Effects of Commercial Kelp Extract and Plant Growth Regulators on Growth of *Gracilaria gracilis* in Culture

by

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DECLARATION

I declare that this thesis is my own work, unaided work. Experimental work discussed in this thesis was carried out under the supervision of Professor J.J. Bolton of the Department of Botany, University of Cape Town, and Dr. Rob Anderson of the Seaweed Unit, Marine and Coastal Management, Cape Town.

Material presented here is all original work by the author and has not been submitted in this or any other form to another University. Where use has been made of research of others, it has been duly acknowledged in the text.

Signed by candidate

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September 2005

DEDICATION

For my dad, no longer with us, for his inspiration and for my mother for her encouragement, inspiration, patience and loving support.

CONTENTS

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ABSTRACT

The addition of a local commercial seaweed extract (Kelpak®) to crop plants has proven to be beneficial as it improves growth and yields. Its efficiency has been attributed to its production method that involves a cold process, resulting in a product containing significant amounts of plant growth regulators (auxins and cytokinins). The aim of this study was therefore to investigate the effects of this commercial seaweed concentrate (Kelpak®) on the growth of *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine *et* Farnham, with a view to the potential in mariculture, especially as this red seaweed is currently under cultivation in South Africa as feed in abalone aquaculture.

A laboratory experiment was carried out by growing excised 5 mm, 10 mm and 15 mm apical segments of *G. gracilis* in culture dishes containing Provasoli Enriched Seawater (ES) medium to which various concentrations of Kelpak® concentrate were added. Treatment of *G. gracilis* apical segments in ES medium with 1:1000 Kelpak® dilution gave the significantly higher growth rate, after 15 days in culture, independently of the initial length of *G. gracilis* apical segments. Apical segments of different initial lengths growing in ES medium with 1:500 Kelpak® dilution had the second highest increase in growth compared to the control. Kelpak® at a dilution of 1:2500 also improved the growth of *G. gracilis* significantly, irrespective of the initial length of the apical segments. The most concentrated Kelpak® dilution of 1:100 inhibited growth of *G. gracilis* apical segments of different initial length. A further experiment was conducted to determine if the plant growth regulators present in Kelpak® would have the same effect on the growth of *G. gracilis* apical segments as Kelpak®. The effects of the auxin indole-3-acetic acid

(IAA) and the cytokinin 6-benzylaminopurine (BA), both singly and in combination, on the growth of *G. gracilis* apical segments over a range of concentrations, equivalent to the reported levels of auxins and cytokinins in experimental Kelpak® dilutions, were investigated. Auxin applied alone at a dilution of 1:1000 and 1:500 significantly improved growth of *G. gracilis* apical segments. Cytokinin tested alone at a dilution of 1:1000 caused significant increase in growth of *G. gracilis* apical segments. The combined auxin and cytokinin treatments significantly increased growth of *G. gracilis* apical segments when applied at 1:1000 and 1:500 dilutions. *G. gracilis* apical segments growing in combined auxin and cytokinin dilutions, as well as in cytokinin dilutions, had higher growth rates than apical segments growing in auxin dilutions.

Lastly, the effect of various dilutions of Kelpak® concentrate on the growth of *G. gracilis* in abalone wastewater was investigated experimentally at Jacobsbaai Sea Products Ltd abalone farm on the South African west coast. *G. gracilis* was grown in effluent water from commercial abalone (*Haliotis midae*) culture. After 15 days, a pronounced inhibitory effect on the yield and on the specific growth rate of *G. gracilis* was observed with the application of the strongest Kelpak® concentrate dilution (1:100), but there was no difference between growth rates in the other treatments or the controls. After 30 days, there were no significant differences between growth rates in any of the treatments or the controls, indicating no apparent effect of different dilutions of Kelpak®. Thereafter the experiment was discontinued as *G. gracilis* colour changed from red to pale yellow, indicating possible nutrient limitation in the experimental conditions. The significance of these findings and the possible relationship between effect of Kelpak® concentrate and plant growth regulators present in Kelpak® concentrate on growth of *G. gracilis* growth is discussed.

GENERAL INTRODUCTION AND OBJECTIVES

There is a long history of seaweeds being used by humans. Ancient Chinese literature dating back thousands of years records the consumption of seaweeds by humans and this practice continues today. In the west, seaweeds have historically been used as soil fertilizers and animal fodder. Since the Twentieth Century, seaweeds have been harvested for their hydrocolloids that are incorporated into many foods, pharmaceutical and cosmetic products. Today, approximately 1 million tones of wet seaweed are harvested annually and extracted to produce hydrocolloids. Total hydrocolloid production is in the region of 55 000 tones per year, with a value of US $585 million (FAO, 2004). Furthermore, over the last 20 years a number of large projects have investigated the possibility to use seaweeds as an indirect source of fuel. That is, to grow large quantities of seaweed in the ocean and then ferment the biomass to generate methane gas for use as fuel (Israel *et al.,* in press).

Members of the genus *Gracilaria* (*sensu lato*) are among the most important algae for the production of agar, a commercially useful polysaccharide. Furthermore, they have been used for human consumption, mostly in salads and soups (Arasaki and Arasaki, 1983), as feed for marine invertebrates aquaculture, such as abalone (Ajisaka and Chiang, 1993), as likely candidates for nutrient removal in waste-water (Fralick *et al*., 1981) and in biomass production for energy (Ryther *et al*., 1979; Hanisak and Ryther, 1986; Flowers and Bird, 1990). Of all of these, their use as raw material for agar production is by far the most significant and the basis of a multimillion-dollar industry (Oliveira *et al*., 2000). The large and increasing international demand for *Gracilaria* as a raw material has necessitated a shift away from utilization of limited

natural stocks towards cultivation of *Gracilaria* on a large scale (Oliveira *et al*., 2000). The development of *Gracilaria* cultivation has probably also been stimulated by the failure to cultivate other groups of agarophytes, namely the Gelidiales that are known to produce a more valuable colloid (McHugh, 1991). Successful commercial *Gracilaria* farming ventures are currently established in many parts of the world including Chile, Venezuela, Namibia, Taiwan and China, and a wide variety of cultivation methods have been employed (Dawes, 1995), including intensive cultivation in tanks, cultivation ponds and cultivation in the sea. The characteristics of *Gracilaria* that make them desirable for cultivation are fast growth rates, good agar yield, quality, and the relative ease of cultivation (Buschmann *et al*., 1995). However, the most important attribute is that almost all cultivated *Gracilaria* species reproduce solely through fragmentation resulting in a high regenerative capacity (Hurtado-Ponce, 1990; Santelices and Varela, 1995).

The biofiltering capacity and the vegetative form of reproduction of *Gracilaria* make it a suitable candidate for mariculture. South Africa, however, has few protected areas, bays and lagoons, where aquaculture can take place in situ, and in some of the protected sites, there is conflicting use of the area (Wakibia *et al*., 2001). On the west coast, harmful algal blooms can lead to the production of H_2S that can even kill seaweeds and have serious implications for mariculture (Pitcher, 1999). Therefore, the potential for successful cultivation of *Gracilaria* is in land-based facilities. In South Africa, growth of commercial abalone farming is creating an increasing demand for freshly harvested kelp (*Ecklonia maxima* (Osbeck) Papenfuss), a common brown seaweed species. The abalone is fed mainly on fresh kelp as it improves the flesh taste, therefore kelp harvests have increased in the last eight years

(Anderson *et al*., 2003a). The abalone farms are currently increasing their capacity hence increase the productivity, leading to a greater demand on fresh kelp. Studies on cultured abalone have indicated that abalone in South Africa exhibit an increase in growth when the diet is supplemented with other algae, such as *Gracilaria* or *Ulva* (Simpson and Cook, 1998). Therefore, farms should cultivate *Gracilaria* or *Ulva* to supply part of their own seaweed requirements. This has already begun on two abalone farms on the South African south coast, where no fresh kelp is available for feed due to the absence of kelp beds in the region. One of the farms is 'Wild Coast Abalone' at Haga Haga near East London, which currently grow more than one ton of *Ulva* per working day using abalone effluent medium. The seaweed is then used as feed for the abalone. An objective of the present study was to increase the growth and yield of *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine *et* Farnham grown commercially as abalone feed.

In agriculture, liquid seaweed extracts are used extensively as plant growth supplements. With the rising popularity of organic farming, the usefulness of these products has become more widely recognized. The liquid seaweed extract can be produced in concentrated form for dilution by the user, and it can either be applied directly onto the plants or it can be watered in around the root areas. Several scientific studies have proved the effectiveness of these products, and seaweed extracts are now widely accepted in the horticultural industry. When applied to fruit, vegetable and flower crops, improvements have included higher yields, improved seed germination and growth, increased uptake of soil nutrients, increased resistance to frost, fungal and insect attack, reduced storage loss in fruit and increased nutrient uptake from the soil (Mooney and van Staden, 1986). The reasons for the effectiveness of the seaweed

extracts are still unclear. The presence of trace elements has been put forward as a possible explanation. However, in view of the low rates of application necessary to elicit a physiological response, it has been suggested that organic compounds rather than mineral elements are responsible for yield increases. Recent research has shown that seaweed products contain certain plant growth regulators and at present, many observed effects are ascribed to these constituents. That plant growth regulators, and in particular cytokinins and auxins, may be involved was suggested by Booth (1966). This conclusion was reached as many of the responses obtained from seaweed application were found to be similar to those following the application of plant growth regulators to plants. Further evidence supporting this hypothesis was the detection of cytokinin-like activity in a number of marine algae and later in commercial seaweed preparations (Brain *et al.*, 1973; Mooney and van Staden, 1987; Crouch and van Staden, 1993; Stirk and van Staden, 1997).

While cytokinins and auxins in liquid seaweed extracts may contribute to increased biomass production of terrestrial plants, less is known about the effectiveness of extracts on the growth of seaweeds. Based on this conclusion, the objective of this study was to determine the effects of different dilutions of the commercial seaweed concentrate Kelpak® on the growth of *Gracilaria gracilis* under controlled conditions in the laboratory. The plant growth regulators, auxins and cytokinins, have been identified in Kelpak® concentrate and are thought to be the active ingredients causing improved growth in land plants. Therefore, the applications of plant growth regulators with concentrations identical to the ones present in Kelpak® concentrate might have similar effects on *Gracilaria gracilis*. A secondary objective of this study was to investigate the feasibility of using nutrient-enriched

abalone effluent water in combination with different Kelpak® concentrate dilutions to grow *Gracilaria gracilis* in culture tanks in a pilot scale aquaculture system on an abalone farm.

CHAPTER 1

LITERATURE REVIEW

1.1. RHODOPHYTA

The family Gracilariaceae, Phylum Rhodophyta, order Gracilariales, *sensu* Fredericq and Hommersand (1989a, 1989b), includes the genera *Gracilaria* Greville, *Gracilariopsis* Dawson, and *Polycavernosa* Chiang and Xia (Bird and Kain, 1995). Red algae (Rhodophyta), as the name suggests, are characterized primarily by a rosy, purplish or reddish brown colour, attributable to the presence of the phycobilin pigments phycocyanin, phycoerythrin and allophycocyanin. The red coloration of these plants varies considerably according to the amount of light and nutrients available to the plant, with shaded, nutrient-replete tissue being darker while well illuminated, nutrient-depleted fronds are lighter or greenish to yellowish. Phycoerythrin masks the green colour of chlorophyll a, a major photosynthetic pigment. Photo-destruction of phycoerythrin may cause red algae to exhibit a wide range of colours including violet, yellow and green (Bold and Wynne, 1985). Furthermore, in a few species, the phycobilin pigments may be present in such small quantities that chlorophyll predominates, the alga appears green and may be interpreted as a polymorphic colour variant that is genetically determined (Plastino *et al.*, 1999). This phenomenon is also occasionally found in individuals of normally red species because of genetic mutation (Bird and McLachlan, 1992). Such green plants of *Gracilaria gracilis* have been found on occasion in Saldanha Bay, South Africa (Govender, 2001).

The basic classification of algae is based on pigmentation, storage products, mode of reproduction, life history and the presence or absence of flagellate stages. Bold and Wynne (1985) noted that the division Rhodophyta could be distinguished from the other groups of macroalgae by the following combination of characteristics:

i. complete absence of flagellate states;
ii. presence of accessory photosynthetic pigments called phycobilins;
iii. occurrence of non-aggregated photosynthetic lamellae or thylakoids within the chloroplasts;
iv. algal cells containing starch grains in the cytoplasm, referred to as floridean starch.

Red algae demonstrate considerable structural diversity and various degrees of complexity between species. Almost all are multicellular, ranging from delicate filaments to crustose, foliose or frondose forms (Bird and McLachlan, 1992). Like all algae, *Gracilaria* species have a relatively simple organisation of thallus compared to terrestrial plants. Though multicellular, these macroalgae do not have differentiated true leaf, stem nor root structures. Oliveira and Plastino (1994) described the genus *Gracilaria* as, 'macroscopic algae, with terete thallus, compressed or flattened to foliose, branching dichotomous, alternate to irregular, little to extensively branched, structurally composed of a solid pseudoparenchyma, with large medullary cells and gradual sharp transition to smaller subcortical and cortical cells'. Red seaweeds have distinctive nutrient reserves and cell wall polysaccharides. Agar polymers are present in the cell walls of *Gracilaria* species. The major biological function of agars is to maintain the integrity of the algal cells and provide mechanical strength to the algal thalli, hence the alga being referred to as an agarophyte (McLachlan, 1985). Dixon

(1973) concluded that 'it is not generally appreciated how little is actually known about most marine and freshwater algae and the Rhodophyta is probably the least well known of any algal group'.

1.2. TAXONOMY OF *GRACILARIA*

The Gracilariaceae is an important component of the flora on most tropical to cool temperate shores. Due to the increased commercial interest in phycocolloids, the Gracilariaceae, an important source of agar and a potential feed for abalone in aquaculture (Anderson *et al.*, 2003a), has been the subject of much research in recent decades throughout the world, resulting in detailed generic descriptions (Fredericq and Hommersand, 1989a, 1989b; Gurgel *et al.*, 2003). Diagnostic characters at the generic level in Gracilariaceae are predominantly based on morphological and developmental characters of the female reproductive system (Iyer *et al.*, 2004). However, some studies (Bird *et al.*, 1986; Abbott *et al.,* 1991) have shown that these features are not reliable for infrageneric discrimination (Iyer *et al.,* 2004). Furthermore, gross morphological characters have been the main means of identification and incorrect applications have led to a number of misidentifications of species within the Gracilariaceae (Iyer *et al.,* 2004, 2005b).

