 6 had significantly higher chlorophyll at 60mM sucrose (Fig. 3.2B Scheffe's test). However, clone 3 experienced the greatest mean chlorophyll content at 30mM sucrose (Fig. 3.2A Scheffe's test).

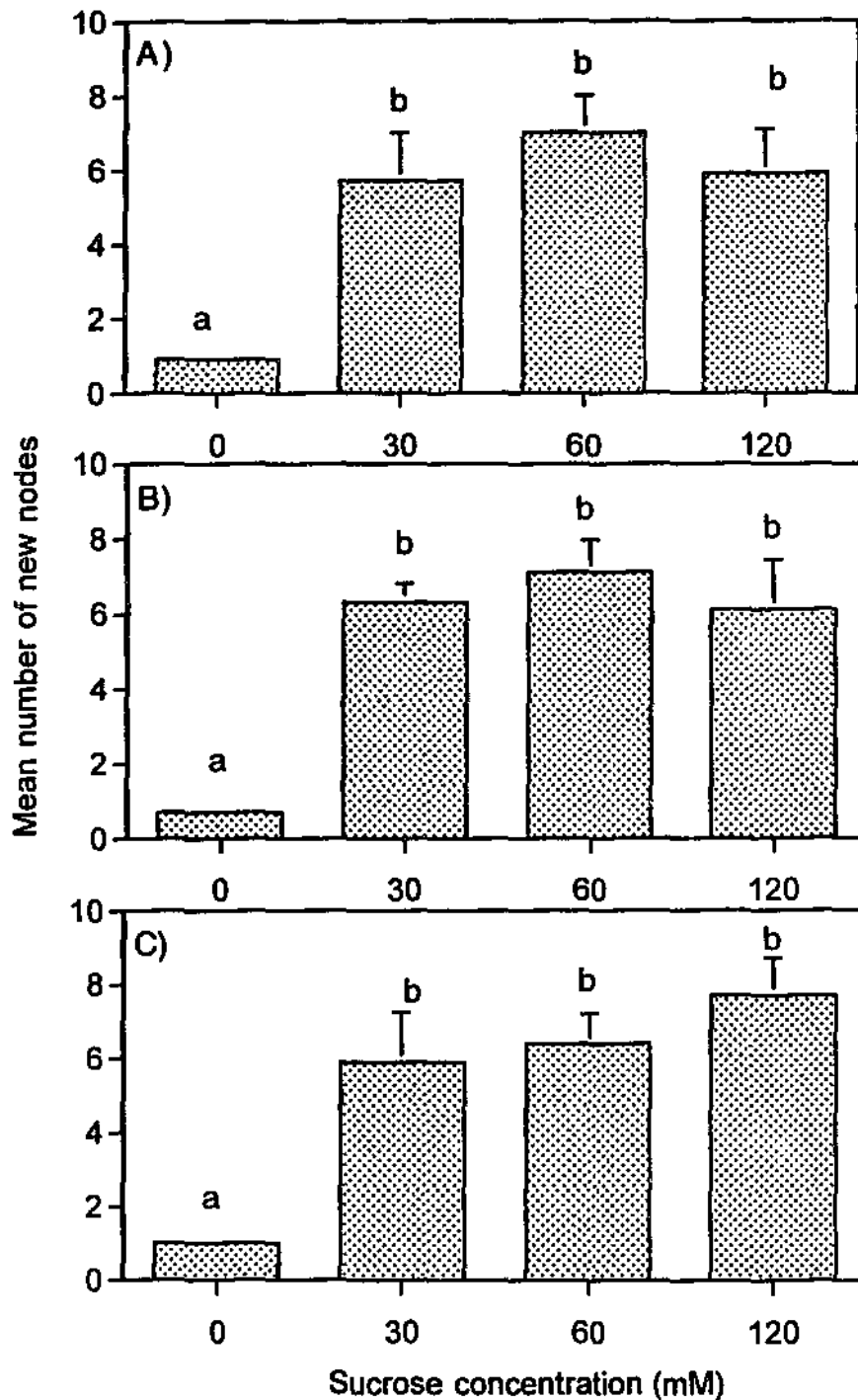


Figure 3.1. Mean number of new nodes for *H. ovalis* produced for A) clone 3, B) clone 6 and C) clone 12, grown in sucrose concentrations of 0mM, 30mM, 60mM or 120mM for 28 days. Vertical bars represent standard errors of the mean, different lower case letters indicate differences between means using Scheffe test ($P < 0.05$).

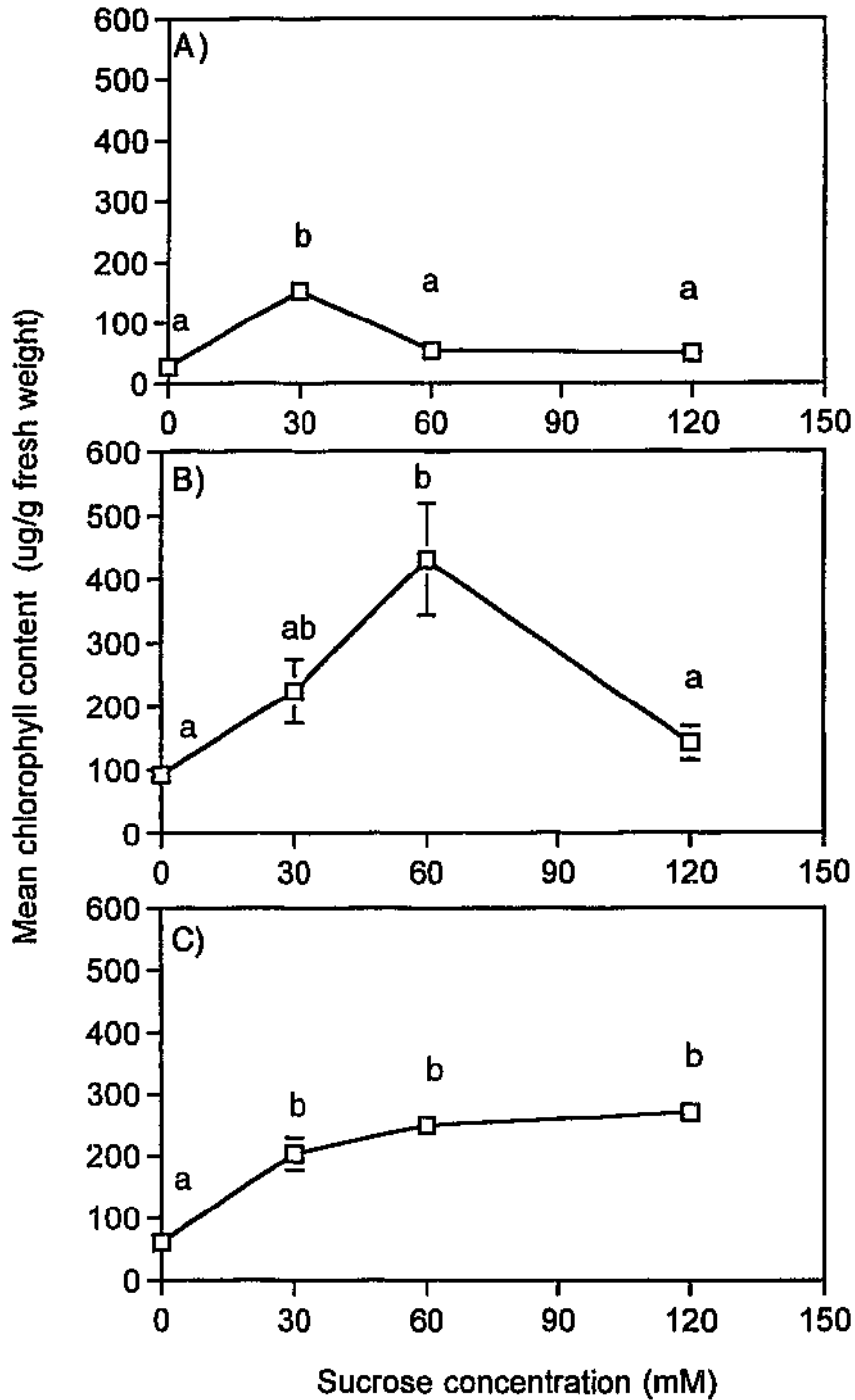


Figure 3.2. Mean chlorophyll content ($\mu\text{g/g}$ fresh weight) for *H. ovalis* of A) clone 3, B) clone 6 and C) clone 12, grown in sucrose concentrations of 0mM, 30mM, 60mM or 120mM for 28 days. Vertical bars represent standard errors of the mean, different lower case letters indicate differences between means using Scheffe test ($P < 0.05$).

3.3.2 Experiment 2. The effect of medium pH on *H. ovalis* growth.

The growth of *H. ovalis* was not significantly affected by the four pH treatments (5, 6, 7 and 8) ($P=0.056$). There was no significant difference between clones (0.299) for the mean number of new nodes (Fig. 3.3).

No significant difference was observed between clones ($P=0.115$) or between treatments ($P=0.082$) for chlorophyll content. However, both clone 2 and 9 showed the highest values in chlorophyll content above pH 7 (Fig. 3.4A and B), but for clone 12 (Fig. 3.4C), the highest value was observed above pH 6. There was very little difference in the initial medium pH after autoclaving. However, at completion of the experiment, the medium pH in all treatments fell to as low as 4 (Table 3.1.).