More than 150 species of *Gracilaria* are currently recognized (Iyer *et al.,* 2005a), however, interpretation of the genus has been described as chaotic (Bird *et al.,* 1982; Bird and McLachlan, 1984; Fredericq and Hommersand, 1989b; Bird and Rice, 1990; Oliveira and Plastino, 1994; Steentoft *et al.,* 1991). Species recognition is problematic due to instances where apparently similar morphologies, described as one species, in

reality contain more than one distinct species or genus (Bird *et al.,* 1992; Fredericq and Hommersand, 1989a, 1989b). *Gracilaria* species are particularly difficult in their taxonomy due to (according to Abbott, 1983):

i. poorly understood species limits;
ii. large amount of variation in morphological features selected for taxonomy;
iii. large numbers of taxa mostly previously studied in narrow geographic ranges;
iv. misapplication of species names due to the lack of reference to type specimens.

Based on these conclusions, DNA sequence analysis has been the most used molecular technique to understand phylogenetic relationships at the species level within the Gracilariaceae (Iyer, 2002; Iyer *et al.,* 2005a).

In past years, *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine *et* Farnham was known as *Gracilaria confervoides* (L.) Greville (Steentoft *et al.,* 1995) in a number of countries, including South Africa. The terete British material was referred to as *Gracilaria confervoides*, and was regarded as the type species of *Gracilaria*. *Gracilaria confervoides* was later renamed *Gracilaria verrucosa* (Hudson) Papenfuss (Papenfuss, 1950). Steentoft *et al*. (1991) determined that the lectotype of *Gracilaria confervoides* is in fact a *Gracilariopsis*. Furthermore, according to Steentoft *et al*. (1995) *Gracilaria gracilis* and *Gracilariopsis longissima* (S. Gmelin) Steentoft, Irvine *et* Farnham, are superficially similar species which had long been confused under the same name *Gracilaria verrucosa* in Britain. In order to prevent multiple renaming of species, of both *Gracilaria* and *Gracilariopsis*, and to achieve nomenclature stability, Steentoft *et al*. (1991) proposed the conservation of *Gracilaria compressa* (C. Agardh) Greville as lectotype of the genus *Gracilaria*. The specific epithet *verrucosa*

was rejected with the neotypification of *Gracilaria longissima* (Gmelin) Steentoft, Irvine *et* Farnham (Steentoft *et al*., 1995). Dawson (1949) separated *Gracilaria* from *Gracilariopsis* on the following basis:

1. *Gracilaria*: gonimoblast irregular, consisting of a few, large vacuolated cells;
   Gracilariopsis: dome-like gonimoblast consisting of many small, no vacuolated cells.
2. *Gracilaria*: sparse protoplasm;
   Gracilariopsis: dense protoplasm.
3. *Gracilaria*: carposporangia in clusters and short chains;
   Gracilariopsis: carposporangia in well-marked radiating chains.
4. *Gracilaria*: nutritive filaments present;
   Gracilariopsis: nutritive filament absent.

Studies by Fredericq and Hommersand (1989a, 1989b), based on the characters identified by Dawson (1949) and several others, distinguished between terete *Gracilaria* (as *Gracilaria verrucosa*) and *Gracilariopsis* (*Gracilariopsis lemaneiformis*), which they recorded for the first time in the British Isles. These studies clearly indicate that terete British gracilarioids (terete, stringy, highly branched members of the Gracilariaceae) have been regularly misidentified and there has been some confusion in the application of the genera *Gracilaria* and *Gracilariopsis*. Following detailed work on British gracilarioid taxa, the name *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine *et* Farnham was provided for *Gracilaria* species that had been confused under the name *Gracilaria verrucosa* (Steentoft *et al*., 1995). Subsequent to molecular work using 18S rDNA sequence data of Bird *et al*. (1992), Bird and Kain (1995) suggested that southern African

gracilarioids should also be assigned to *Gracilaria gracilis* (formerly *G. verrucosa*). This led Stegenga *et al*. (1997) to record two species of terete Gracilariaceae on the South African west coast, *Gracilaria gracilis* and *Gracilariopsis lemaneiformis* (Bory) Dawson, Acleto *et* Foldvik. However, South African *Gracilariopsis*, previously referred to as *Gracilariopsis lemaneiformis*, was confirmed to be conspecific with European *Gracilariopsis longissima* (Gurgel *et al*., 2003; Iyer *et al*., 2005a, 2005b). Furthermore, Iyer *et al*. (2005a) described a new species of *Gracilariopsis* from Southern Africa, *Gracilariopsis funicularis* Iyer, Bolton *et* Coyne, based on morphological and anatomical data.

It has been demonstrated (Iyer, 2002; Iyer *et al*., 2005a) that both *Gracilaria gracilis* and *Gracilariopsis longissima* are present in South Africa and had been previously described as a single species under the names *Gracilaria confervoides* and more recently *Gracilaria verrucosa* (Isaac, 1956; Simons, 1977). Studies by Govender (2001) based on 18S rDNA sequences demonstrated that the two gracilarioid species, *Gracilaria gracilis* and *Gracilariopsis longissima* co-exist within the Saldanha Bay-Langebaan Lagoon systems and St. Helena Bay. *Gracilaria* from Saldanha Bay has been described by Simons (1977) as consisting of 'ramifying, stringy streamers and looks like branching, reddish-brown bootlaces'. This description also applies to many other species of *Gracilaria* and to some species of *Gracilariopsis*. *Gracilaria gracilis* can be distinguished from *Gracilariopsis longissima* by characters of the female reproductive structures described by Fredericq and Hommersand (1989a, 1989b). However, the morphological plasticity of the Gracilariaceae and the rarity of fertile material often led to confusion about the precise

identity of material determined by anatomical methods, causing a hindrance to algal taxonomists (Bird, 1995; Fredericq and Hommersand, 1989a, 1989b).

A recent morphological and taxonomic study by Iyer *et al.* (2004) provided a comprehensive reappraisal and revision of the South African species and recognized only two *Gracilariopsis* species and nine *Gracilaria* species. Furthermore, based on molecular evidence, the southern African gracilarioid complex (stringy, terete, elongate members of the Gracilariaceae) was resolved into three species: *Gracilaria gracilis*, *Gracilariopsis longissima* and *Gracilariopsis funicularis* (Iyer *et al.*, 2005b).

1.3. LIFE CYCLE OF *GRACILARIA*

Red seaweeds typically have complex haploid-diploid life cycles that are sometimes referred to as triphasic. The three phases are a diploid (tetrasporophyte) phase, a haploid (gametophyte), usually dioecious phase and an additional diploid, zygote-derived sporangium (carposporophyte) phase (Engel *et al.*, 2001). *Gracilaria* has a life history that follows the basic pattern of most red algae, the three-phase '*Polysiphonia*-type' (McLachlan and Edelstein, 1977), with an alternation of morphologically inseparable yet genetically distinct generations, with separated sexes in the gametophyte phase (Kain and Destombe, 1995) (Figure 1.1).

As in all sexual life cycles, the three phases are interconnected by meiosis and syngamy. The gametophyte and tetrasporophyte stages are independent; and the carposporophyte stage develops on the gametophyte thallus. Fertilization (syngamy) occurs on the female gametophyte and the female gamete fertilized by spermatium

(male gamete) develops into a parasitic carposporophyte. Cystocarps are macroscopic hemispherical swellings observed on the surface of female branches, within which the carposporophyte produces thousands of diploid carpospores, which are released into the environment. The carpospores attach to a substratum and each carpospore can develop into a diploid tetrasporophyte. Finally, completing the cycle, meiosis takes place on the tetrasporophyte plants, giving rise to haploid tetraspores. The tetraspores develop into gametophytes that produce gametes by mitosis.

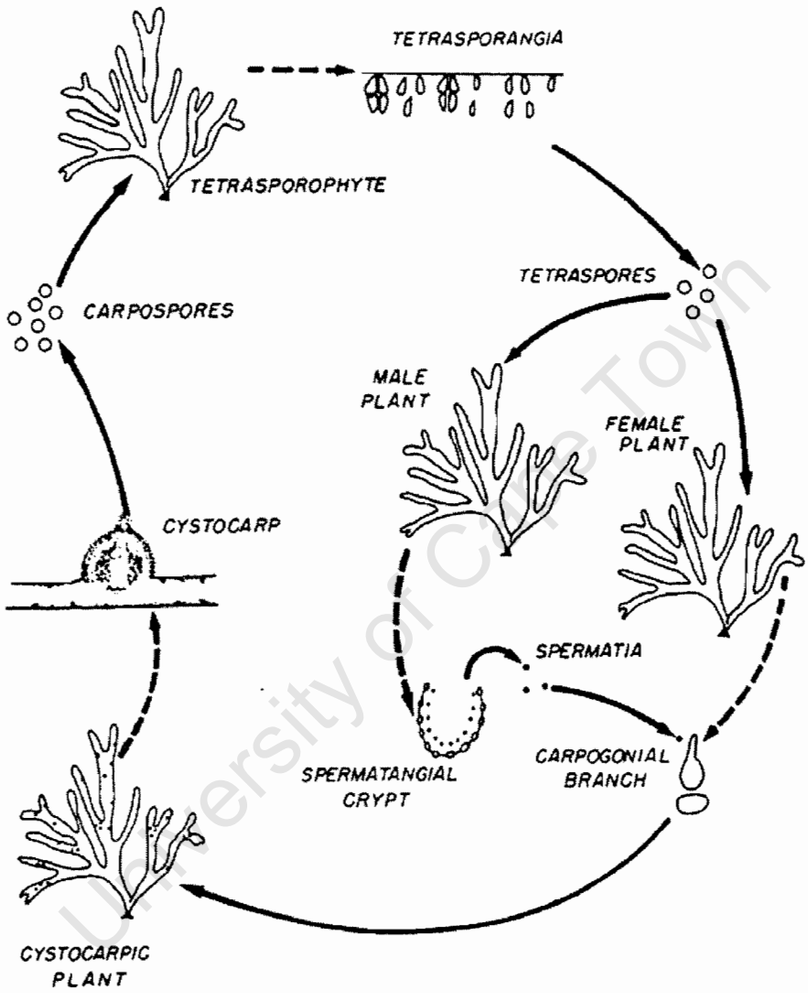


Figure 1.1. A diagram of a *Polysiphonia*-type life history as reported in most species of *Gracilaria* (Rhodophyta, Gracilariales). (From Oliveira and Plastino, 1994).

1.4. BIOGEOGRAPHY AND ECOLOGY OF *GRACILARIA*

Members of the genus *Gracilaria* are widely distributed geographically, with the majority of species concentrated in the warmer waters of the northern hemisphere and with no more than a few species (*Gracilaria chilensis* Bird, McLachlan *et* Oliveira and *Gracilaria gracilis*) established in more temperate waters (Oliveira and Plastino, 1994; Oliveira *et al*., 2000). The distribution of *Gracilaria* species in the world, extends in the Atlantic Ocean from South Africa (Papenfuss, 1940) to Norway (Rueness *et al*., 1987) and from Argentina to Canada (Oliveira, 1984); in the Pacific Ocean from New Zealand (Nelson, 1987) and Australia (May, 1948) to northern Japan (Yamamoto, 1978), and from southern Chile (Romo *et al*., 1979) to British Columbia (Saunders and Lindsay, 1979) *Gracilaria* is common in the Mediterranean (Gargiulo *et al*., 1985) and widespread in the Indian Ocean (Umamashewara Rao, 1972; Silva *et al*., 1996), and also found off the Antarctic Peninsula (Wiencke and Clayton, 2002).

The broad geographical distribution of *Gracilaria* is probably due to the large number of species, including many sibling species, and the broad tolerances that some species have for salinity (Oliveira *et al*., 2000). The areas occupied can be subject to considerable variation in ecological conditions. *Gracilaria* can tolerate salinities up to 60 ppt (Yokoya and Oliveira, 1992a) and even fresh water (Macchiavello, 1994). Some *Gracilaria* species have wide temperature tolerances and can survive temperatures from 30 ^{o}C (Yokoya and Oliveira, 1992b) down to freezing and can withstand being frozen for a few months (Titlyanov *et al*., 1995). Furthermore, some can survive burial by sand and mud for a few months (Santelices and Doty, 1989). With respect to vertical distribution, most *Gracilaria* species can be found in the

lower intertidal with only a few species going to deeper waters or surviving long periods of exposure to air (Critchley, 1993). *Gracilaria* species are common in wave sheltered areas, and rarely grow in areas of extreme wave action but can be found in partially turbulent waters where they develop attached to shells, loose stones or sedentary animals such as the ascidian *Pyura stolonifera* (Santelices and Doty, 1989; Critchley, 1993). In the tropical warm waters, *Gracilaria* male, female and tetrasporic specimens are found side by side year round, with low biomass populations and are always attached. This is not the case for cold-water *Gracilaria* species, where most plants remain infertile and multiply by vegetative propagation, and populations generally have higher biomasses and may be free-living (Critchley, 1993).

The most economically important *Gracilaria* beds, based on agar yield and quality, occur as free-living, monospecific stands on mud or sand, and the main species include *Gracilaria chilensis* and *Gracilaria gracilis* (McLachlan and Bird, 1986; Kautsky, 1989; Molloy and Bolton, 1995; Oliveira *et al*., 2000). On the southern African coast, *Gracilaria gracilis* generally occurs in subtidal regions, not growing deeper than about 10 m and maximum percentage cover is normally associated with water less than 7 m deep (Anderson *et al*., 1993). The South African *Gracilaria* does not possess holdfasts and the algae are perennial and can regenerate readily from thallus pieces. The commercial species are up to about 70 cm in length, cylindrical and highly branched. Along the southern African coastline, apart from Lüderitz Lagoon in Namibia (Molloy, 1992) and St. Helena Bay (north of Saldanha Bay), Saldanha Bay is the only other coastal area that yields significant commercially utilisable beach-casts of *Gracilaria gracilis* (Anderson *et al*., 1993). Natural *Gracilaria* populations are found in Small Bay where the sandy bottom slopes

upwards towards the north. *Gracilaria gracilis* also grows extensively in the shallow Langebaan Lagoon of the Saldanha Bay complex (Christie, 1981; Rotmann, 1990; Molloy and Bolton, 1995), although these populations remain unutilized, as the area is now part of the West Coast National Park.