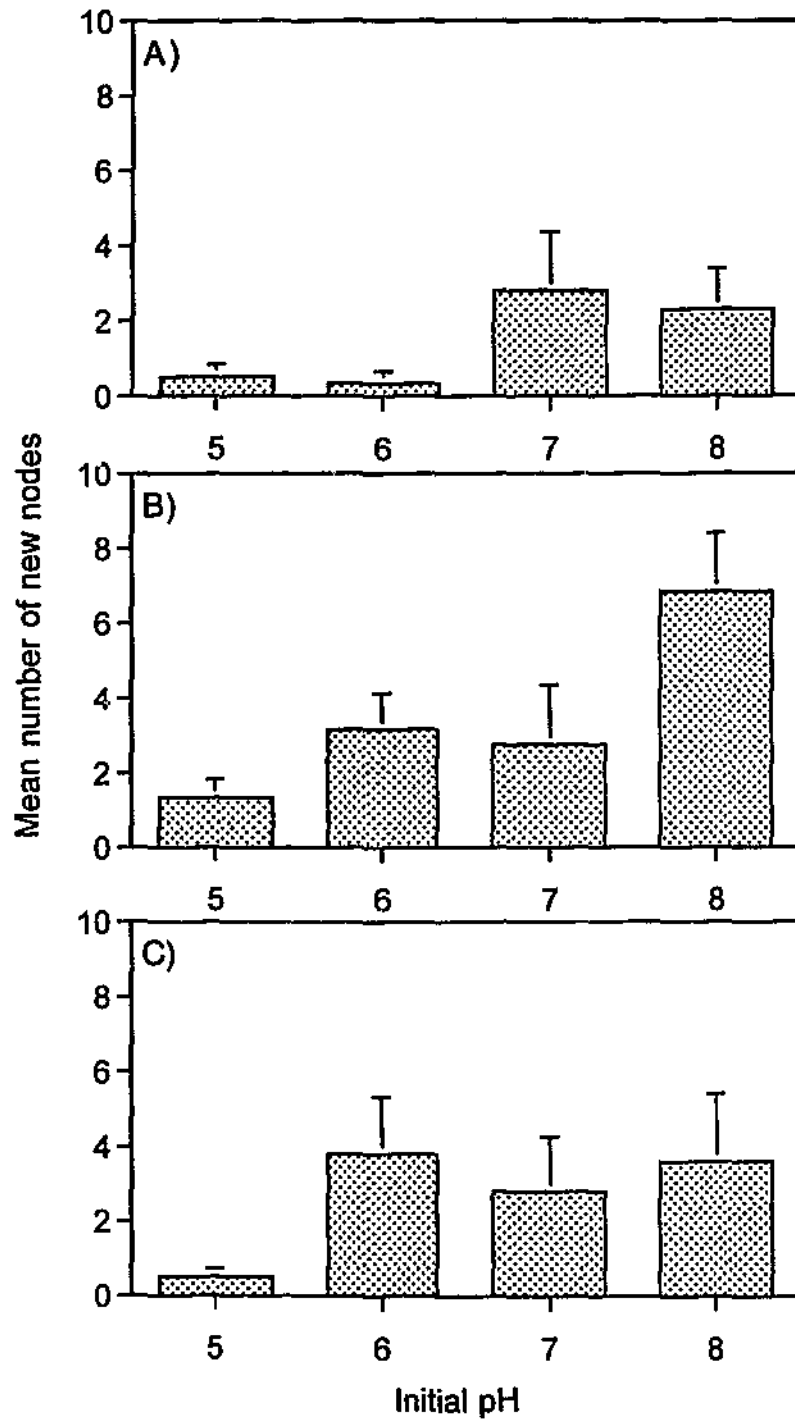


Figure 3.3. Mean number of new nodes of *H. ovalis* produced for A) clone 2, B) clone 9 and C) clone 12, grown for 28 days in unbuffered medium at pH 5, 6, 7, and 8. Vertical bars represent standard errors of the mean.

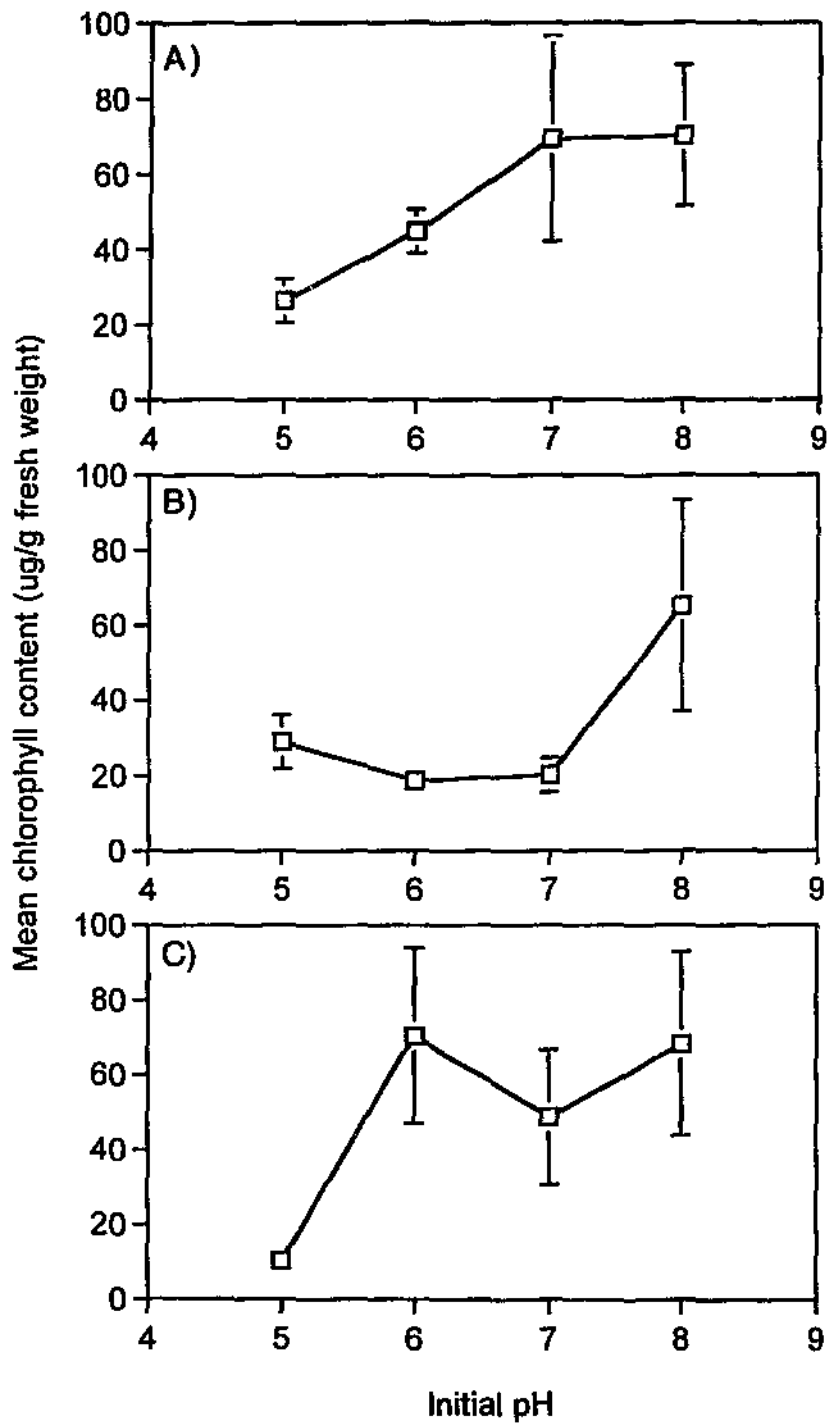


Figure 3.4. Mean chlorophyll content ($\mu\text{g/g}$ fresh weight) of *H. ovalis* for A) clone 2, B) clone 9 and C) clone 12, grown for 28 days in unbuffered medium at pH 5, 6, 7, and 8. Vertical bars represent standard errors of the mean.

Table 3.1: Unbuffered pH before and after autoclaving, and mean pH recorded at the end of the experiment.

pH (before autoclaving)	pH (after autoclaving)	pH (after experiment)
5	5.5	3.9 ± 0.04
6	6.4	3.9 ± 0.12
7	7.1	4.2 ± 0.39
8	7.4	4.0 ± 0.15

3.3.3 Experiment 3. The effect of buffered medium pH on *H. ovalis* growth.

Two way analysis of variance indicated that there was a significant difference due to treatments ($P=0.001$), but no significant difference between clones ($P=0.992$) for mean number of new nodes. Clone 3 was the only clone for which an effect of treatment was significant ($P=0.003$), with the pH 7 buffered treatment producing more new nodes than the pH 5 buffered and the pH 8 unbuffered (Fig. 3.5A Scheffe's test). For both clones the highest multiplication values were obtained on buffered media from pH 6 to 8 (Fig. 3.5A, B).

Mean chlorophyll content was significantly different between clones ($P=0.021$) and treatments ($P=0.000$). Clone 3 showed a significant difference between treatments ($P=0.000$), with the highest chlorophyll content contained on media buffered at pH 6, 7 or 8, compared to buffered pH 5 and unbuffered pH 8 (Fig. 3.6A Scheffe's test), however, no significant difference was observed for clone 12 (Fig. 3.6B; $P=0.207$).

There was little difference in the initial pH after autoclaving. However, at the completion of the experiment, the medium pH was more stable between buffered treatments pH 6 to 8, than in buffered pH 5 and unbuffered pH 8 (Table 3.2.)

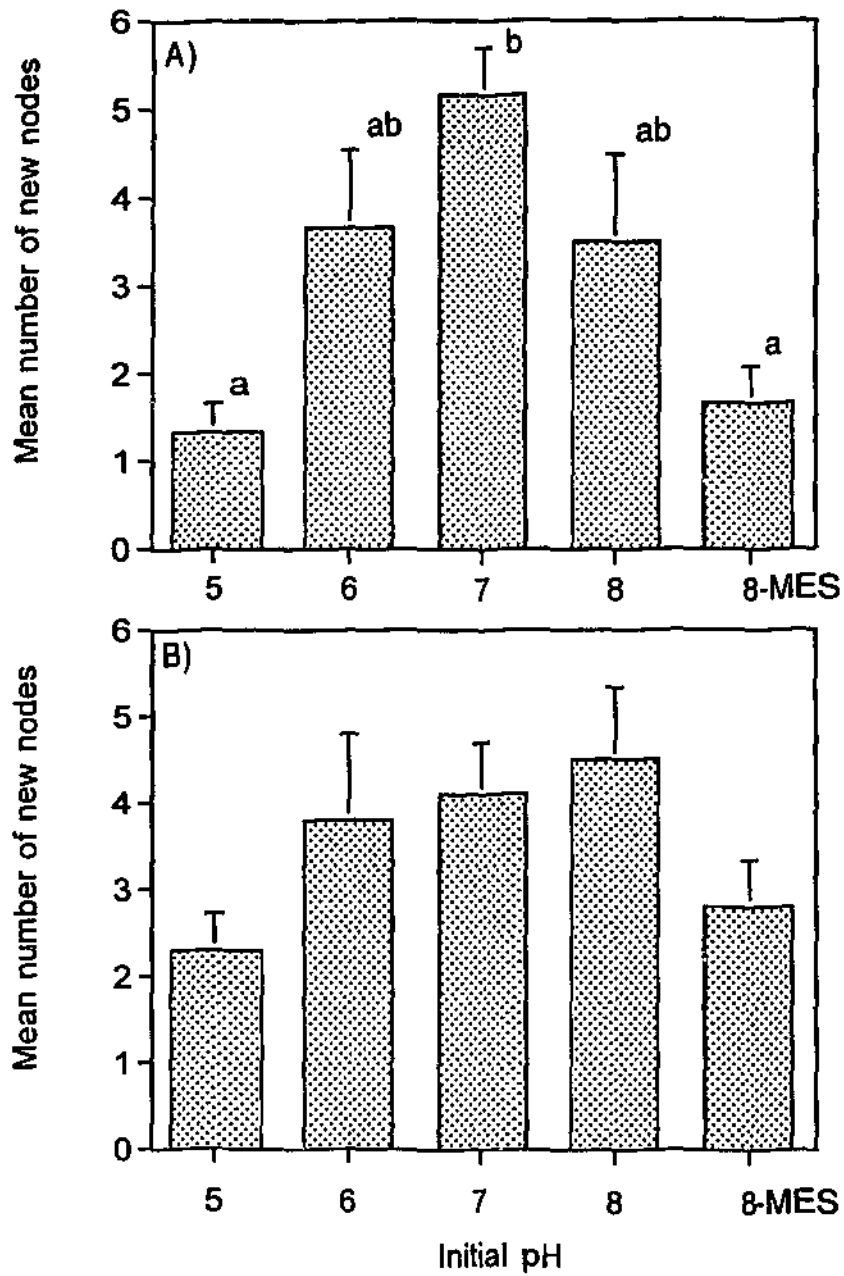


Figure 3.5. Mean number of new nodes of *H. ovalis* produced for A) clone 3 and B) clone 12, grown in buffered (10mM MES) medium pH 5, 6, 7, and 8 and unbuffered medium pH 8 for 21 days. Vertical bars represent standard errors of the mean, different lower case letters indicate differences between means using Scheffe test ($P < 0.05$).

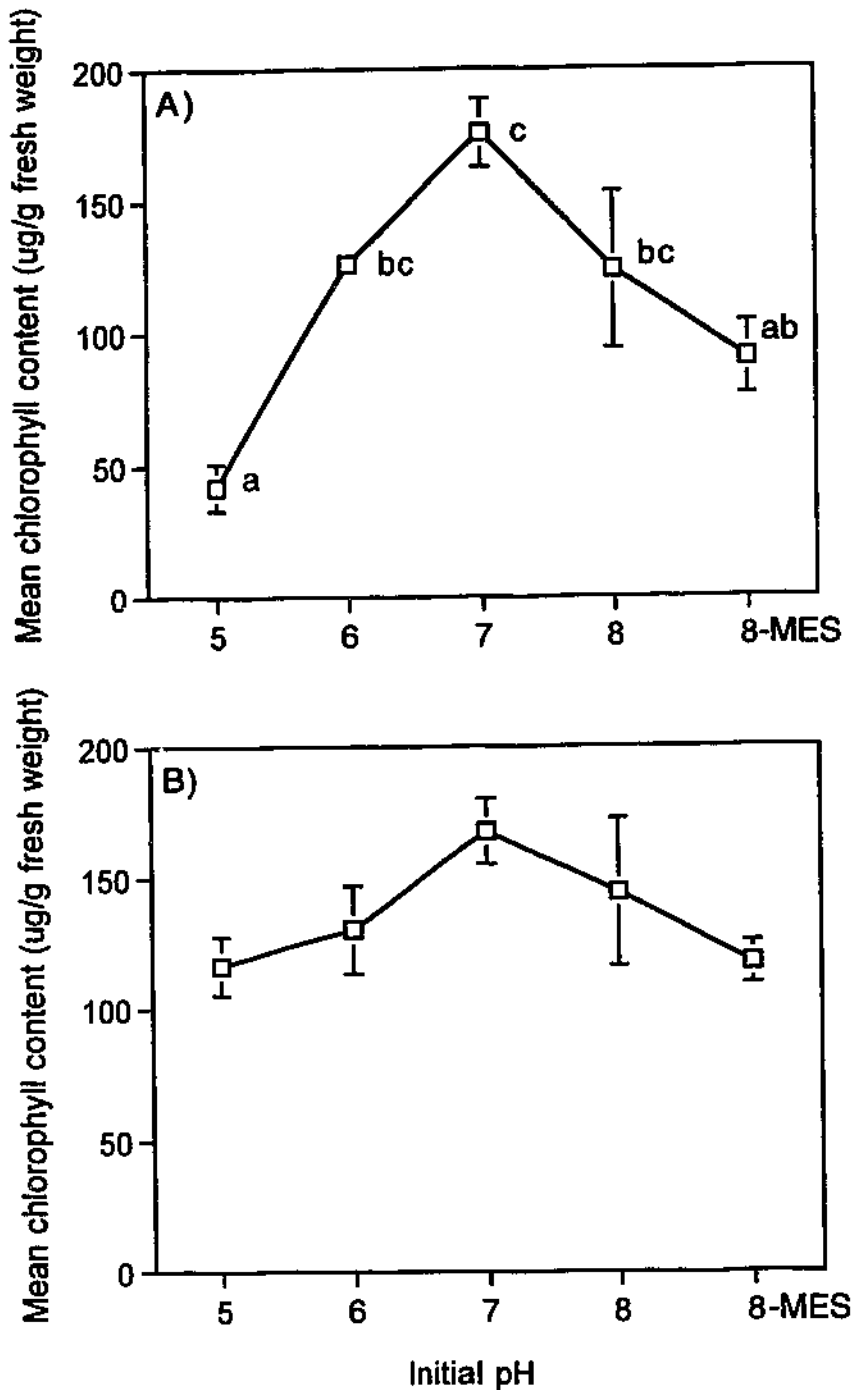


Figure 3.6. Mean chlorophyll content ($\mu\text{g/g}$ fresh weight) of *H. ovalis* produced for A) clone 3 and B) clone 12, grown in buffered (10mM MES) medium pH 5, 6, 7, and 8 and unbuffered medium pH 8 for 21 days. Vertical bars represent standard errors of the mean, different lower case letters indicate differences between means using Scheffe test ($P < 0.05$).

Table 3.2: Medium pH before and after autoclaving, and mean pH recorded at the end of the experiment when buffered (10mM MES). (*) represents unbuffered treatment.

pH (before autoclaving)	pH (after autoclaving)	pH (after experiment)
5	5.2	3.8 ± 0.08
6	6.0	5.4 ± 0.17
7	6.8	6.1 ± 0.06
8	7.1	6.1 ± 0.04
8*	7.5*	5.5* ± 0.26

3.3.4 Experiment 4. The effect of the length of time between subculture on the growth of *H. ovalis*

The length of subculture showed no significant difference between treatments ($P=0.274$) or between clones ($P=0.808$) for mean number of new nodes. With regard to multiplication (Fig. 3.7), one way analysis of variance indicated a significant difference for clone 6 ($P=0.043$), however, Scheffe's test found no difference between treatments.