1.5. COMMERCIAL IMPORTANCE OF *GRACILARIA*

Members of the red algal genus *Gracilaria* are among the most economically important seaweeds having a variety of uses. The prime commercial importance of *Gracilaria* is as a source of phycocolloids called agars. Agar is used in microbiological media for cultures, plant nutritional studies (Bornman and Barnard, 1993), and for food preparations. It is used in the food industry in baking jellies, meringues, pie fillings and various other types of confectionery. It is also used for the manufacture of dental impression media (Renn, 1997). Kain (1995) found that agar was the phycocolloid commanding the highest price on the world market.

Some species of *Gracilaria* have been used for human consumption as a green vegetable (Levring *et al*., 1969; Smith *et al*., 1984; Santelices and Doty, 1989), as a consumable in tablet form in Japan (Armisen, 1995), as fertilizer (Zaneveld, 1959), in the tertiary treatment of sewage (Ryther *et al*., 1979), and in biogas production (Hanisak, 1981). *Gracilaria* has also been used as a source of food, either as a major feed or as a supplementary feed for invertebrates in aquaculture (Chiang, 1981).

Worldwide, the cultivation of *Gracilaria* has increased steadily as natural stocks became fully exploited or even over-harvested. Cultivation offers the best

potential for meeting demands for agarophytes and their products (Hansen *et al.*, 1981; Oliveira *et al.*, 2000). The genus *Gracilaria* has great potential as a mariculture species because of its ability to give high yields and produce commercially valuable extracts (Lapointe and Ryther, 1978). Therefore, *Gracilaria* availability has greatly increased mainly through the development of cultivation techniques in several countries (Critchley, 1993). Successful large-scale cultivation has followed laboratory studies where the physiological characteristics of *Gracilaria* were studied (Lignell and Pedersén, 1987; Friedlander *et al.*, 1990). Commercial farming of *Gracilaria* is currently taking place in China, Chile, Taiwan and Namibia (Critchley and Ohno, 1998). Brazil, Israel, Mexico, the Philippines, South Africa and Venezuela are assessing the possibility of farming the genus (Critchley and Ohno, 1998).

In Southern Africa, commercial interest in seaweeds developed out of shortages imposed during World War II. Embargoes and disruption of shipping led to global shortages in algal derived colloids, including agar. This in turn resulted in a survey of gels from local seaweed stocks (Anderson *et al.*, 1989). The South African seaweed industry developed largely out of the collection of *Gracilaria gracilis* beach cast material, with minor utilisation of fresh harvested plants (Anderson *et al.*, 1989). However, the *Gracilaria* industry collapsed in 1974, as beach casts ceased, probably due to the construction of a breakwater and ore-jetty (Anderson *et al.*, 1989). Since then, the yields have been drastically reduced, and the collections have never recovered to previous levels and have collapsed several times. Collapses have been explained in terms of the presence of oligotrophic water in summer (Anderson *et al.*, 1996a), the presence of numerous grazers (Anderson *et al.*, 1993) and possible degradation of the thalli by superficial bacteria (Anderson *et al.*, 2003a). The

inconsistencies in collections have made the resource unpredictable and unreliable for export and local agar production (Anderson *et al.*, 1993) thereby impeding further development of the seaweed industry (Anderson *et al.*, 1996a).

In an effort to develop a stable *Gracilaria* industry in South Africa, the mariculture of *Gracilaria gracilis* was experimentally investigated in Saldanha Bay and St. Helena Bay (Figure 1.2). Saldanha Bay and St. Helena Bay are the only two localities where commercial quantities of *Gracilaria* and *Gracilariopsis* grow naturally and which are sheltered enough for cultivation in South Africa (Anderson *et al.*, 1996a). Suspended cultivation of *Gracilaria gracilis* in Saldanha Bay has been experimentally investigated and relatively high average growth rates were obtained for many years (Anderson *et al.*, 1996a). However, two subsequent attempts at commercial farming were abandoned. In both, growth was poor during summer due to oligotrophic surface waters but good in spring and satisfactory in winter (Anderson *et al.*, 1996b). For suspended cultivation of *Gracilaria* to succeed in Saldanha Bay, additional nutrients would have to be supplied in summer, either by providing slow-release inorganic fertilizer, or by using the bottom layer of nutrient-rich water that is present in summer (Anderson *et al.*, 1996b). Apart from being a sheltered bay, Saldanha Bay is also a very active ore and oil-loading harbour, and the bay is important for fishing and sailing therefore there is likely to be conflict over water space (Anderson *et al.*, 2003b).

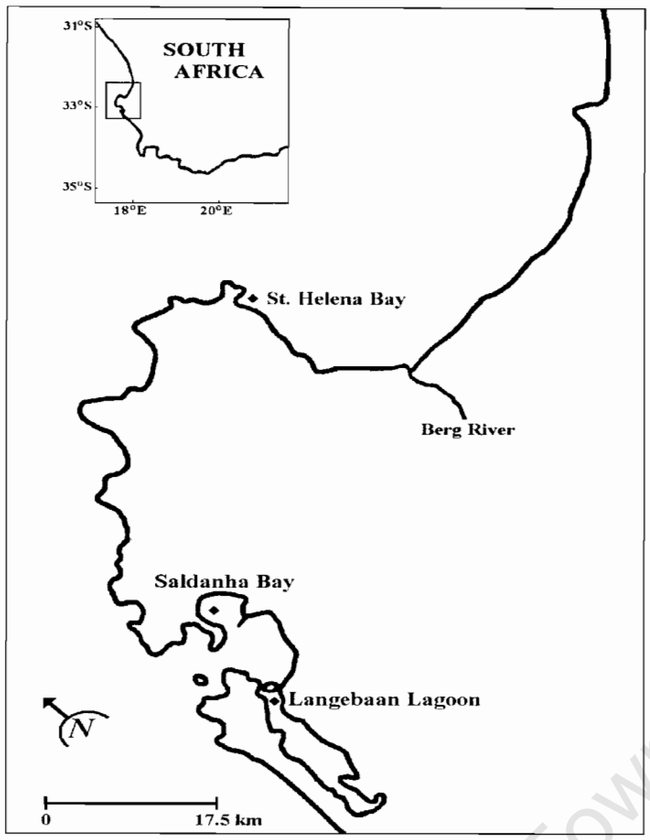


Figure 1.2. Map showing localities where commercial quantities of *Gracilaria* and *Gracilariopsis* grow naturally, Saldanha Bay and St. Helena Bay (marked by dots). (From Anderson *et al*., 2003b).

St. Helena Bay is a large, shallow embayment, protected from the prevailing westerly swell, and has a consistent upwelling of cold, nutrient-rich water in summer (Anderson *et al*., 2003a). The water exchange is good all year and there is no thermal stratification (Wakibia *et al*., 2001) creating a suitable site for *Gracilaria* cultivation. Pilot-scale experiments on suspended *Gracilaria* cultivation have shown that yields are high during summer and growth remains good throughout the year (Wakibia *et al*., 2001; Anderson *et al*., 2003b). However, in the last decade there have been three natural low-oxygen events that have led to the production of toxic levels of hydrogen sulphide in the seawater, and subsequent anaerobic decomposition of organic matter

in the sediment and water column (Mathews and Pitcher, 1996), killing the seaweeds. Such events may have implications for mariculture in the region. Pilot-scale pond cultivation of *Gracilaria* has also been tested at Kleinzee in the Northern Cape Province (Anderson *et al*., 2003a).

Outside of South Africa, in Namibia (Lüderitz Bay) *Gracilaria* has been commercially exploited since 1981 (Rotmann, 1987; Critchley *et al*., 1991). Dried beach cast of *Gracilaria gracilis* is sold abroad for the production of agar (Anderson *et al*., 1989; Critchley *et al*., 1991; Critchley and Rotmann, 1992; Molloy, 1992). The prohibition of direct harvesting from the Namibian *Gracilaria* beds coupled with the unreliability of beach cast material justified the need for cultivation of *Gracilaria* to ensure consistent supply of this seaweed and to meet the ever increasing demands for agar and its derivatives (Critchley *et al*., 1991; Molloy and Bolton, 1992, 1995). So far only *Gracilaria gracilis* is being cultivated, but other species of potential economic importance may also be considered in the future (Molloy and Bolton, 1992).

1.6. SIGNIFICANCE OF AGAR

Agar is one of the most important colloids extracted from seaweeds. The word 'agar' comes from the Malay term 'agar-agar' and was originally used for gel extracted from *Eucheuma*, which has subsequently been identified to belong to a different group of phycocolloids, the carrageenans (Yaphe, 1984; Critchley, 1993).

The most important properties of agars are that they form aqueous solutions at low concentrations, form thermo-versatile gels, are relatively inert, have a significant degree of hysteresis, retain moisture and resist hydrolysis by terrestrial microorganisms (Renn, 1997). Traditionally, agar from *Gelidium* is the source of bacteriological grade agar because of the gel strength. Agar from *Gracilaria* species is normally used as 'food grade' and is found in canned hams as the jelly-like substances that prevent meat from adhering to the walls of the metal can. Furthermore, agar from *Gracilaria* can be combined with sugars and not lose the gel-strength (Abbott, 1995).

Agar production worldwide is valued at US $132 million annually (FAO, 2004). It is mainly produced from red seaweeds since the 1960s, but on a much larger scale since 1990. Agar is a water-soluble polysaccharide, which constitutes the matrix component of cell walls of marine red algae known as agarophytes (Armisen, 1995). Agar consists of two different components, agarose and agaropectin. Agarose is a natural polysaccharide with a linear structure of repeated units of the disaccharide agarobiose, which consist of 3, 6-L-galactose and D-galactose. Agaropectin is an acid polysaccharide containing a sulphate ester, pyruvid acid and D-glucuronic acid in addition to agarobiose (Marinho-Soriano and Bourret, 2005).

Agar is mostly obtained from five genera (*Gelidium*, *Gelidiella*, *Gracilaria*, *Gracilariopsis* and *Pterocladia*) in two orders of red algae. However, two genera, *Gelidium* and *Gracilaria*, account for most of the raw material used, with *Gelidium* species giving the higher quality agar. All *Gelidium* used for commercial agar extraction comes from natural resources, principally from France, Indonesia, the Republic of Korea, Mexico, Morocco, Portugal and Spain (FAO, 2004). *Gelidium* is a

small, slow-growing plant and, while efforts to cultivate it in tanks and ponds have sometimes been successful, these efforts have generally proved uneconomic. *Gracilaria* species tend to give good yields of agar but with poor gel strength, as a result of which the species was once considered unsuitable for agar production (Critchley, 1993).

In the 1950s, however it was found that pre-treatment of the seaweed with alkali before extraction lowers the yield but increases the gel strength, giving a good-quality agar (McHugh, 1991). Once the alkali process was industrialized, *Gracilaria* became a useful source of agar and its value on the international market increased. This allowed expansion of the industry that had been previously limited by the available supply of *Gelidium*, and led to the harvesting of a variety of wild species of *Gracilaria* in countries such as Argentina, Chile, Indonesia and Namibia (Avila and Seguel, 1993; Critchley, 1993). Development of successful cultivation methods has led to increased availability of *Gracilaria* as a source of agar and its use now exceeds that of *Gelidium* (Critchley, 1993). These methods have since spread from Chile to China, Indonesia, the Republic of Korea, Namibia, the Philippines and Vietnam, usually using species of *Gracilaria* native to each particular country (FAO, 2004). Today, *Gracilaria* is growing in importance owing to its abundance in biomass in the wild and its successful cultivation, particularly in Chile.

The commercial importance of *Gracilaria* is in the rapid and relative ease of growth, broad tolerances of many forms, and the diversity of species that occur in tropical and warm temperate waters of the world (Abbott, 1995; Bird, 1995; Smit and Bolton, 1999). In addition, some species of *Gracilaria* yield good grade agarocolloids.

whereas others produce agar or agaroids that have different, but unique properties. With the understanding of the chemical structure and physical properties of agarocolloids, new uses may be found for these potentially valuable *Gracilaria* extracts (Murano, 1995). A number of South African companies are currently showing interest in the cultivation of *Gracilaria gracilis* for agar, or as a possible feed for the most lucrative culture of abalone (Engledow and Bolton, 1992; Njobeni, 2005).

1.7. COMMERCIAL CULTIVATION OF *GRACILARIA*

Cultivation is fundamental for the sustained production of *Gracilaria* as natural stocks are limited. Management of natural beds is subject to social-economic pressure (Oliveira and Miranda, 1998), and little success has been achieved with habitat manipulation (Oliveira *et al*., 2000). Hence the cultivation of the agarophytic red alga *Gracilaria* has become of major importance in several parts of the world, such as Asia, South America and southern Africa (Santelices and Doty, 1989).

1.7.1. Cultivation in the Sea

Gracilarioids have been cultivated in open sea, usually in the protected bays and estuaries, using various planting techniques such as bottom planting systems, the direct insertion of thalli in soft substrata, or suspended systems using ropes and/or nets hung horizontally or vertically in the water column. To be grown in the sea, the seaweed has to be stationary with the water circulating around it to supply nutrients and remove excreted metabolites (Oliveira *et al*., 2000). Several techniques are used to maintain the seaweed in place, the seaweed can be tied or induced to sporulate on

some sort of substrate, or planted directly in the sediment. *Gracilaria* cultured in open waters produces high yields of good quality agar. The main problem with seaweed cultivation in open water systems is how to obtain a sustainable production in a fluctuating environment (Westermeyer *et al*., 1993). Furthermore, cultivation in open water systems has the disadvantage associated with unpredictable changes in the natural environment, tidal currents that can cause damage to the system of ropes and anchors, grazing, sediment accumulation and epiphytism. However, some of these disadvantages can be overcome through careful site selection.

Bottom Planting Systems

In the natural environment *Gracilaria* can either settle on a mixture of substrates or it can be anchored in sand. Several bottom-planting techniques have been developed to attach *Gracilaria* to the substratum (Alveal, 1986). The selection of the best technique depends on the species of gracilarioid algae selected, the conditions at the cultivation site and labour costs (Oliveira *et al*., 2000).

Vegetative propagation of thalli attached to ropes is widespread and has been utilized in many places (Oliveira *et al*., 2000). Vegetative thalli already attached to substrate particles can be transplanted into areas where growth is needed (Buschmann *et al*., 1995) or plants can be artificially secured to hard substrates. Algae can be attached to pieces of wood, rocks, or dead corals using rubber bands, raffia or other kind of tape. Alternatively the seaweeds can be planted directly into the floor sediment, such as has been done successfully on a large scale in Chile (Oliveira *et al*., 2000). The direct method consists of a direct insertion of *Gracilaria* thalli into the sandy bottom mainly in intertidal areas, using different types of tools such as forks or

weights (Santelices and Doty, 1989). However, earlier studies have shown that only a few species of *Gracilaria* can tolerate direct planting in the sediment (Santelices and Doty, 1989; Oliveira *et al*., 2000). The species most adapted to this technique is *Gracilaria chilensis*, which can tolerate some degree of burial (Santelices and Doty, 1989). One of the advantages of the direct planting method on soft substrata is that if harvesting is done by cutting the thallus at about 30 cm above ground, then the new bed can develop as an anthropogenic bed that satisfies the effect of exploitation (Oliveira *et al*., 2000).

Gracilaria can be grown by the inoculation of spores onto ropes, a bottom planting system known as rope seeding (Smit, 1998). Seeding ropes with spores is more recent and has been used commercially only for bottom cultivation. This method is initiated in indoor tanks and in the inoculation chamber that contains the fertile thalli supplied with compressed air to keep the sporelings healthy. Following seedling establishment, the seeded material can then be replanted. The cultivation of ropes seeded with spores seems to have been commercially successful only in Chile (Alveal *et al*., 1997). In Molokai, Hawaii, an experimental hatchery was developed to produce spore-seeded rocks that were sold to locals, who placed them in the sea and harvested the *Gracilaria* later (Oliveira *et al*., 2000). The thalli were then used to produce more spores or grown in cages for sale as food.

A particular problem associated with bottom planting systems is that the algae remain at a fixed depth in the water column, which means that light intensity fluctuates with tidal oscillations (Oliveira *et al*., 2000). Furthermore, herbivores and fouling can be a serious problem where the control is usually cumbersome and

difficult. Another problem is the risk of mortality when plants are transplanted from one environment to another and the thalli may become dislodged from their substrata, which in turbulent waters may cause a significant decrease in yield (Buschmann *et al.*, 1995).

Suspended Systems

In a suspended cultivation system, the seaweed is attached to a line or enclosed in a cage or net, with the system held above the sea bottom (Oliveira *et al.*, 2000). Numerous methods have been developed and most of these try to maximize the light and water flow around the thallus, while avoiding benthic grazers. One of the most common methods in suspended systems is to attach the thalli, individually, to a substrate either ropes or monolines. Strips of raffia or nylon string can be used to attach pieces of thalli to the rope or monoline, or the ropes can be inoculated with tetraspores or carpospores. Once the rope is stocked with the *Gracilaria*, the ropes are then suspended, stretched under tension, between sticks buried in the sediment or supported at different levels by buoys on a raft system (Critchley, 1993). The ropes are set parallel to tidal currents. Floating rafts are advantageous as the thalli remain at a consistent depth irrespective of tidal fluctuations, so maximizing the light intensity. Furthermore, advantages of rope farming include the positioning of algae in optimal environments, crop predictability and control, surface harvesting, no diving requirements, and possible epiphyte and grazing control (Dawes, 1995).

Cultivation on ropes, or nets, on the bottom, or floating at specific water depth have been attempted in many countries (Molloy, 1998; Buschmann *et al.*, 1995; Smith *et al.*, 1984). These experiments however did not go further than the pilot stage or

have led only to modest production. At Saldanha Bay in South Africa, Anderson *et al.* (1996a) conducted a pilot study on the performance of raft cultivation. Average annual growth rates for *Gracilaria* of 5 % d^{-1} were recorded over several years, with a potential commercial yield of approximately 39.6 t dry wt ha^{-1} y^{-1}, but subsequent attempts at commercial scale farming were not successful (Anderson *et al.*, 1996a). In both experiments, growth of suspended *Gracilaria* was best in spring and reasonably good in winter. The major problem in Saldanha Bay was the low nitrogen levels in the surface waters in summer, during periods when strong thermal stratification is formed in the water column. However, improved growth rates were obtained at a site subject to increased N-levels from fish-waste discharge, even when growth rates were poor at a control site (Anderson *et al.*, 1999). While this site may be better for cultivation of *Gracilaria*, there is limited water space (Anderson *et al.*, 2003b) and there may be a risk from *Ulva* blooms.

Gracilaria has also been grown experimentally in suspended open-water cultivation in St. Helena Bay, the only other site on the west coast of South Africa sufficiently sheltered from the swell to cultivate seaweed. Here, consistent upwelling of cold, nutrient–rich water keeps the N-levels in the surface water high during most of summer, and growth is generally higher then in Saldanha Bay (Anderson *et al.*, 2003b).

Suspended cultivation of *Gracilaria gracilis* from Saldanha Bay and Langebaan Lagoon and *Gracilariopsis* from St. Helena Bay has been tested in St. Helena Bay. Relatively good growth rates and agar quality throughout the year were obtained by Wakibia *et al.* (2001). However, in the last decade there have been three

natural low-oxygen events severe enough to lead to the production of toxic levels of H_2S, killing seaweeds (Anderson *et al.*, 2003b). Another problem with suspended cultivation is related to the stability of the floating structures in the sea they should be designed to resist rough conditions during periods of strong winds that can occur even in protected bays.

1.7.2. Land-Based Cultivation

Commercial cultivation of *Gracilaria* is done on a very large scale in several countries, such as Chile (Avila and Seguel, 1993), China (Ren *et al.*, 1984) and Taiwan (Chiang, 1981). *Gracilaria* can be cultivated in land-based systems, that is pond or tank culture systems. Outdoor pond and tank cultivation of unattached *Gracilaria* may be divided into intensive and non-intensive cultivation systems (Friedlander and Levy, 1995). The non-intensive ponds are usually made of uncovered earthen construction and are always without an artificial water agitation system, while the intensive cultivation ponds are made of a concrete or plastic structure with a water agitation system.

Pond Cultivation

Gracilaria has been cultivated in ponds in a number of countries in the world (Oliveira and Alveal, 1990; Santelices and Doty, 1989; Critchley and Ohno, 1998), but commercial success has only been achieved in Taiwan, southern China and Vietnam (Ohno *et al.*, 1997). Ponds include natural lagoons or artificial excavations, which vary in length (0.7 to 1.0 ha), and depth (less then a metre) (Critchley, 1993). *Gracilaria* ponds are generally located in areas not exposed to strong prevailing winds, with connections to the sea so that water is exchanged by tidal flow. When

exchange rates are low, a source of fresh water is required to prevent the salinity in the ponds rising too high as a result of evaporation (Oliveira *et al*., 2000) and ponds must therefore be situated near the sources of both freshwater and seawater (Critchley, 1993). Ponds are usually small since thalli tend to be blown into concentrated areas in larger ponds. In large ponds thalli may be tied to bamboo poles or covered with netting. The ponds should be shallow to ensure sufficient sunlight and uniform light absorption by the thalli. Furthermore, the water depth is used as a mechanism of modifying temperature changes. Water exchange rate in ponds is used as a means to adjust for salinity and mineral nutrient supplies for algal growth. Fertilizers or plant growth regulators may be added to the ponds to enhance the quality of *Gracilaria*. *Gracilaria* in the ponds is usually free floating and evenly distributed over the bottom. Once sufficient biomass has been reached, the plants are removed from the ponds using either rakes or nets.

Santelices and Doty (1989) stated that perhaps the most important limitation of this cultivation system is that only relatively low value food-grade agar is produced. Increased temperatures in ponds might be responsible for the low gel strength values of agar produced from the crop (Critchley, 1993). One of the major problems associated with *Gracilaria* pond farming is the development of epiphytes. Epiphytes have to be removed periodically by hand. Ponds are easy and simple to operate and with some degree of environmental and biotic parameter control, they can make potential *Gracilaria* cultivation sites (Oliveira *et al*., 2000). The only inputs in pond cultivation are water pumping and nutrient addition hence it is regarded as a cheap method for cultivation of *Gracilaria*. However, artificial ponds and intensive pond farming can become expensive when it involves the excavation of the pond, addition

of fertilizers, aeration and water movement, labour for farming and subsequent processing. In South Africa, pilot-scale pond cultivation of *Gracilaria* is being tested at Kleinzee in the Northern Cape Province. Seawater pumped to wash diamond gravel, is first led through a large shallow pond, in which *Gracilaria* is planted either on the bottom or on suspended lines (Anderson *et al.*, 2003a).

Tank Cultivation

Tank systems may be very productive, but they require high energy input and capital investment. To ensure high and sustainable production, it is necessary to have considerable rates of seawater replacement, good supply of nutrients, especially nitrogen and phosphorous (Oliveira *et al.*, 2000), and if possible some control of the light and temperature as well as the epiphytes and predators. It is also essential to continually move the plants using compressed air or paddle-wheels. Under intensive cultivation and with a high productivity, control of CO_2 supply and pH, and sometimes temperature and light, may also be necessary (Craigie, 1999). Consequently for tank systems to be effective they should have a very high biomass in order to have light as the only limiting factor to growth (McLachlan, 1991).

Intensive tank cultivation of free-floating seaweeds, among all the techniques available for the production of *Gracilaria*, provides the greatest productivity per unit area compared to any other type of farming. Furthermore, it is possible to control and mechanize its major operations. Consequently, this is also the most expensive form of cultivation and usually limited to situations where capital return is the principal benefit. Seawater tank systems provide the possibility to be used as biofilters for fishpond and other effluents. This allows a choice between internal re-circulation of

water and release of water back to the sea (Shpigel and Neori, 1996). The disadvantage of this method is its high cost compared to other cultivation methods. The high cost is mainly because of the energy needed for seawater pumping and water movement, and because of necessary CO_2 enrichment (Friedlander and Levy, 1995).

Tanks have been used in several countries, usually on an experimental level (Bird and Ryther, 1990; Oliveira and Alveal, 1990; Critchley, 1993; Friedlander and Levy, 1995; Martinez and Buschmann, 1996; Troell *et al.*, 1999). Some studies on the cultivation of *Gracilaria gracilis* in South Africa have indicated that tank cultivation is suitable in integrated abalone and *Gracilaria* cultivation systems where the seaweed is used to feed the animals (Smit, 1998).

1.7.3. Integrated Aquaculture

Aquaculture has grown rapidly in recent decades to become one of the most important means of obtaining food from the sea (Kautsky *et al.*, 2001). The coastal zones in the world are facing increasing depletion of resources, pollution problems and overcrowding. With dramatic population increases in the coastal areas, food requirements have increased and food production should increase to meet present and future requirements without using vast amounts of land. Aquaculture systems are needed which are more integrated with the environment, can be more realistically community-based and have less negative impact on the environment yet which can produce sufficient food for today's requirement in an efficient manner (Brzeski and Newkirk, 1997).

Commercial aquaculture is practiced using large monocultures. Like all types of monocultures, large-scale, and especially intensive aquaculture will result in negative environmental impacts and make the surrounding waters less suitable or even unusable for other proposes, including further culturing and harvesting of natural stocks (Kautsky *et al*., 2001). Therefore, as an alternative to monocultures and high-tech pollution treatment, the practice of integrated aquaculture has been proposed as an environmentally friendly way of recycling wastes, especially those produced through the cultivation of high trophic level species, which require exogenous energy (food). The concept of integrated aquaculture is not new. In China, Japan and South Korea traditional integrated aquaculture has been practiced for many years in lagoons and bays placing fish net pens, shellfish and seaweed cultures next to each other in an apparently optimized balance (Kautsky *et al*., 2001).

Integrated aquaculture or polyculture occurs when an output from one subsystem in an integrated farming system, which otherwise may have been wasted, becomes an input to another subsystem resulting in a greater efficiency of output of desired products from the land or water under the farmer's control. It is a prerequisite however that a successful sustainable integrated farming system mimics as much as possible the way the natural ecosystem functions. Therefore, by integrating organisms such as biofilters, which feed off each other's waste, one can effectively mimic the flow of nutrients that takes place during natural ecosystem functioning and thereby reduce the unnatural stress that monocultures place on their associated ecosystems. The concept of integrated aquaculture aims at reducing, in an economically and socially beneficial manner, the adverse environmental impacts of aquaculture (freshwater, saline or marine) on the coastal environment. A land-based or open-water

integrated aquaculture system may involve the integration of fed organisms, fish or shrimp, the extractive algae (seaweeds) and filter-feeders (mussels, oysters). In the land-based integrated aquaculture, each organism is cultured in their own pond or tank at medium to high levels of intensity. The water recycles between them. In open-water integrated aquaculture, wastes from fish-net pens and agricultural runoff are used to supply cultured seaweed with dissolved nutrients.

Wastewater from land-based fish and shrimp cultivation has proven to be a suitable nutrient source for the culture of seaweeds and filter-feeders (Kautsky *et al.*, 2001). Many commercial bivalves such as oysters and mussels are filter feeders that remove particulate organic nutrients (Troell *et al.*, 2003). Seaweeds have the ability to use sunlight to extract from the water dissolved inorganic nutrients (Rawson *et al.*, 2002). Both bivalves and seaweeds are known as extractive species as they remove nutrients from the water. Thus, when integrated with fed aquaculture of fish or shrimp, extractive organisms turn wastes into productive resources (Rawson *et al.*, 2002; Troell *et al.*, 2003). The integrated aquaculture systems can use multiple species from different trophic levels, for reducing costs through recycling of wastes, while increasing total productivity in weight and in value with respect to feed input and pollution output (Troell *et al.*, 2003). An integrated system will also secure income by resulting in a more diversified production of commercially attractive species (Troell *et al.*, 1999).