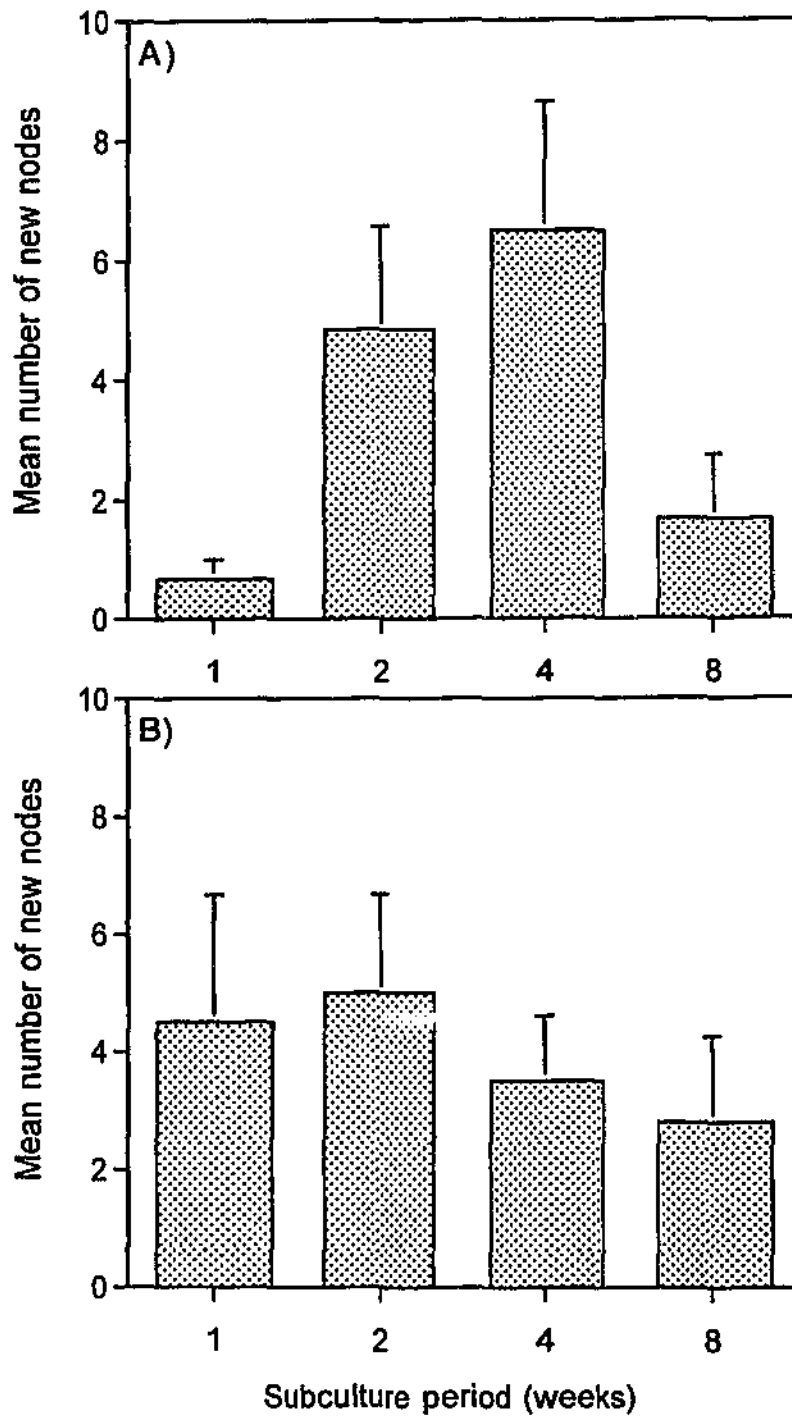


Figure 3.7. Mean number of new nodes of *H. ovalis* produced for A) clone 6 and B) clone 12, subcultured at one, two, four and eight weeks respectively. Vertical bars represent standard errors of the mean.

3.3.5 Experiment 5. The effect of cytokinins on the growth of *H. ovalis*

There was no significant difference ($P=0.177$) between the five cytokinin treatments ($0\mu\text{M}$, and $5\mu\text{M}$ kinetin, 2iP, BAP or zeatin). However, a significant difference was observed between clones 3 and 12 ($P=0.001$) with regard to multiplication, with clones 3 and 6 producing approximately 6 new nodes per explant, and clone 12 producing only 2 new nodes per explant (Fig. 3.8).

There were no significant differences observed for mean chlorophyll content between clones ($P=0.101$) or between treatments ($P=0.231$) (Fig. 3.9).

3.3.6 Experiment 6. The effect of cytokinins on the growth of *H. ovalis* and *R. megacarpa*

There was no significant difference between clones 6 and 12 ($P=0.494$) or between the five cytokinin treatments ($0\mu\text{M}$, $5\mu\text{M}$ kinetin, 2iP, BAP and zeatin) ($P=0.720$) for the mean number of new nodes produced for *H. ovalis* (Fig. 3.10A and B). Similarly, for *R. megacarpa* there was no difference between treatments ($P=0.178$) for the mean number of new nodes (Fig 3.10C).

A significant difference for mean chlorophyll content was observed between clones ($P=0.013$) but not between treatments ($P=0.278$) for *H. ovalis* (Fig. 3.11A and B). *R. megacarpa* was not observed to show a significant difference ($P=0.088$) for mean chlorophyll content within treatments (Fig. 3.11C).

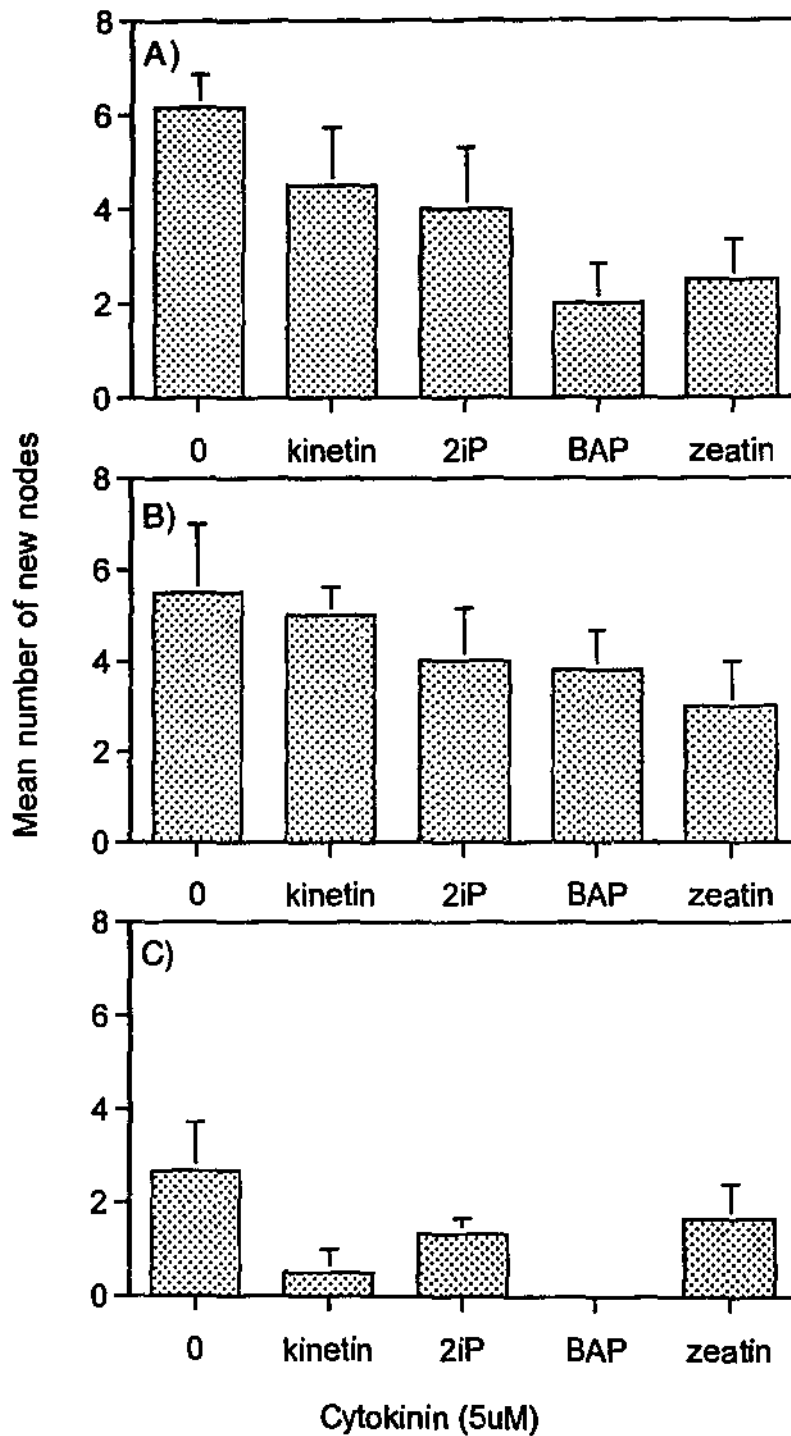


Figure 3.8. Mean number of new nodes of *H. ovalis* produced for A) clone 3, B) clone 6 and C) clone 12, grown in no cytokinin (control) and 5 μM kinetin, 2iP, BAP or zeatin for 28 days. Vertical bars represent standard errors of the mean.

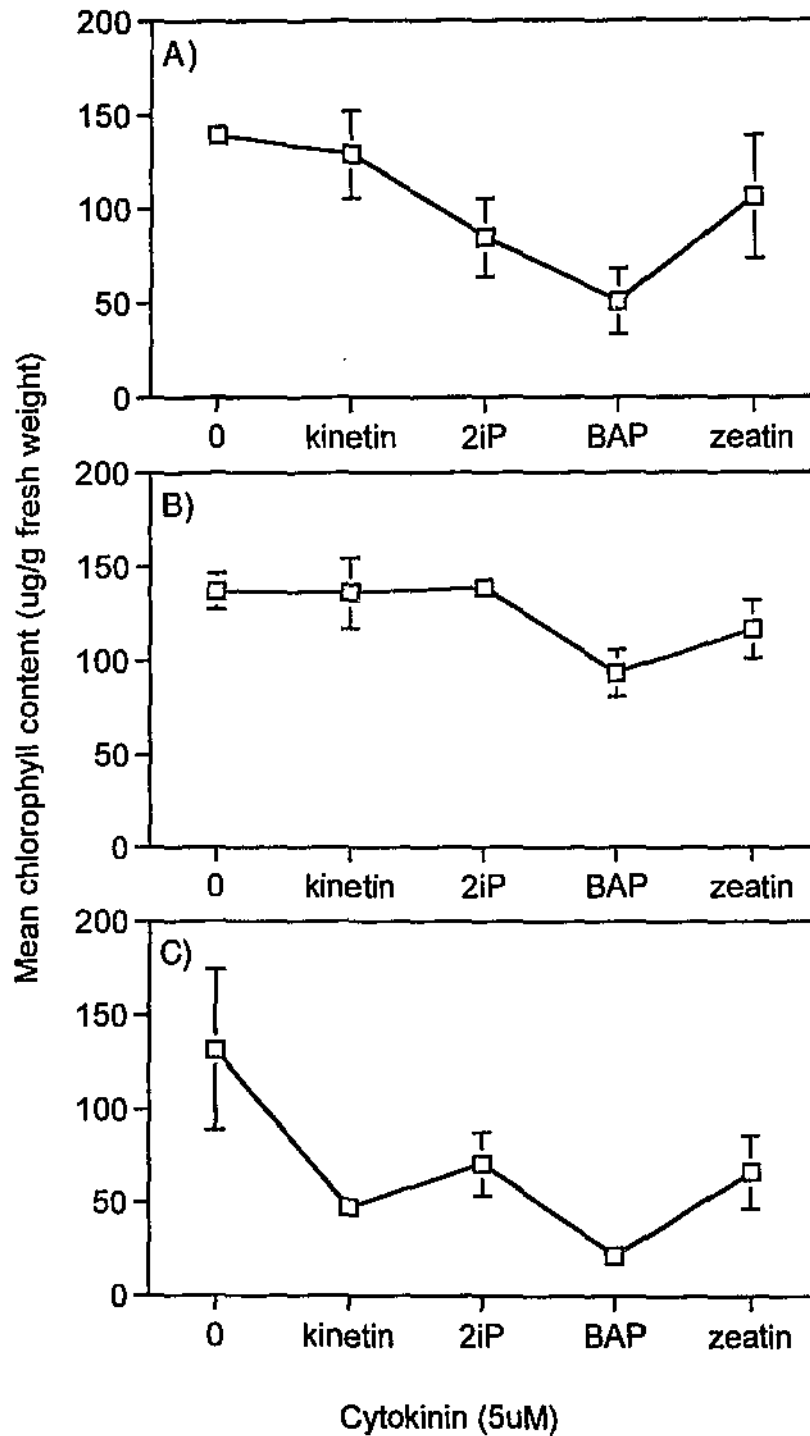


Figure 3.9. Mean chlorophyll content ($\mu\text{g/g}$ fresh weight) of *H. ovalis* produced for A) clone 3, B) clone 6 and C) clone 12, grown no cytokinin (control) and 5uM kinetin, 2iP, BAP or zeatin for 28 days. Vertical bars represent standard errors of the mean.

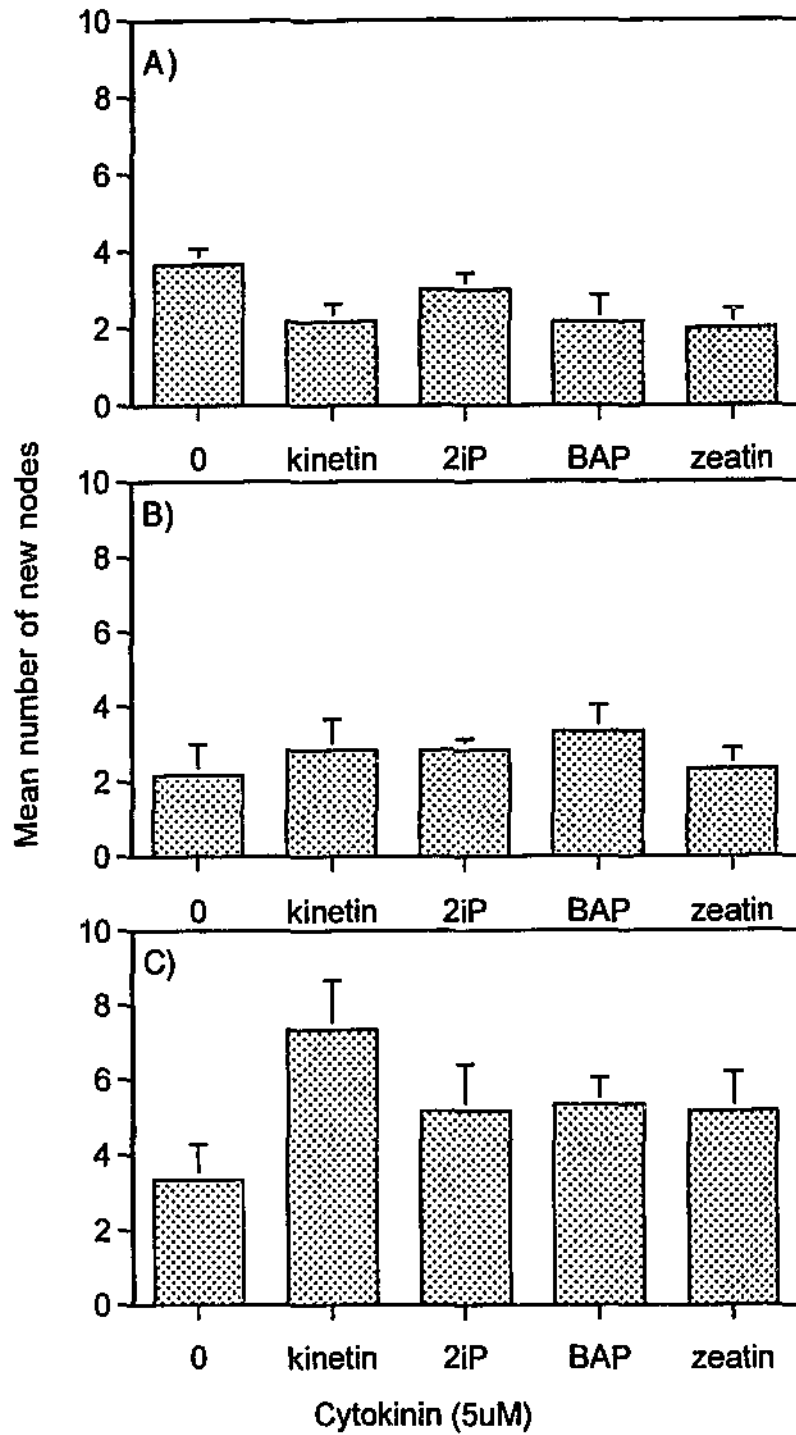


Figure 3.10. Mean number of new nodes of *H. ovalis* for A) clone 6, B) clone 12 and of C) *R. megacarpa* grown in no cytokinin (control) and 5 μ M kinetin, 2iP, BAP or zeatin for 14 days. Vertical bars represent standard errors of the mean.

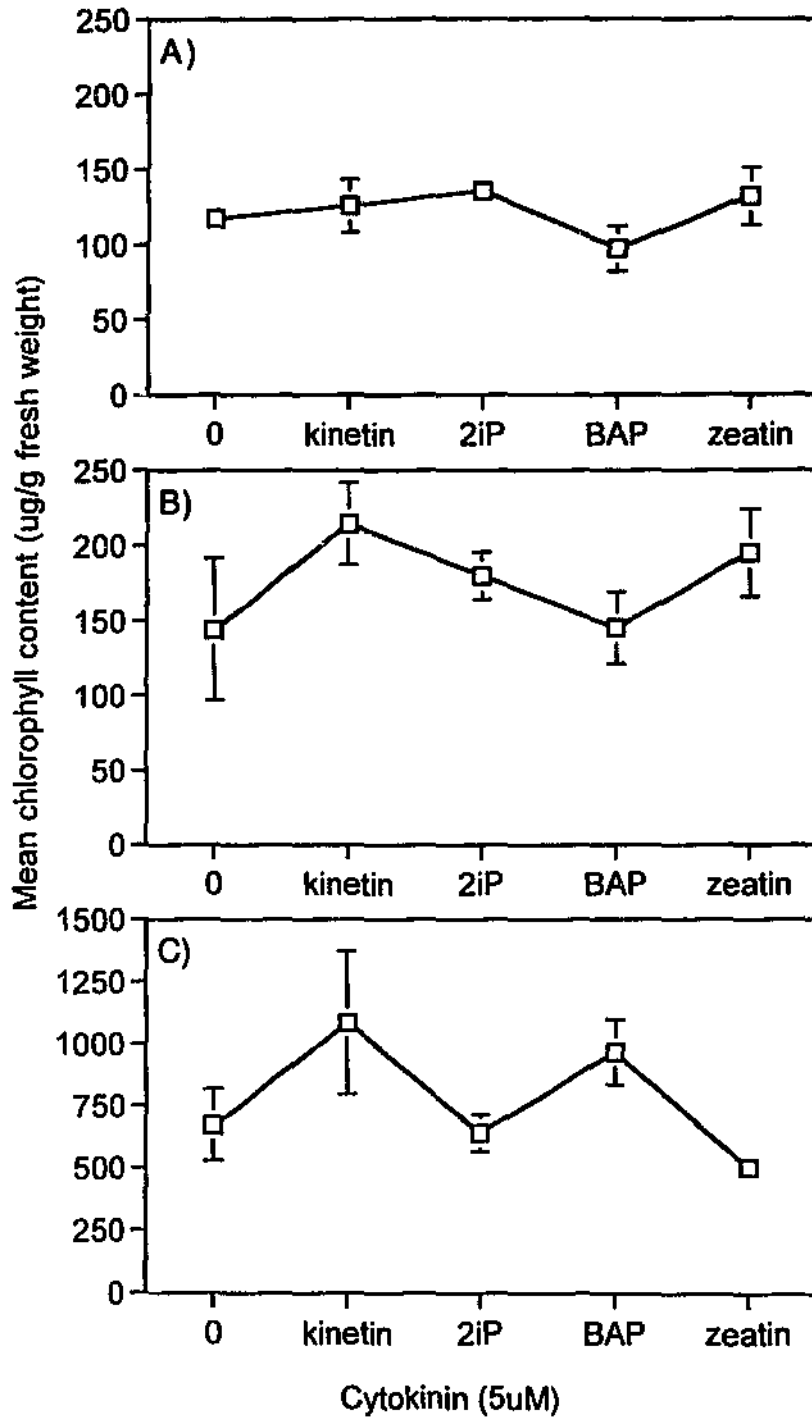


Figure 3.11. Mean chlorophyll content ($\mu\text{g/g}$ fresh weight) of *H. ovalis* for A) clone 6, B) clone 12 and of C) *R. megacarpa* grown in no cytokinin (control) and $5\mu\text{M}$ kinetin, 2iP, BAP or zeatin for 14 days. Vertical bars represent standard errors of the mean.