Seaweed monocultures in land-based systems are generally non-viable in terms of commercial levels as many seaweed species are generally of less economic value than the cultured marine animals (Shpigel and Neori, 1996) and there are high

expenses in running a monoculture system. Hence the need to cultivate seaweeds in integrated culture systems. Nowadays, several studies have acknowledged the potential of seaweeds as nutrient scrubbers in integrated aquaculture of finfish, shellfish and crustaceans.

Farming of South African abalone, a highly valued herbivore, is one example of a successful and commercial land-based aquaculture system with excellent potential for integrated seaweed cultivation (Halling, 2004). The environmental and economic benefits of integrated seaweed culture systems are many in comparison to monocultures. There is the improved resource use in integrated systems, achieved by the re-circulation of excreted nutrients that are transformed into seaweed biomass. Thus integration makes production of one or several additional products possible without supplementary input (Folke and Kautsky, 1992). In an integrated culture the continuous nutrient supply optimizes seaweed growth rate and productivity. By selecting economically valuable species for integration, the farmer's production potentially adds to overall economy (Buschmann *et al.*, 1996).

1.8. SEAWEEDS AND PLANT GROWTH REGULATORS

The use of marine algae as manures and fertilizers dates back to the Ancient Greeks. As early as the twelfth century, algae from the phylum Phaeophyta (brown seaweeds) were used as manure on the coastal lands of Europe (Crouch, 1990). Furthermore with an increased awareness of environmental issues such as excessive fertiliser, herbicide and pesticide use, and an escalation of energy prices, there is a need to find alternative methods to improve crop yield (Metting *et al.*, 1990). One

such well-established practice is the use of seaweed concentrates as biostimulants (Stirk and van Staden, 2004). The use of different seaweed extracts and concentrates as soil drenches and foliar sprays on agricultural plants is increasing in popularity, even though the literature on seaweed extracts is unbalanced and contradictory. Well-documented studies (Crouch *et al.*, 1990; Featonby-Smith and van Staden, 1983a, 1983b, 1987), mostly from universities and research institutes, report that seaweed extracts improve growth and yield of plants, as well as prevent pests and improve quality of the product. One report indicated that seaweed extracts often have no effect on plant growth (Verkleij, 1992). Besides these studies, there are leaflets and reports from producers of seaweed extract publicizing the benefits of their products. The value of this information is hard to assess since often a statistically valid experimental design is lacking. However, the significance of scientific evaluation should not be overestimated considering the experience of many farmers, who use seaweed extracts to their satisfaction.

The algae are regarded as a less advanced group of organisms than higher plants. However, algae have a number of morphogenic events in their life cycle, including germination, branching, reproduction events and senescence that require some level of organization. Although the morphology is simpler than that of higher plants, many of the processes of development are similar. It is thus to be expected that plant growth regulators play a role in controlling some of these events as they do in higher plants (Stirk *et al.*, 2003). Cytokinin metabolism and regulation has developed into a much more complex system in higher plants to control a wider range of morphogenic events such as cell division, enlargement and differentiation, chloroplast and vascular tissue development, shoot growth, fruit and flower development, apical

dominance and senescence (Auer, 1997). The synthesis and metabolism of plant growth regulators and the physiological responses of higher plants to these compounds have been extensively investigated, although many questions still remains unanswered. Similarly, information on the plant growth regulators in algae are limited.

In algae, growth is an orientated process. The polarities in cells and thalli are established from the start and are maintained through development. Probably, the apico-basal polarity and the apical dominance in seaweeds are caused by growth hormones (Lobban and Harrison, 1994) also known as plant growth regulators. Plant growth regulators, specialized chemical substances produced by plants, are main internal factors controlling growth and development. The auxins, cytokinins, gibberellins, abscisic acid, and ethylene have all been identified in plant tissues. Biosynthesis mutants have in every case established that these endogenously synthesised substances play a functional role, but the precise function is still a matter of debate and argument (Lobban and Harrison, 1994).

Plant growth regulators are synthesised at one site in the plant and transported to a different site, where they are effective at very low concentrations. Depending on the target tissue, a growth regulator may have different effects. One current view of their functions is that they regulate the rate at which individual parts of the plant grow, integrate growth of those parts from the whole organism, and control reproduction (Bradley, 1991). Plant growth regulators also allow mature plants to respond to changes in the environment. In 1958, Bentley postulated that algae might also have similar biochemical systems to the 'more advanced' higher plants. Since

then, there has been accumulating evidence that some of the plant growth regulators that operate in higher plants could have a similar role in the algae. Much of this speculation arose due to observations made when bioassays, performed to determine the presence of plant hormone-like substances, resulted in a positive responses from seaweed material.

Many studies have looked at the effect of applying plant growth regulators, such as auxins, cytokinins and gibberellins, to seaweeds (Bradley and Cheney, 1990; Lobban and Harrison, 1994; Yokoya *et al*., 1999, 2003; Yokoya and Handro, 1996, 1997; Huang and Fugita, 1997). Many other studies have attempted to extract and characterize seaweed compounds with growth-regulatory effects (Albetz, 1980; Finnie and van Staden, 1985; Munda and Gubensek, 1975; Mooney and van Staden, 1986; Stephenson, 1968). Auxins, abscisic acid (ABA), cytokinins and gibberellins have been identified in seaweeds by using a technique such as high performance liquid chromatography (HPCL) (Blunden and Wildgoose, 1977; Crouch *et al*., 1992a). However no technique has been able to show that the compound is active as a growth substance, neither could they allow recognition of a growth substance that did not fall into one of the classic substances group (Lobban and Harrison, 1994).

Evans and Trewavas (1991) pointed out that microorganisms synthesize representatives of all the five plant growth substance groups as secondary metabolites. Consequently, there is a possibility that microorganisms could provide the precursors for growth regulator biosynthesis by associated algae. Therefore, according to Bradley (1991) if bacteria are a source of plant growth substances, one can speculate that changes in environmental conditions might affect their growth, which in turn might

change the plant hormone supplied to the seaweed on which they live. Growth substances have also been identified in seawater, although their origin has not been traced (Pedersén, 1973; Evans and Trewavas, 1991). Nevertheless, plant growth substances have been identified in commercial seaweed liquid fertilizer although the response is still unclear. Seaweed concentrate has been reported to promote increase in yields of many agricultural plants (Crouch, 1990; Featonby-Smith and van Staden, 1983a, 1983b, 1987; Finnie and van Staden, 1985).

1.9. SEAWEED CONCENTRATES

Seaweed concentrates are generally made from kelps due to their large biomass and availability rather than their chemical suitability (Mooney and van Staden, 1986). They are processed using different methods such as hot water extraction, the alkaline caustic soda and dehydration process or the cell burst method (Stirk and van Staden, 1997). The seaweed concentrates are applied to crops as root dips, soil drenches or foliar sprays. *Ecklonia maxima* blades and stipes have been commercially harvested at Kommetjie, South Africa, since 1979 and are processed at a factory in Simon's Town using a patented Cell Burst Method. The freshly washed *Ecklonia maxima* is passed through a series of cutters. The resulting fine particles are subjected to high pressure and then passed at high velocity through a low-pressure area. The released energy causes the cell walls to expand and exceed their elastic limit, thereby rupturing and releasing their cellular contents (Stirk and van Staden, 1997). The Cell Burst Method does not involve the use of heat, chemicals and dehydration steps that could be detrimental to the active ingredients. This seaweed concentrate is marketed under the trade name Kelpak®. This process uses relatively

low amounts of kelp (approximately 60 tonnes dry mass annually) but is a high value product (Anderson *et al.*, 1989, 2003a).

Seaweed concentrates, including Kelpak®, are effective biostimulants in many crops including vegetables, trees, flowering plants and grain crops (Metting *et al.*, 1990). The beneficial effects of seaweed concentrate include improved seed germination, seedling establishment, flowering, fruit and crop yield, shelf life and enhanced resistance to frost, pests and diseases (Crouch and van Staden, 1994). The physiological responses include improved nutrient mobilisation and partitioning, increased chlorophyll content and leaf area, the development of a vigorous rooting system, and senescence retardation (Metting *et al.*, 1990).

Cytokinins and auxins have recently been quantified in Kelpak® using the most recent available analytical methods (Stirk and van Staden, 2004). The total cytokinin content was about 5 μmol ml^{-1} seaweed concentrate (Crouch and van Staden, 1992a). A number of different cytokinin metabolites were identified. These included isoprenoid zeatin and isopentenyladenine derivatives and aromatic benzyladenine and topolin derivatives (Stirk and van Staden, 1997). The total auxin content was ten times higher at 34 μmol ml^{-1} seaweed concentrate of which a third was indole-3-acetic acid (IAA). Seven other indole conjugates, including some indole-amino acids were also quantified (Stirk *et al.*, 2004). Furthermore, using GC-MS, Crouch and van Staden (1992a) identified IAA, indole-3-carboxylic acid (ICA), N'N-dimethyltryptamine, indole-3-aldehyde (IAId) and *iso*-indole,1,3-dione (N-hydroxyethylphthalimide) in Kelpak®. Cytokinins and auxins do occur in seaweeds (Stirk *et al.*, 2004), and even though not as extensively investigated as land plants, the

literature shows that the cytokinin-free bases of iso-pentenyladenine (iP) and zeatin (Z) as well as their riboside conjugates are most commonly identified in seaweeds (Auer, 1997; Jameson, 1993).

1.10. Plant Growth Regulators and Seaweed Concentrates

Seaweed extracts are used extensively in agriculture as plant growth supplements (Stirk and van Staden, 1997). There are numerous reports in the literature showing that application of seaweed extracts increases crop yields, improves growth, increases plant resistance to frost, fungal and insect attack, causes a reduction in red spider, aphid and nematode infestation (Crouch, 1992; Nelson and van Staden, 1986). Furthermore it reduces storage loss in fruit and increases inorganic nutrient uptake from the soil of a wide range of crops including tomatoes, sugar beet, beetroot potatoes, grasses, citrus and beans (Mooney and van Staden, 1986; Featonby-Smith and van Staden 1987; Beckett and van Staden, 1990; Crouch *et al*., 1990).

Low rates of application of seaweed concentrates elicit a physiological response from the crop. The active compounds present in the seaweed concentrates need to be effective at low concentrations, hence it has been suggested that organic compounds rather than mineral elements are responsible for improved yield (Crouch and van Staden, 1993; Stirk and van Staden, 1997). It is thought that plant growth regulators may be one of the active constituents in seaweed products as many responses obtained with application of seaweed concentrates to plants mimic those observed when cytokinins are applied (Crouch and van Staden, 1993). Due to the wide range of elicited physiological responses obtained with the application of

seaweed extracts, it is probable that more than one group of plant growth regulators is involved (Crouch and van Staden, 1993; Stirk and van Staden, 1997). Many physiological responses shown by crop plants are thought to be due to cytokinins. Cytokinins have been identified in some seaweed concentrates (Crouch and van Staden, 1993; Stirk and van Staden, 1997; Stirk *et al.*, 2003), such as Kelpak®, Maxicrop and Marinure. Auxins are also likely to be active ingredients in seaweed concentrates as they promote rooting upon application (Crouch and van Staden, 1994).

Most studies have concentrated on the identification of cytokinins in the Phaeophyta because of their economic value as supplementary growth stimulants. Nevertheless many studies on the extraction and identification of cytokinins in algae report these in the green (Zhang *et al.*, 1991), the red (Zhang *et al.*, 1991) and the brown algae (Mooney and van Staden, 1987; Farooqui *et al.*, 1990; de Nys *et al.*, 1990; Featonby-Smith and van Staden, 1984a; Kingman and Moore, 1982). *Ecklonia maxima* (Osbeck) Papenf. (Featonby-Smith and van Staden, 1984a), *Sargassum heterophyllum* (Turn.) J. Agardh (Mooney and van Staden, 1984, 1987), and *Ascophyllum nodosum* (L.) Le Jollies were the species most commonly used (Duan *et al.*, 1995). In addition, detection of cytokinins in seaweed extracts (Tay *et al.*, 1985, 1987), seawater (Pedersén, 1973) and marine bacteria (Maruyama *et al.*, 1986) has also been documented (Duan *et al.*, 1995). Very few studies have investigated the physiological responses of algae to plant growth regulators such as auxins and cytokinins (Yokoya *et al.*, 1999, 2003; Yokoya and Handro, 1996, 1997; Huang and Fugita, 1997).

Plant growth regulators, and in particular cytokinins, have been implicated in improved growth, since responses obtained with the application of exogenous cytokinins were similar to those obtained with the application of seaweed extracts (Stirk and van Staden, 1997). However, the wide range of physiological responses obtained with application of seaweed extracts implies that more than one group of plant growth regulators may be involved (Crouch and van Staden, 1993). Cytokinins have important physiological roles in flowering plants (Zhang *et al*., 1991) by promoting plant cell division and elongation (Duan *et al*., 1995). Nonetheless, there is only limited information on the function of cytokinins in algae (Zhang *et al*., 1991). Since the review of the existence of cytokinins in terrestrial plants and their physiological function by Skoog and Armstrong (1970), many papers have been published on land plants, and some summaries have also been presented on algae (Augier, 1978; Mooney and van Staden, 1986; Zhang *et al*., 1993). Cytokinins have been shown to promote growth in *Porphyra* (Iwasaki, 1965), *Ectocarpus* and *Pylaiella* (Pedersén, 1968, 1973), and *Ecklonia* and *Hypnea* (Jennings, 1969). Cytokinins were also found to regulate the regeneration and morphogenesis of *Ulva* (Provasoli, 1958), *Ectocarpus* (Pedersén, 1973), *Fucus* (Borowczak *et al*., 1977), *Sphacelaria* (Dworetzky *et al*., 1980), and *Myagropsis* (Kim and Lee, 1985).

Furthermore, synthetic kinetin stimulated cell division in the gametophytes of the brown alga *Ecklonia radiata* and stimulated growth of excised branch apices and offset the degradation of the red pigment phycoerythrin in *Hypnea musciformis* (Wulfen) Lamour (Jennings, 1969; Jennings *et al*., 1972). Burkiewicz (1987) found that exogenous kinetin and isopentenyladenine (iP) increased cell number by stimulating cell division in some unicellular algae. Growth was stimulated in

Macrocystis pyrifera (L.) C. Ag. by zeatin (Z) and iP (de Nys *et al*., 1991). *Ectocarpus confervoides* f. hiemalis Kjellman and *Pylaiella litoralis* f. rupincola Kjellman showed stimulated growth when grown in seawater known to contain iP (Pedersén, 1973). Hence, evidence from the literature indicates that cytokinins are found in macroalgae and that they seemingly play a role in growth and morphogenesis of algae.

Indole-3-acetic acid (IAA) is the most abundant naturally occurring auxin (Bartel, 1997; Normanly, 1997). Land plants maintain most IAA in the inactive conjugate from which it is then converted to biologically active IAA by hydrolysis (Bartel, 1997). These conjugates perform different functions such as storage, transport, protection against peroxidation and catabolism (Normanly, 1997). The study conducted by Stirk *et al*. (2004) using two seaweed concentrates made from the kelps *Ecklonia maxima* and *Macrocystis pyrifera* concluded that both seaweed concentrates had cytokinin- and auxin- like activity. Using highly effective efficient mass spectrophotometer technology, both seaweed concentrates were found to contain more cytokinin metabolites, including aromatic cytokinins, than previously detected. According to the study conducted by Stirk *et al*. (2004), IAA was the dominant auxin in both seaweed concentrates. Furthermore, this confirms the findings of Crouch *et al*. (1992) who also positively identified IAA and three other indole precursors in *Ecklonia maxima* derived concentrate. However the origin of the indoles in seaweed concentrates is not known. Perhaps they are either present in the fresh kelp or the free amino acids may join with free indoles during the processing of the kelps (Stirk *et al*., 2004).

1.11. CONCLUSION

From the above account, it is obvious that *Gracilaria* is a commercially important algal genus and its growth and cultivation has been extensively researched. Furthermore, there is a vast body of information concerning the beneficial effects of seaweed products and plant growth regulators on plant growth. However, the link between *Gracilaria*, seaweed products and plant growth regulators has not been studied. The use of commercial seaweed products in the agriculture and horticultural sector is fast becoming an accepted practice. However, there have been only a few scientific studies (Leitao, 2001; Robertson-Andersson, 2004; Robertson-Andersson *et al.*, in press) on the effect of seaweed extracts on the cultivation of commercial seaweed species. This study aims to investigate this using the extract from the South African kelp *Ecklonia* and the commercially important red alga *Gracilaria gracilis*.

CHAPTER 2

EFFECTS OF SEAWEED CONCENTRATE KELPAK® ON GROWTH OF *GRACILARIA GRACILIS* IN LABORATORY CULTURE

2.1. INTRODUCTION

Members of the genus *Gracilaria* have been harvested around the world as raw material for agar (Oliveira, 1984). Cultivation has been proposed not only to avoid devastation of the natural populations, but also to enhance agar quality through genetic selection and improvement (Kain, 1991). The characteristics of *Gracilaria* that make them desirable for cultivation are fast growth rate, good agar yield and quality and relative ease of growth (Buschmann *et al*., 1995; Smit and Bolton, 1999). Furthermore, one of the most important features of *Gracilaria* is that almost all species important in mariculture reproduce solely by vegetative means through fragmentation. This leads to very high regenerative capacity (Hurtado-Ponce, 1990; Santelices and Varela, 1995) and eliminates the need to raise plants from spores.

Seaweed extracts and seaweeds have been used as fertilizers and soil conditioners for centuries (Crouch, 1990). Initially, enhanced plant growth with seaweed application was attributed to its soil conditioning properties. However, later it was found that increased trace element supply could only explain some of the beneficial effects of seaweed (Francki, 1960). Low rates of seaweed extract could also promote plant growth significantly (Crouch and van Staden, 1992b, 1993), hence the

active compounds in the seaweed concentrate need to be effective at low concentrations. Thereafter, it was suggested that organic compounds, rather than mineral elements, were responsible for improved growth (Brain *et al.*, 1973; Blunden, 1977; Crouch and van Staden, 1993; Finnie and van Staden, 1985). In recent years, it has been demonstrated that seaweed extracts contain not only most of the major and minor nutrients, amino acids, and vitamins B_1, B_2, C, E, but also plant growth regulators (Albetz, 1980; Finnie and van Staden, 1985; Munda and Gubensek, 1975; Mooney and van Staden, 1986; Stephenson, 1968; Nelson and van Staden, 1985) and the stimulating effects of seaweed extracts may be attributed to these components, especially cytokinins and auxins (Blunden and Wildgoose, 1977; Crouch *et al.*, 1992). Recent studies have shown the presence of cytokinins and auxins in Kelpak® seaweed concentrate and a number of cytokinins and auxins were detected and identified (Crouch *et al.*, 1992; Stirk *et al.*, 2004). Furthermore, cytokinin- and auxin-like activity were detected in Kelpak® concentrate (Stirk *et al.*, 2004).

Seaweed extracts exhibit various beneficial effects on plant growth and development (Crouch, 1990; Crouch *et al.*, 1990; Metting *et al.*, 1990). They may enhance nutrient uptake, regulate plant growth substances, increase chlorophyll content, protein synthesis and cell division, promote root and shoot growth and improve seed germination (Beckett and van Staden, 1990; Button and Noyes, 1964; van Staden *et al.*, 1994; Yan, 1993). Seaweed extracts when applied to plants stimulate shoot growth and branching (Temple and Bomke, 1989), increase root growth and lateral root development (Metting *et al.*, 1990), improve nutrient uptake (Yan, 1993), enhances resistance to diseases (Featonby-Smith and van Staden, 1983a) and environmental stresses such as drought and salinity (Nabati *et al.*, 1994).

Far less is known about the effect of seaweed extracts and plant growth regulators on the growth of seaweeds. According to Bradley (1991), some plant growth regulators are able to affect growth and development in seaweeds. Several studies have been conducted on the effects of plant growth regulators on seaweed growth (Bradley and Cheney, 1990; Lobban and Harrison, 1994; Yokoya *et al*., 1999, 2003; Yokoya and Handro, 1996, 1997; Huang and Fugita, 1997). A study conducted by Yokoya and Handro (1996), indicate that plant growth regulators have a role in controlling growth, callus formation and plant regeneration in *Grateloupia dichotoma* J. Ag., moreover some effects were comparable with those observed in vascular plants. Furthermore, a study conducted by Yokoya *et al*. (2004) indicated that auxins and cytokinins have a regulatory role in the growth and morphogenesis in *Gracilaria tenuistipitata* Chang *et* Xia and *Gracilaria perplexa* Byrne *et* Zuccarello (Gracilariales, Rhodophyta).

Kelpak® is a commercially available seaweed extract and it is acknowledged as a plant growth stimulator due to its hormonal content (Featonby-Smith and van Staden, 1987). The addition of Kelpak® has proven successful in agriculture and horticulture plants, however its use to grow seaweeds is unknown. In South Africa, research on *Gracilaria* mariculture has been concentrated on open-water cultivation in the only two suitable bays on the west coast, Saldanha Bay and St. Helena Bay (Anderson *et al*., 2003b) Cultivation methods were adapted from those successfully used in Namibia for suspended cultivation (Dawes, 1995). Pilot-scale pond cultivation of *Gracilaria* is still being tested in a few abalone farms. Low nutrients reduce or prevent the growth of *Gracilaria*, but can be enhanced by the application of a slow release fertilizer, such as have been tested in Japan with *Undaria* cultivation (Ogawa

and Fujita, 1997). Ponds can be fertilized with inorganic agricultural fertilizers or manure. Therefore we hypothesize that as Kelpak® extract improves aspects of land plant growth (possibly due to plant growth regulators) it may have similar effects on seaweeds such as *Gracilaria*. The aim of this study was to test the effects of the seaweed concentrate Kelpak® (at different dilutions) on the growth in laboratory culture of *Gracilaria gracilis* apical segments of different lengths.

2.2. MATERIALS AND METHODS

2.2.1. Plant Material

The material was collected on the day prior to the start of the experiment. Infertile plants of the red algae *Gracilaria gracilis* were collected from Saldanha Bay on the South African west coast and brought to the University of Cape Town phycology laboratory. In the laboratory *Gracilaria* was washed with running fresh water and sterile seawater and brushed with a soft brush, to remove contaminants. The darkest thallus fragments were selected, since darker thallus is nutrient-replete hence healthier. Unbranched apical segments from random plants were cut in a range of sizes of 5 mm, 10 mm and 15 mm. The range in length was chosen to find which segments would contribute more to the overall enlongation of *Gracilaria*. Also, because smaller segments were unbranched, cleaner from epiphytes and had less contamination. Each length of *Gracilaria gracilis* apical formed a separate treatment.

2.2.2. Experimental Design

In agriculture, commercial seaweed extracts are primarily applied as a spray directly onto the leaves, although sometimes onto the soil. The liquid extracts are diluted in volume by a factor 20 to 500 and are applied at a rate of diluted product of 500 to 1000 l ha^{-1}, corresponding to a rate of undiluted product of 1 to 50 l ha^{-1} and to a rate of seaweed dry matter of 0.08 to 4.0 kg ha^{-1} (assuming a dry matter content of 8%) (Verkleij, 1992). The frequency of application is crop-dependent, however the seaweed extract should be applied several times during the growing season, as the effects appear to be gradual and cumulative. For this experiment, the *Gracilaria* tips were permanently in the dilute Kelpak® concentrate, however the Kelpak® dilutions fall within the range of those used in agricultural crops.

One-third strength standard Provasoli Enriched Seawater (ES) medium was prepared according to a standard recipe (Appendix 1) (Starr and Zeikos, 1987). The treatments were: control (no Kelpak®), 1:100, 1:250, 1:500, 1:1000, 1:2500, and 1:5000 Kelpak® added to one third strength ES. The culture medium was changed every 2 days, which maintained constant value for the salinity and pH. The small amount of material per culture dish prevented problems with pH changes.

The experiments were carried out at 15 ^{o}C with an irradiance of 50–80 μ mol photons m^{-2} s^{-1} provided by cool white fluorescent tubes and a photoperiod of 16 hours (light) : 8 hours (dark). Culture vessels were crystallizing dishes to which 200 ml of ES medium was added. There were four replicates for each of the three treatments (5, 10 and 15 mm), each containing one apical segment. The crystallizing

dishes were rotated on a daily basis to ensure they were all exposed to similar light conditions. The experiment was run for 15 days and repeated four times.

2.2.3. Seaweed Concentrate

The commercially available seaweed concentrate used in this study was Kelpak®. Kelpak® is manufactured by Kelp Products (Pty) Ltd., Simons Town, Republic of South Africa. The seaweed concentrate is manufactured from the stipes and fronds of the brown alga *Ecklonia maxima* (Osbeck) Papenfuss, using a cell burst process. This process involves the use of pressure on fresh material to compress the cells in the absence of air or water followed by the sudden release resulting in the rupture of the cell walls and release of the contents. That is, the seaweed is progressively reduced in particle size, and the particles pass under extremely high pressure into a low pressure chamber where they disintegrate, resulting in the liquid extract. The process excludes the use of heat, chemicals or dehydration that could affect some of the organic components of the concentrate (Verkleij, 1992).

The Kelpak® concentrate used in this study was the concentrated version, produced for commercial agriculture, rather than the more diluted version of Kelpak® concentrate produced for home gardens.

2.2.4. Data Analysis

Individual measurements of thallus length (mm) were made using a Vernier calliper and the branches counted every five days. The initial and final biomass (in fresh weight) was determined. The specific growth rate (SGR) was calculated using the following formula:

$$SGR\ (\%\ day^{-1}) = (100 \ln N_t / N_o) / t$$

For the specific growth rate using length, t is time in days, N_o is the initial length (mm) and N_t is the length (mm) at time t. For the specific growth rate using the fresh biomass, t is time in days, N_o is the initial weight (mg) and N_t is the weight (mg) at time t (as used by Engledow and Bolton, 1992).

2.2.5. Statistical Analysis

The effects of different Kelpak® dilutions on the growth rate of *Gracilaria gracilis* was analysed using ANOVA, the single factorial analysis of variance (p = 0.05) using the statistical package STATISTICA 7, to test the null hypothesis that the means of the specific growth rate of all tested Kelpak® dilutions were not significantly different. The least significance difference (LSD) test or planned comparison test was conducted at the 95 % confidence level, to distinguish significantly different results.

2.3. RESULTS

2.3.1. *Gracilaria gracilis* apical segments with 5 mm initial length

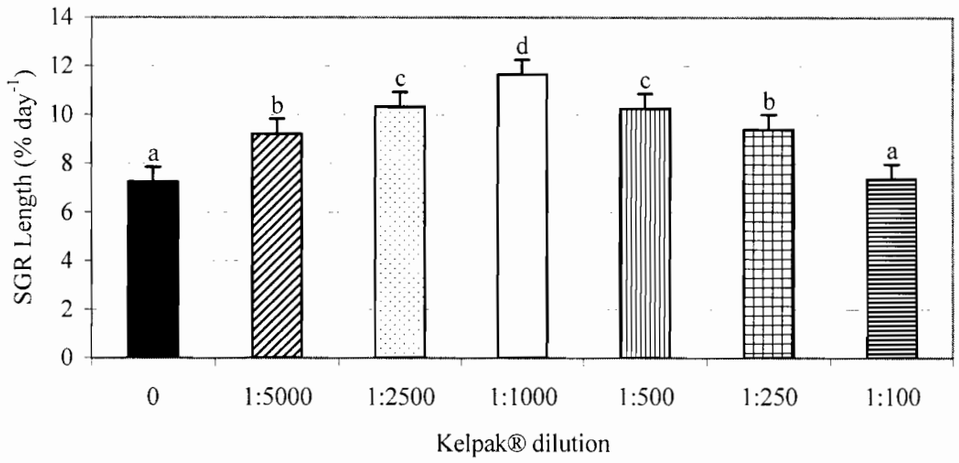
In the first 7 days, the main axes of *Gracilaria gracilis* apical segments had a higher specific growth rate (in terms of length) in all the treatments except in 1:100 Kelpak® dilution, compared to the control (Figure 2.1 A). *Gracilaria gracilis* apical segments had significantly higher specific growth rates (in terms of length) in 1:1000

Kelpak® dilution (11.65 % day^{-1}), followed by 1:2500 and 1:500 Kelpak® dilutions (10.32 % day^{-1} and 10.25 % day^{-1}, respectively) compared to the control (7.24 % day^{-1}) and other treatments. Apical segments growing in 1:250 and 1:5000 Kelpak® dilutions (9.39 % day^{-1} and 9.21 % day^{-1}, respectively) had significantly higher SGR (in terms of length) when compared to the control and 1:100 Kelpak® dilution. There was no significant difference in SGR (in terms of length) of apical segments growing in 1:100 Kelpak® dilution compared to the control.