3.4 DISCUSSION

3.4.1 Sucrose

Increased growth was shown to be dependent on the addition of sucrose to the medium, however, there was little or no difference in growth responses to different concentrations of sucrose. Once the sucrose concentration doubled from 60mM to 120mM, growth decreased in clones 3 and 6, but increased in clone 12. This would suggest that a concentration around 60 mM sucrose is optimal for growth. Bird *et al.* (1996) found that the growth of *R. maritima* was high in medium with 2iP addition at a sucrose concentration of 29.21 mM. However, this may have been attributed to other contents of the medium, and may also have been a response specific to *R. maritima*.

The decreased growth for clones 3 and 6, and the increased growth observed for the clone 12 at the higher sucrose concentration, may have been associated with their differing abilities to adjust osmotically to the rapid increase in sucrose. There is limited published data to compare with, other than the examination by Loques *et al.* (1990) of the effect of sucrose concentrations on *P. oceanica* macromeristem survival. That study found that a concentration of 263 mM was adequate to maintain cultures for up to 4 months. However, the cultures were short-lived, which may have been due to the high sucrose concentration, and the subsequent high osmotic level of the medium.

The differences in chlorophyll content between treatments correlate with differences in growth. The variation between clones is an indication of their genetic differences, resulting in different physiological requirements for sucrose. The results signify greater health and growth of plants in medium with sucrose addition.

3.4.2 Effect of pH

The lack of effect of unbuffered pH medium on both the growth and chlorophyll content of *H. ovalis*, signifies the importance of buffer addition, in order to stabilise medium pH. The low level of medium pH observed for all treatments at the end of the experiment would have decreased the ability of the plant to take up nutrients and organics, thereby reducing both plant growth and health. This is observed in tissue culture of terrestrial plants, when pH drops from 5.8 to 4.5 (Williams 1990; Woodward & Bennett, 1996).

The stability of medium pH was dependent on the addition of buffer. Although, the level of MES buffer was only adequate in maintaining a medium pH 6.1, it was an improvement on the unbuffered medium, where medium pH was approximately 4.0 after the experiment. The greatest multiplication for both clones (3 and 12), was found in the buffered treatments pH 6-8. This was most likely because of the availability of nutrients and organics to the plants was not retarded.

The difference in chlorophyll content between treatments, again, signifies the effect of adequately buffered pH in maintaining plant fitness. The greater growth experienced at pH 6 corresponds to the higher chlorophyll content data indicative of plant health.

With regard to published data of *in vitro* seagrass culture, there is no reference to buffered medium pH, consequently there is nothing with which a comparison can be made. In the sea, pH is approximately 8 (Invers *et al.*, 1997), however, the buffer applied in this experiment was only adequate in stabilising pH at approximately 6. The pH range suitable for buffering by MES, lies between 5.5 and 6.7. Consequently, it would have been useful to compare other buffers used in plant and animal cell culture, to include: N-[2-Hydroxyethyl]piperazine-N-3-propanesulfonic acid (EPPS) (7.1-8.5), N-[2-Hydroxyethyl]piperazine-N-[2-ethanesulfonic acid] (HEPES) (7.2-8.6) or 3-[N-tris(Hydroxymethyl)methyl-amino]-2-hydroxypropane-sulfonic acid (TAPSO) (6.8-8.0), in order to find one which would stabilise medium pH 8.

3.4.3 Subculture periods

The growth of *H. ovalis* clones (6 and 12) was not affected by the time between subculture on basal medium (Chapter 2.). However, there was a visible increase in the health of plants subcultured after two weeks, in comparison to the other periods. This would suggest that the standard

culture medium was adequate to support both the growth and vigour of *H. ovalis* plants for intervals of two weeks.

3.4.4 Cytokinins

The addition of cytokinins to the medium had no significant affect on either growth or chlorophyll content of three *H. ovalis* clones and one *R. megacarpa* clone.

These results differ from those of Koch and Durako (1991) who found that cytokinin additions of kinetin, BAP, 2-iso-pentyladenine and zeatin resulted in a three to four-fold increase in the growth of *in vitro* *R. maritima*. Cultures were found to respond in a dose-dependent manner to 2-iso-pentyladenine, but in a broad dose-dependent way to the other cytokinins.

However, these results were found to be similar to the results reported by Bird *et al.* (1996). They discovered *R. maritima* growing *in vitro* did not respond to the addition of BAP in a sucrose-based medium, but they did find that 2iP stimulated greater growth than BAP. Their experiments examined concentrations ranging from 0 to 25 μ M for BAP and 2iP, however only 14.8 μ M 2iP was found to stimulate an increase in growth of cultures.

As the experiments applied in this study only examined cytokinins at a concentration of 5 μ M, this may have explained the lack of stimulation of growth of *H. ovalis* and *R. megacarpa*.

In the first test, there were problems associated with removing the leaves of *H. ovalis*, which reduced the sample size. The experiment was repeated, in order to reduce any experimental error that might have occurred in the first test, this time using both *H. ovalis* and *R. megacarpa*, with leaves intact.

There were differences observed in the first test between *H. ovalis* clones, that were not observed during the second test. However, as three clones were used in the first of the cytokinin experiments, but only two in the second cytokinin experiment, this may suggest reasons for the change in trends. The experiment using *R. megacarpa*, was restricted because the material examined was of the one clone.

The results do not comply with those found in other studies and suggest, that unlike terrestrial plants, cytokinin may not have an affect on seagrasses. If time permitted, it would have been useful to apply different concentrations of cytokinins, as further examination may have indicated an affect on growth.

These results indicate that there may be substantial differences in the requirements of seagrasses compared to terrestrial plants. The addition of

sucrose to the medium, like that of terrestrial plants, increases the rate of growth. The pH favoured by most terrestrial plants in tissue culture is 5 to 6. As this is lower than that for seagrasses, further investigation of buffers adequate to stabilise medium pH 8, is necessary. The addition of cytokinins also needs further examination, to determine if it was the concentration of cytokinins affecting growth, or whether cytokinins play a different role in seagrasses to those observed in terrestrial plants.

CHAPTER 4. *POSIDONIA CORIACEA*

4.1 INTRODUCTION

Surface sterilization procedures frequently used for terrestrial plant material have not been adequate for obtaining axenic seagrass material. Seagrasses possess an undeveloped cuticle and harbor a large diversity of surface endophytic bacteria and fungi. Consequently, surface sterilization which is non-deleterious to the plant has not been easily achieved (Koch & Durako, 1991).

Tissue culture of the genus *Posidonia* has been least developed of all the groups examined in this work. One of the major problems has been achieving sterile cultures without damage to the plant. Loques *et al.* (1990) achieved sterile macromeristems of *P. oceanica*, but the cultures died after 4 months. Accordingly, a major hurdle for the *in vitro* culture of seagrasses has been the type of plant material used in sterilization.

Success in obtaining axenic cultures of *R. maritima* (Koch & Durako, 1991), *T. testudinum* (Moffler & Durako, 1984), *H. engelmannii* (Jewett-Smith & McMillan, 1990) and *H. ovalis* for this work, has been obtained by the surface sterilization of seed.

An examination of three sterilization techniques, two of which were modified from published data, were applied in order to initiate *P. coriacea*

into axenic culture. The techniques were applied to rhizomal tissue, and the most successful of these techniques were applied to fruits with and without the pericarp intact.

4.2 MATERIALS AND METHODS

4.2.1 Experiment 1. Surface sterilization of rhizome

Sampling and storage conditions of plant material are outlined in Chapter 2.1.

The rhizomes were washed and then cut into single nodes. The nodes were placed in clean aerated artificial sea water. Three sterilization treatments were followed, each with 100 replicates of one node.

In treatment one the explants were placed in sterile milli Q water for 20 minutes and intermittently swirled, and then placed in 2% benzalkonium chloride (zephiran) in 10% ethanol for 5 minutes with swirling.

The second treatment was modified from Loques *et al.*, (1990) and Hudson (1981). The explants were placed in 70% ethanol for 30 seconds, and then in calcium hypochlorite (70g L^{-1}) for 25 minutes with intermittent swirling.

In the third treatment, the explants were placed in 70% ethanol for 30 seconds, and then 1% sodium hypochlorite for 25 minutes and periodically swirled. This technique is also a modification of Loques *et al.* (1990) and Hudson (1981).

The final steps for all three sterilization techniques involved rinsing the rhizomes three times in sterile artificial seawater, re-trimming the tissues, and placing the explants in basal medium (Chapter 2.) under sterile conditions. The cultures were maintained in a growth cabinet set at 21.5°C and monitored for contamination, survival, and growth.

4.2.2 Experiment 2. Surface sterilization of fruit

Fruits were collected as detailed in Chapter 2.1. Forty fruits were sterilized with the pericarp intact and forty fruits were sterilized without the pericarp. The third sterilization treatment (Chapter 4.2.1), was followed as it had previously given a higher number of uncontaminated cultures. The seeds were placed in basal medium (Chapter 2.) under sterile conditions and were stored in a growth cabinet set at 21.5°C. They were monitored for contamination, germination, and growth.

4.3 RESULTS

4.3.1 Experiment 1. Surface sterilization of rhizome

There was a significant difference between the three sterilization treatments ($\chi^2 = 0.05$) applied to the rhizomal tissue of *P. coriacea*. Of the 300 *P. coriacea* rhizomes sterilized, 23 appeared sterile. The greatest number of sterile cultures was obtained using treatment three (12%), followed by one (8%) and treatment 2 (3%) detailed in Table 4.1. However, there was a large degree of bacterial contamination overall. The cultures were discarded approximately one month after treatment as no signs of growth were observed, and the tissue appeared dead.

4.3.2 Experiment 2. Surface sterilization of fruit

A significant difference was observed between the two sterilization treatments ($\chi^2 = 0.05$) applied to *P. coriacea* seed. The greatest sterility was obtained in the first treatment, after the seed was sterilized with the pericarp intact (62.5%), whereas contamination was observed in all of the seeds sterilized without the pericarp (Table 4.2.). The contamination observed appeared to be bacterial.

In the first treatment, 19 of the uncontaminated seeds germinated. To date, 13 have remained alive and have continued to grow leaves. They are subcultured on a fortnightly basis onto basal medium (Chapter 2.). The

other six cultures experienced a browning of the leaves and ceased growing.