After 15 days in culture (Figure 2.1 B), the apical segments growing in 1:1000 Kelpak® dilution had significantly the higher SGR (in terms of length) of 8.39 % day^{-1} when compared to the control (6.36 % day^{-1}) and the other treatments. *Gracilaria gracilis* apical segments had significantly higher specific growth rates (in terms of length) of 7.43 % day^{-1}, 7.29 % day^{-1} and 6.90 % day^{-1} in 1:500, 1:2500 and 1:250 Kelpak® dilutions, when compared to the control. There was no significant increase in the SGR (in terms of length), of apical segments growing in 1:5000 and 1:100 Kelpak® dilutions compared to the control.

After 7 days in culture, the SGR (in terms of fresh mass) of *Gracilaria gracilis* apical segments growing with different Kelpak® dilutions showed no significant difference throughout the treatments and the control (Figure 2.2 A). After 15 days in culture (Figure 2.2 B), *Gracilaria gracilis* apical segments treated with 1:1000 Kelpak® dilution in ES medium, had a significantly higher SGR (in terms of fresh mass) of 13.10 % day^{-1} compared to the control (11.29 % day^{-1}) and 1:100 Kelpak® dilution (11.41 % day^{-1}). All the other treatments showed no significant difference in the SGR (in terms of fresh mass) compared to the control.

A



B

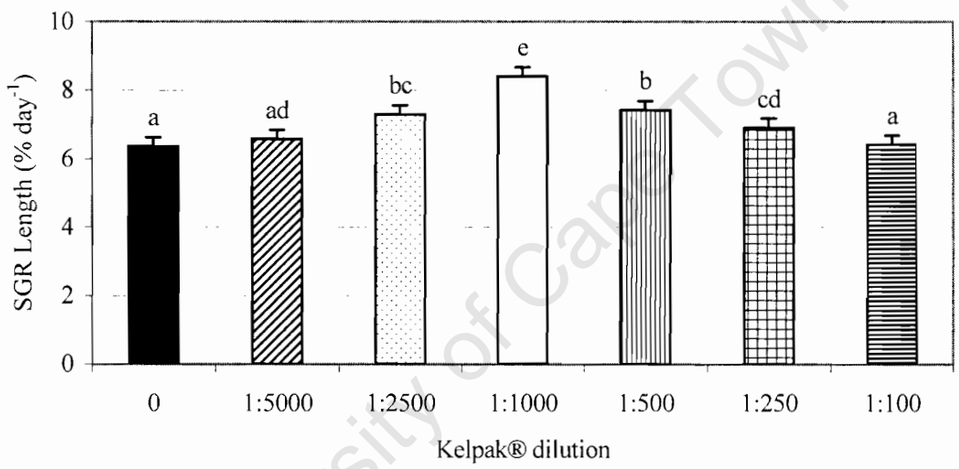


Figure 2.1. The effect of various Kelpak® dilutions on the specific growth rate, SGR (% day^{-1}) determined from average length (mm), of apical segments of *Gracilaria gracilis* with initial length of 5 mm. A. After 7 days in ES culture medium. B. After 15 days in ES culture medium. Vertical lines represent ± one Standard Error, $p = 0.05$. Values of bars marked by a different letter are significantly different according to the LSD post-hoc test ($p < 0.05$).

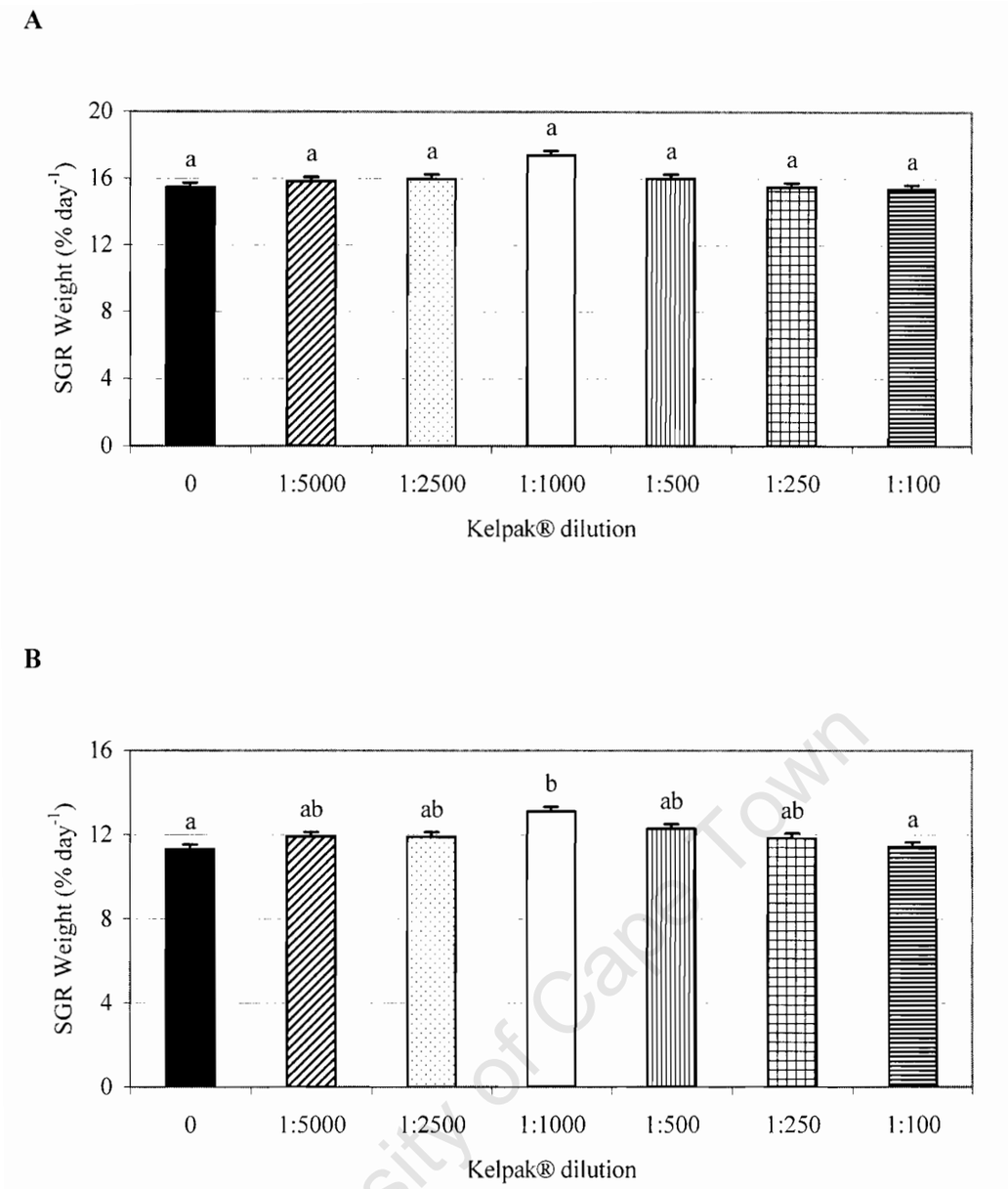


Figure 2.2. The effect of various Kelpak® dilutions on the specific growth rate, SGR (% day^{-1}) determined from fresh mass (mg), of apical segments of *Gracilaria gracilis* with initial length of 5 mm. A. After 7 days in ES culture medium. B. After 15 days in ES culture medium. Vertical lines represent ± one Standard Error, $p = 0.05$. Values of bars marked by a different letter are significantly different according to the LSD post-hoc test ($p < 0.05$).

2.3.2. *Gracilaria gracilis* apical segments with 10 mm initial length

Apical segments of *Gracilaria gracilis* in ES medium after 7 days (Figure 2.3 A) had a significantly higher SGR (in terms of length) in 1:500 and 1:1000 Kelpak® dilutions (3.53 % day^{-1} and 3.50 % day^{-1}, respectively) compared to the control (2.90 % day^{-1}) and 1:5000, 1:250 and 1:100 Kelpak® dilutions. Apical segments growing in 1:2500 Kelpak® dilution had a significantly higher SGR (in terms of length) of 3.30% day^{-1} compared to the control (2.90 % day^{-1}) and 1:100 Kelpak® dilution (2.87 % day^{-1}). There was no significant difference in SGR (in terms of length) of apical segments in the control and 1:5000, 1:250 and 1:100 Kelpak® dilutions. However, apical segments growing in 1:250 Kelpak® dilution had a significantly higher SGR (in terms of length) when compared to apical segments growing in 1:100 Kelpak® dilution.

After 15 days in culture, the significantly higher SGR (in terms of length) of the apical segments of *Gracilaria gracilis* (Figure 2.3 B) was found in Kelpak® dilutions of 1:1000 (6.12 % day^{-1}), 1:2500 (4.68 % day^{-1}) and 1:500 (4.62 % day^{-1}) compared to the control (3.64 % day^{-1}) and the 1:5000 and 1:100 Kelpak® dilutions (3.80 % day^{-1} and 3.54 % day^{-1}). The SGR (in terms of length) of *Gracilaria gracilis* apical segments growing in 1:250 Kelpak® dilution was significantly higher when compared to 1:100 Kelpak® dilution, however not significantly different from the control. There was no significant difference in SGR (in terms of length) of the apical segments growing in 1:5000 and 1:100 Kelpak® dilutions and control.

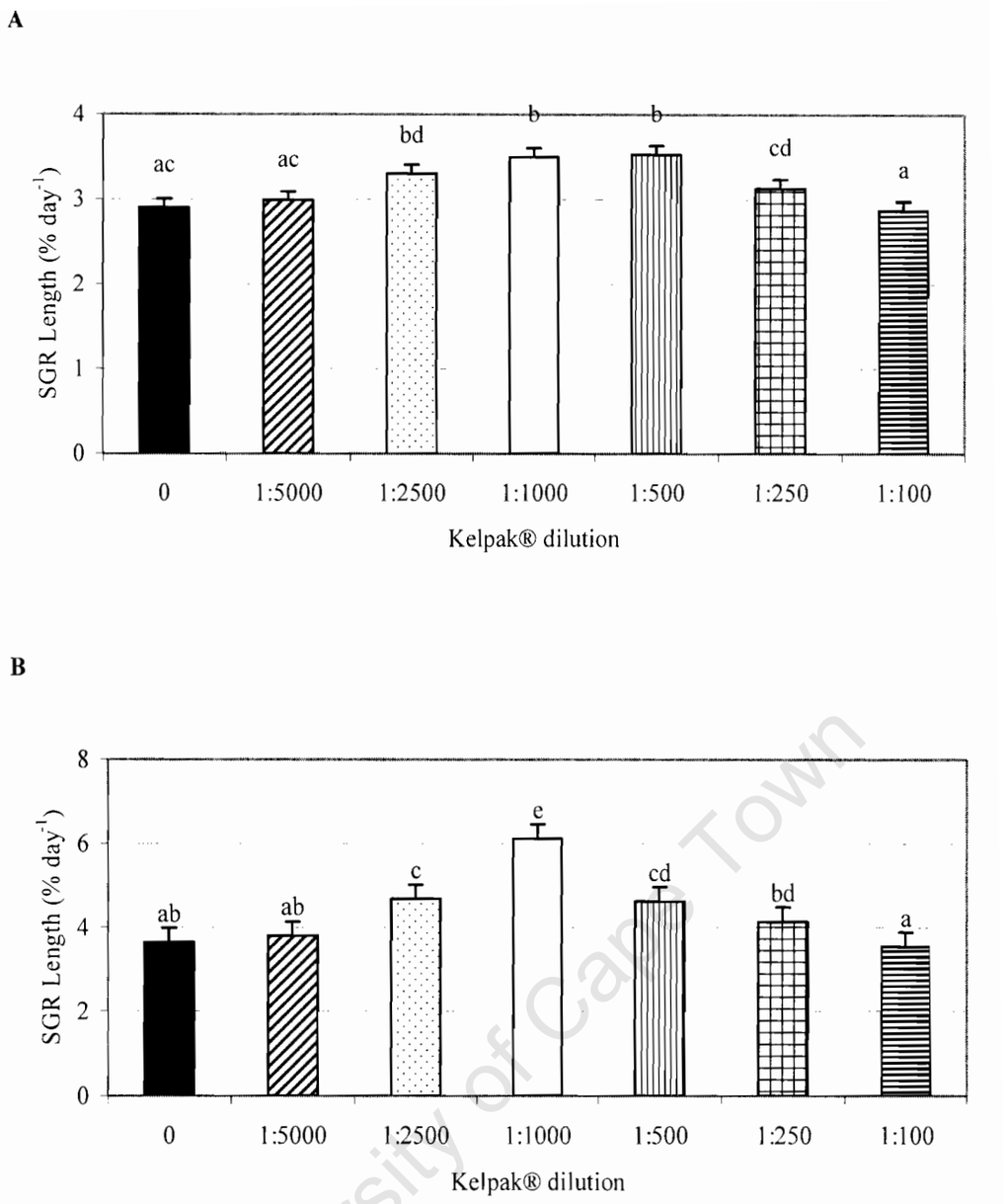


Figure 2.3. The effect of various Kelpak® dilutions on the specific growth rate, SGR (% day^{-1}) determined from average length (mm), of apical segments of *Gracilaria gracilis* with initial length of 10 mm. A. After 7 days in ES culture medium. B. After 15 days in ES culture medium. Vertical lines represent ± one Standard Error, $p = 0.05$. Values of bars marked by a different letter are significantly different according to the LSD post-hoc test ($p < 0.05$).