Of the 40 seeds from the second treatment, six germinated, however these became contaminated within a week of sterilization. In another trial, 275 seeds were sterilized using the second treatment, however none germinated and eventually became contaminated.

Table 4.1: Mean percentage contamination (% contamination) of *Posidonia coriacea* rhizomes using three sterilization treatments of 100 replicates each. There was a significant difference between treatments ($\chi^2 = 0.05$) one month after sterilization.

Treatment	% Contamination
1	92
2	97
3	88

Table 4.2: Mean percentage contamination (% contamination) of *Posidonia coriacea* seeds using two sterilization treatments of 40 replicates each. There was a significant difference between treatments ($\chi^2 = 0.05$) recorded one month after sterilization.

Treatment	% Contamination
1	62.5
2	100

4.4. DISCUSSION

4.4.1. Surface sterilization of rhizome

The first and third treatments were most suitable in obtaining axenic *P. coriacea* cultures using the rhizome material. However, the rhizomes were not successful in initiating *P. coriacea* in tissue culture. Unlike seed, the rhizome material has no protective barrier to prevent harm to the internal tissues. These results do not compare with Loques *et al.* (1990), in which they initiated *P. oceanica* in tissue culture by the surface sterilization of macromeristems. However, the cultures only survived for four months. This may have been as a direct result of the plant material used, as the rhizome material may not be able to tolerate those concentrations of chemicals applied during the sterilization procedure.

4.4.2. Surface sterilization of fruit

The most suitable material in obtaining axenic cultures of *P. coriacea* was the seed. Seeds of *P. coriacea* are surrounded by a fleshy pericarp which protects the embryo from the deleterious affects associated with vigorous sterilization. This has also been found to be a successful mechanism of initiating axenic cultures of *R. maritima* (Koch & Durako, 1991), *T. testudinum* (Moffler & Durako, 1984), *H. engelmannii* (Jewett-Smith & McMillan, 1990), and *H. ovalis* for this work.

The seed sterilized without the pericarp would have come in contact with bacteria and fungi, once the pericarp was removed, which may have survived the sterilization procedures. The seed sterilized with the pericarp intact had more protection against the chemicals applied in the sterilization process and would have also to a lesser degree been exposed to possible bacterial and fungal infection.

These results indicate that there are substantial differences in the type of plant material used to initiate seagrasses in tissue culture. The rhizome material was not successful in obtaining sterile cultures of *P. coriacea*, nor was seed that was sterilized without the pericarp. However, successful axenic cultures were obtained by sterilizing seed with the pericarp intact.

CHAPTER 5. GENERAL DISCUSSION

This work illustrates that there are both differences and similarities between the tissue culture requirements for the marine seagrasses, and those that are suitable for the tissue culture of terrestrial plants. The similarities can be found in the range of responses that were observed between clones of *H. ovalis*, sucrose requirements and the relative ease of establishment from seed compared to rhizomes. Differences appear in the frequency of subculturing required, the effects of cytokinins on axillary bud stimulation and, perhaps most importantly, the need to stabilise a relatively high pH in the culture medium.

Differences between clones were obtained in experiments on sucrose. This raises a question that may arise in the further development of a protocol for this group of plants; how much should the protocol be adjusted for individual clones? This is a major problem for the tissue culture of many terrestrial plants as, in many cases, considerable effort is put into producing elite clones. At this stage of research into seagrasses, this should not cause too many problems as there has not been any extensive development of the genetic material. In addition, it will be a major step forward simply to be able to reliably grow some seagrasses for whatever purpose they are needed.

The sucrose concentrations required for seagrasses appear to be in line with what might be expected for other plants, as they are usually in the

range of 60-90mM. There does not appear to be any justification for the high levels used by Loques *et al.* (1990), as they found that a concentration of 263mM sucrose was adequate in maintaining macromeristem cultures of *P. oceanica*. However, this may have caused osmotic shock, as the cultures only survived for four months. The limited published data makes it difficult to compare these findings.

The variation in pH is the component of this work that appears to vary the greatest from that of work on other plants. The responses in unbuffered media is similar to that seen in other plants where there is a drop (often very rapid) in the pH down to the values of about pH 4. For many terrestrial plants *in vitro*, this is not important and the required responses can be obtained despite this drop. However, in terrestrial plants that have in the past been considered to be recalcitrant, this is beginning to be investigated as a way to overcome their recalcitrance (George, 1993; Woodward and Bennett, 1996). The drop that is experienced in these plants from an initial pH of between 5 and 6 is not nearly as great as the drop experienced in the tissue culture of these seagrasses which have an optimum pH around 8. The drop to pH 4 is likely to have substantial effects on the capacity of the tissues/explants to survive and grow. While, in many cases, this causes problems with regard to nutrient absorption this is not likely to be the case in liquid media, however, it is likely to be significant with regard to many of the metabolic activities that are affected by pH.

Unlike terrestrial *in vitro* plant cultures (prefer pH 5 to 6), it appears that aquatic marine plants *in vitro* grow best at a higher pH. This is most likely due to the environmental conditions normally experienced in the field by seagrasses. As the pH of the sea is approximately 8, it would not be optimal for the seagrasses at a lower pH, and this would retard the availability of nutrients and organics to the plant. Further experiments could examine buffers which would be better equipped to stabilise a medium of pH 8.

Frequency of subculture might be expected to be from 4 to 6 weeks for most plants. The more frequent subculture of these seagrasses may be due to the rapid growth (multiplication rates of six times as high; a multiplication rate of three times is considered to be the minimum for commercial production of other plants (Hartney & Barker, 1983). It has also been shown in other plants that cultures grown in liquid medium rather than solid medium more rapidly utilise the nutrients in the medium (George 1993; George 1996). This may lead to faster growth but obviously leads to the need for more frequent subculture.

The apparent lack of response to cytokinins in the medium was unexpected as these have been reported to increase growth in related species. This may be due to the lack of concentrations examined but these were similar to those that were reported before. The ranges that are used for tissue culture, and in particular axillary bud stimulation vary greatly. George (1996) illustrates that ranges used for terrestrial plants can

be from about 1 μM to 73 μM . Clearly, there is a requirement to further investigate of the use of cytokinins for seagrass multiplication.

As found by others (Koch & Durako, 1991; Jewett-Smith & McMillan, 1990; Moffler & Durako, 1984), the most successful plant material to initiate seagrasses in tissue culture, has been found with the fruits. These results comply with those found in this examination. *P. coriacea* axenic cultures were achieved by the surface sterilization of seed with the pericarp intact. The success with this method is believed to be as a consequence of the pericarp protecting the internal tissues of the seed.

This may also have to do with the juvenile plant tissue being better suited to overcome the stabilisation period. Most plants once initiated in culture pass through a period of stabilisation (George, 1993; George, 1996). This requires that they adapt to the environmental conditions associated with *in vitro* culture. This phase is usually markedly reduced for explants taken from juvenile tissues and therefore it might be expected that growth from the seed of *Posidonia* will more rapidly obtain this stabilised state.

5.1 Concluding Remarks

These trials endeavoured to determine whether there were differences in the tissue culture of terrestrial plants, to that of seagrasses. They indicated that there is potential to develop different tissue culture practices for seagrasses, to those in place for terrestrial plants. In particular, the ability

to buffer medium pH to a higher level than that for terrestrial plants, appeared to be an important factor for future research. Of other relevance to seagrasses, was the application of cytokinins to promote growth. This needs to be investigated more fully.

REFERENCES

- Balestri, E., Piazza, L. and Cinelli, F. (1998). In vitro germination and seedling development of *Posidonia oceanica*. *Aquatic Botany*. **60**, 83-93.
- Bird, K.T. and Jewett-Smith, J. (1994). Development of a medium and culture system for in vitro propagation of the seagrass *Halophila engelmannii*. *Canadian Journal Botany*. **72**, 1503-1510.
- Bird, K.T., Jewett-Smith, J. and Fonseca, M.S. (1994). Use of *in vitro* propagated *Ruppia maritima* for seagrass meadow restoration. *Journal of Coastal Research*. **10**, 732- 737.
- Bird, K.T., Brown, M.S., Henderson, T.T., O'Hara, C.E. and Robbie, J.M. (1996). Culture studies of *Ruppia Maritima* L. in bicarbonate and sucrose-based media. *Journal of Experimental Marine Biology and Ecology*. **199**, 153-164.
- Blaber, S.J.M. and Blaber, T.G., (1980). Factors affecting the distribution of juvenile estuarine and inshore fish. *Journal of Fish Biology* **17**, 143-62.

Cambridge, M.L. (1979). Cockburn Sound Technical Report on Seagrass. Report No. 7. Department of Conservation and Environment, Perth, Western Australia.

Cambridge, M.L. and McComb, A.J. (1984). The loss of seagrasses in Cockburn Sound, Western Australia 1. The time course and magnitude of seagrass decline in relation to industrial development. *Aquatic Botany*. **20**, 229-243.

Cambridge, M.L., Chiffings, A.W., Brittan, C., Moore, E. and McComb, A.J. (1986). The loss of seagrasses in Cockburn Sound, Western Australia 2. Possible cause of seagrass decline. *Aquatic Botany*. **24**, 269-285.

Carruthers, T.J.B. and Walker, D.I. (1997). Light climate and energy flow in the seagrass canopy of *Amphibolis griffithii* (J.M. Black) den Hartog. *Oecologia*. **109**, 335-341.

Clarke, S.M. and Kirkman, H. (1989). Seagrass dynamics. 304-334. In A.W. Larkum, A.J. McComb and S.C. Shepherd (Eds.). *The biology of seagrasses: a treatise on the biology of seagrasses with special reference to the Australian region*. *Aquatic plant studies 2*. Amsterdam: Elsevier Science Publishers B.V.

- Cronquist, A.J. (1981). *An integrated system of classification of flowering plants*. New York: Columbia Univ. Press.
- George, E.F. (1993). *Plant propagation by tissue culture. Part 1. The technology*. Edington: Exegetics Ltd.
- George, E.F. (1996). *Plant propagation by tissue culture. Part 2. In practice*. Edington: Exegetics Ltd.
- Gordon, D.M., Chase, S.C., Grey, K.A. and Simpson, C.J. (1994). *Changes to the structure and productivity of a Posidonia sinuosa seagrass meadow in Princess Royal Harbour, Western Australia, during and after imposed shading*. Technical Series no. 50. Environmental Protection Authority, Perth, Western Australia.
- Hartney, V.J. and Barker, P.K. (1983). The vegetative propagation of eucalypts by tissue culture. *Silviculturae*. **8**, 791-792.
- Hillman, K., Lukatelich, R.J., Bastyan, G. and McComb, A.J. (1991). *Water quality and seagrass biomass, productivity and epiphyte load in Princess Royal Harbour, Oyster Harbour and King George Sound*. Technical Series no. 39. Environmental Protection Authority, Perth, Western Australia.

- Hudson, D.M. (1981). *Salinity tolerance and tissue culture of some Australian seagrasses*. Honours thesis, Murdoch University, Perth, Western Australia.
- Invers, O., Romero, J. and Perez, M. (1997). Effects of pH on seagrass photosynthesis: a laboratory and field assessment. *Aquatic Botany*. **59**, 185-194.
- Jewett-Smith, J. and McMillan, C. (1990). Germination and seedling development of *Halophila engelmannii* Aschers. (Hydrocharitaceae) under axenic conditions. *Aquatic Botany*. **36**, 167-177.
- King, R.J., Adam, P. and Kuo, J. (1990). Seagrasses, mangroves and saltmarsh plants. In M.N. Clayton and R.J. King (Eds.). *Biology of marine plants*. Melbourne, Australia: Longman Cheshire Pty Ltd.
- Kirkman, H. (1989). *Restoration and creation of seagrass meadows with special emphasis on Western Australia*. Technical series no. 30. Environmental Protection Authority, Perth, Western Australia.
- Kirkman, H. and Kuo, J. (1990). Pattern and process in southern Western Australian seagrasses. *Aquatic Botany*. **37**, 367-382.

Kirkman, H. and Walker, D.I. (1989). Regional studies - Western Australian Seagrass. 157-178. In A.W. Larkum, A.J. McComb and S.C. Shepherd (Eds.). *The biology of seagrasses: a treatise on the biology of seagrasses with special reference to the Australian region. Aquatic plant studies 2*. Amsterdam: Elsevier Science Publishers B.V.

Klump, D.W., Howard, R.K. and Pollard, D.A. (1989). Trophodynamics and nutritional ecology of seagrass communities. 394-437. In A.W. Larkum, A.J. McComb and S.C. Shepherd (Eds.). *The biology of seagrasses: a treatise on the biology of seagrasses with special reference to the Australian region. Aquatic plant studies 2*. Amsterdam: Elsevier Science Publishers B.V.

Koch, E.W. and Durako, M.J. (1991). In vitro studies of the submerged angiosperm *Ruppia maritima*: auxin and cytokinin effects on plant growth and development. *Marine Biology*. **110**, 1-6.

Kuo, J. and McComb, A.J. (1989). Seagrass taxonomy, structure and development. 6-67. In A.W. Larkum, A.J. McComb and S.C. Shepherd (Eds.). *The biology of seagrasses: a treatise on the biology of seagrasses with special reference to the Australian region. Aquatic plant studies 2*. Amsterdam: Elsevier Science Publishers B.V.

Lanyon, J.M., Limpus, C.J. and Marsh, H. (1989). Dugongs and turtles: grazers in the seagrass system. 610-627. In A.W. Larkum, A.J. McComb and S.C. Shepherd (Eds.). *The biology of seagrasses: a treatise on the biology of seagrasses with special reference to the Australian region. Aquatic plant studies 2*. Amsterdam: Elsevier Science Publishers B.V.

Larkum, A.W.D. and den Hartog, C. (1989). Evolution and Biogeography of seagrasses. 112-145. In A.W. Larkum, A.J. McComb and S.C. Shepherd (Eds.). *The biology of seagrasses: a treatise on the biology of seagrasses with special reference to the Australian region. Aquatic plant studies 2*. Amsterdam: Elsevier Science Publishers B.V.

Lenanton, R.C.J. (1982). Alternative non-estuarine nursery habitats for some commercially and recreationally important fish species of south-western Australia. *Australian Journal of Marine Freshwater Research*. **33**, 881-900.

Loques, F., Caye, G. and Meinesz, A. (1990). Axenic culture of selected tissue of *Posidonia oceanica*. *Aquatic Botany*. **37**, 171-188.

Masini, R.J., Cary, J.L., Simpson, C.J. and McComb, A.J. (1990). *Effects of light and temperature on the photosynthesis of seagrasses, epiphytes and macroalgae and implications for management of the Albany harbours*. Technical Series no. 32. Environmental Protection Authority, Perth, Western Australia.

McConchie, C.A. and Knox, R.B. (1989). Pollination and Reproductive Biology of Seagrasses. 74-101. In A.W. Larkum, A.J. McComb and S.C. Shepherd (Eds.). *The biology of seagrasses: a treatise on the biology of seagrasses with special reference to the Australian region. Aquatic plant studies 2*. Amsterdam: Elsevier Science Publishers B.V.

McMillan, C. (1980). Culture methods. In R.C. Phillips and C.P. McRoy (Eds.) *Handbook of seagrass biology: an ecosystem perspective*. New York: Garland STPM Press.

McMillan, C. (1987). Seed germination and seedling morphology of the seagrass, *Halophila engelmannii* (Hydrocharitaceae). *Aquatic Botany*. **28**, 179-188.

McMillan, C. (1988). Seed germination and seedling development of *Halophila decipiens* Ostenfeld (Hydrocharitaceae) from Panama. *Aquatic Botany*. **31**, 169-176.

- Meinesz, A., Caye, G., Loques, F. and Molenaar, H. (1991). Growth and development in culture of orthotropic rhizomes of *Posidonia oceanica*. *Aquatic Botany*. **39**, 367-377.
- Moffler, M.D. and Durako, M.J. (1984). Axenic culture of *Thalassia testudinum* Banks Ex König (Hydrocharitaceae). *American Journal of Botany*. **71** (10), 1455-1460.
- Moran, R. and Porath, D. (1980). Chlorophyll determination in intact tissue using N,N-dimethylformamide. *Plant Physiology*. **65**, 478-479.
- Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiologia Plantarum*. **15**, 473-497.
- Orth, R.J. and Van Montfrans, J. (1984). Epiphyte-seagrass relationships with an emphasis on the role of micrograzers: a review. *Aquatic Botany*. **18**, 43-69.
- Poiner, I.R., Walker, D.I. and Coles, R.G. (1989). Regional Studies - Seagrasses of tropical Australia. 279-296. In A.W. Larkum, A.J. McComb and S.C. Shepherd (Eds.). *The biology of seagrasses: a treatise on the biology of seagrasses with special reference to the Australian region. Aquatic plant studies 2*. Amsterdam: Elsevier Science Publishers B.V.

Scott, J.K., Dybdahl, R. and Wood, W.F. (1986). *The ecology of Posidonia seagrass fish communities in Cockburn Sound, Western Australia*. Technical Series no. 11. Department of Conservation and Environment Perth, Western Australia.

Shepherd, S.A., McComb, A.J., Bulthuis, D.A., Neverauskas, V., Steffensen, D.A. and West, R. (1989). Decline of Seagrasses. 346-387. In A.W. Larkum, A.J. McComb and S.C. Shepherd (Eds.). *The biology of seagrasses: a treatise on the biology of seagrasses with special reference to the Australian region. Aquatic plant studies 2*. Amsterdam: Elsevier Science Publishers B.V.

Silberstein, K., Chiffings, A.W. and McComb, A.J. (1986). The loss of seagrass in Cockburn Sound, Western Australia. 3. The effects of epiphytes on productivity of *Posidonia australis* Hook. F. *Aquatic Botany*. **24**, 355-371.

Stapel, J. and Hemminga, M.A. (1997). Nutrient resorption from seagrass leaves. *Marine Biology*. **128**, 197-206.

Thorhaug, A. (1986). Review of seagrass restoration. *Ambio*. **15** (2), 110-117.

- . . -

Walker, D.I. (1989). Regional Studies - Seagrass in Shark Bay, the Foundations of an Ecosystem. 182- 206. In A.W. Larkum, A.J. McComb and S.C. Shepherd (Eds.). *The biology of seagrasses: a treatise on the biology of seagrasses with special reference to the Australian region. Aquatic plant studies 2*. Amsterdam: Elsevier Science Publishers B.V.

Walker, D.I., and McComb, A.J., (1992). Seagrass degradation in Australian Coastal Waters. *Marine Pollution Bulletin*. **25**, 191-195.

Walker, D.I., Sim, C.B. and Penniford, M.G. (1994). The effects of light and temperature on growth of the seagrasses *Posidonia sinuosa* and *Amphibolis antarctica* in culture. 22-53. In D.I. Walker, E. Paling, C.B. Sim, M.G. Pennifield, C. Manning and H. Kirkman. *Relationship between nitrogen and primary production. Perth coastal waters study project E3.1*. Water Authority of Western Australia, Perth.

West, R.J., and Larkum, A.W.D. (1983). Seagrass primary production - a review. *Proceedings of the Linnean Society of N.S.W.* **106** (3), 213-223.

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

Woodward, A.J. and Bennett, I.J. (1996). Optimisation of nitrogen content for micropropagation of *Eucalyptus marginata*. Pp 251-254. In: A. Taji, A. and Williams, R. (eds.). Tissue culture: Towards the next century. Proceedings of the 5th International Association of Plant Tissue Culture.