Table 2.1 shows the effect of different Kelpak® dilutions on the average number of branches of *Gracilaria gracilis* in ES medium. After 15 days, there was no change in the average number of branches of *Gracilaria gracilis* growing in different treatments compared to the control or among the treatments.

Table 2.1. The effect of various Kelpak® dilutions on the average number of branches of apical segments of *Gracilaria gracilis* with initial length of 10 mm after 15 days in ES medium. The same letter denotes no significant differences at 5 percent level (one-way ANOVA and LSD post-hoc test ($p < 0.05$)).

Kelpak® dilution	**Average number of branches**
0	0.3 ± 0.5 a
1:5000	0.3 ± 0.6 a
1:2500	0.3 ± 0.5 a
1:1000	0.3 ± 0.5 a
1:500	0.3 ± 0.5 a
1:250	0.3 ± 0.6 a
1:100	0.1 ± 0.4 a

The specific growth rate (measured as a change in fresh mass) of *Gracilaria gracilis* apical segments growing in different Kelpak® dilutions after 7 days in ES medium is represented in Figure 2.4 A. *Gracilaria gracilis* apical segments growing in 1:1000 Kelpak® dilution had a significantly higher SGR (in terms of fresh mass) of 11.74 % day^{-1}, followed by 1:500 and 1:2500 Kelpak® dilutions of 9.64 % day^{-1} and 8.21 % day^{-1} when compared to the control (5.46 % day^{-1}). There was no significant

increase in the SGR (in terms of fresh mass) of apical segments growing in 1:5000, 1:250 and 1:100 Kelpak® dilutions compared to the control.

After 15 days in culture (Figure 2.4 B), *Gracilaria gracilis* apical segments in ES medium treated with 1:1000 Kelpak® dilution had significantly higher SGR (in terms of fresh mass) of 8.21 % day^{-1} followed by 1:500, 1:250, 1:2500 Kelpak® dilutions (7.69 % day^{-1}, 7.27 % day^{-1} and 7.11% day^{-1}, respectively) compared to the control (5.22 % day^{-1}) and the 1:100 Kelpak® dilution (5.17 % day^{-1}). Apical segments growing in ES medium with 1:5000 and 1:100 Kelpak® dilutions showed no significant difference in SGR (in terms of fresh mass) compared to the control. The highest SGR (in terms of fresh mass) was more than 50% greater than that in the control.

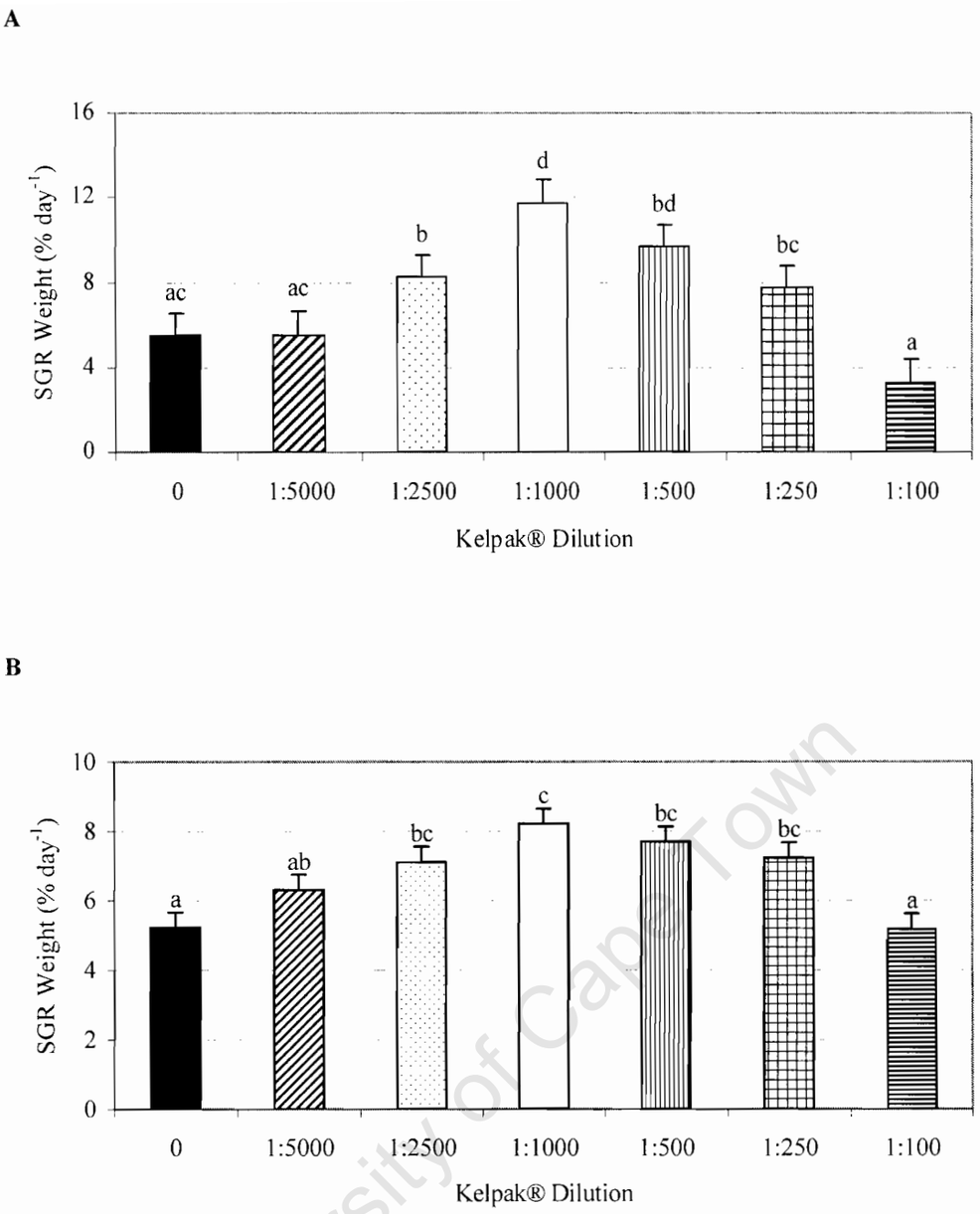


Figure 2.4. The effect of various Kelpak® dilutions on the specific growth rate, SGR (% day^{-1}) determined from fresh mass (mg), of apical segments of *Gracilaria gracilis* with initial length of 10 mm. A. After 7 days in ES culture medium. B. After 15 days in ES culture medium. Vertical lines represent ± one Standard Error, $p = 0.05$. Values of bars marked by a different letter are significantly different according to the LSD post-hoc test ($p < 0.05$).

2.3.3. *Gracilaria gracilis* apical segments with 15 mm initial length

Apical segments of *Gracilaria gracilis* (Figure 2.5 A) showed a significantly higher SGR (in terms of length) after 7 days in ES medium, in 1:1000, 1:500 and 1:2500 Kelpak® dilutions (8.91 % day^{-1}, 8.25 % day^{-1} and 8.25% day^{-1}, respectively) compared to the control (4.46 % day^{-1}) and the other treatments. Apical segments growing in ES medium with 1:5000 Kelpak® dilution had significantly higher SGR (in terms of length) compared to the control and 1:100 Kelpak® dilution.

After 15 days in culture, all concentrations of Kelpak® tested, with the exception of the most concentrated (1:100 Kelpak® dilution) significantly increased growth of *Gracilaria gracilis* (Figure 2.5 B). Apical segments of *Gracilaria gracilis* had a significantly higher SGR (in terms of length) in 1:1000 Kelpak® dilution (6.40 % day^{-1}), followed by 1:500 and 1:2500 Kelpak® dilutions (5.57 % day^{-1} and 5.09 % day^{-1}, respectively) compared to the control (2.89% day^{-1}) and other treatments. Apical segments growing in 1:250 and 1:5000 Kelpak® dilutions had a SGR (in terms of length) of 3.57 % day^{-1} and 3.57 % day^{-1}, significantly higher compared to the 2.89 % day^{-1} from the control and 2.88 % day^{-1} from the 1:100 Kelpak® dilution. When compared with the control the apical segments growing in 1:100 Kelpak® dilution showed no significant difference in the SGR (in terms of length).

Although the 1:5000 Kelpak® dilution treatment showed a increase in branching, there was no significant difference in the number of branches of *Gracilaria gracilis* in the different Kelpak® dilutions compared to the control (Table 2.2). However, branching was significantly reduced in the 1:100 Kelpak® dilution, compared to the 1:5000 Kelpak® dilution.

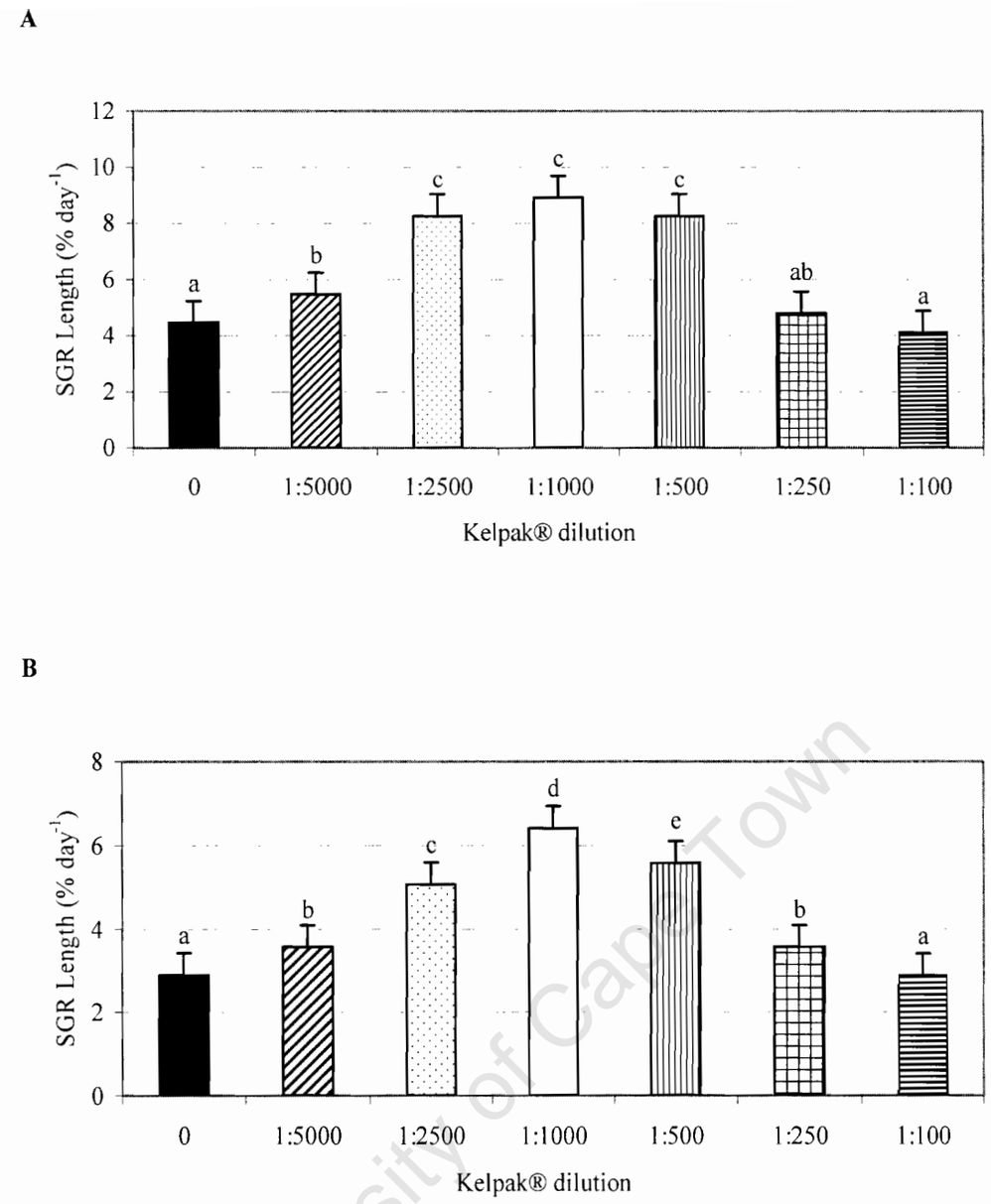


Figure 2.5. The effect of various Kelpak® dilutions on the specific growth rate, SGR (% day^{-1}) determined from average length (mm), of apical segments of *Gracilaria gracilis* with initial length of 15 mm. A. After 7 days in ES culture medium. B. After 15 days in ES culture medium. Vertical lines represent ± one Standard Error, $p = 0.05$. Values of bars marked by a different letter are significantly different according to the LSD post-hoc test ($p < 0.05$).

Table 2.2. The effect of various Kelpak® dilutions on the total number of branches of apical segments of *Gracilaria gracilis* with initial length of 15 mm after 15 days in ES medium. Different letters denote significant differences at 5 percent level (one-way ANOVA and LSD post-hoc test ($p < 0.05$)).

Kelpak® dilution	**Total number of branches**
0	1.4 ± 1.6 ab
1:5000	1.9 ± 1.6 a
1:2500	1.4 ± 1.3 ab
1:1000	1.3 ± 1.8 ab
1:500	0.9 ± 1.7 ab
1:250	0.9 ± 1.5 ab
1:100	0.7 ± 0.9 b

Gracilaria gracilis apical segments after 7 days culture, had significantly higher SGR (in terms of fresh mass) in ES medium with 1:1000 Kelpak® dilution (10.01 % day^{-1}) compared to the control (6.25 % day^{-1}) and 1:5000 and 1:100 Kelpak® dilutions (6.25 % day^{-1} and 5.99 % day^{-1}) (Figure 2.6 A). No significant difference in SGR (in terms of fresh mass) were observed for apical segments growing in the rest of the Kelpak® dilutions compared to the control. After 15 days in culture (Figure 2.6 B), apical segments in ES medium showed a significantly higher SGR (in terms of fresh mass) of 7.18 % day^{-1} in 1:1000 Kelpak® dilution compared to the control (4.07 % day^{-1}) and the rest of the treatments. There was no significant difference in the SGR (in terms of fresh mass) of apical segments growing in the rest of the treatments compared to the control.

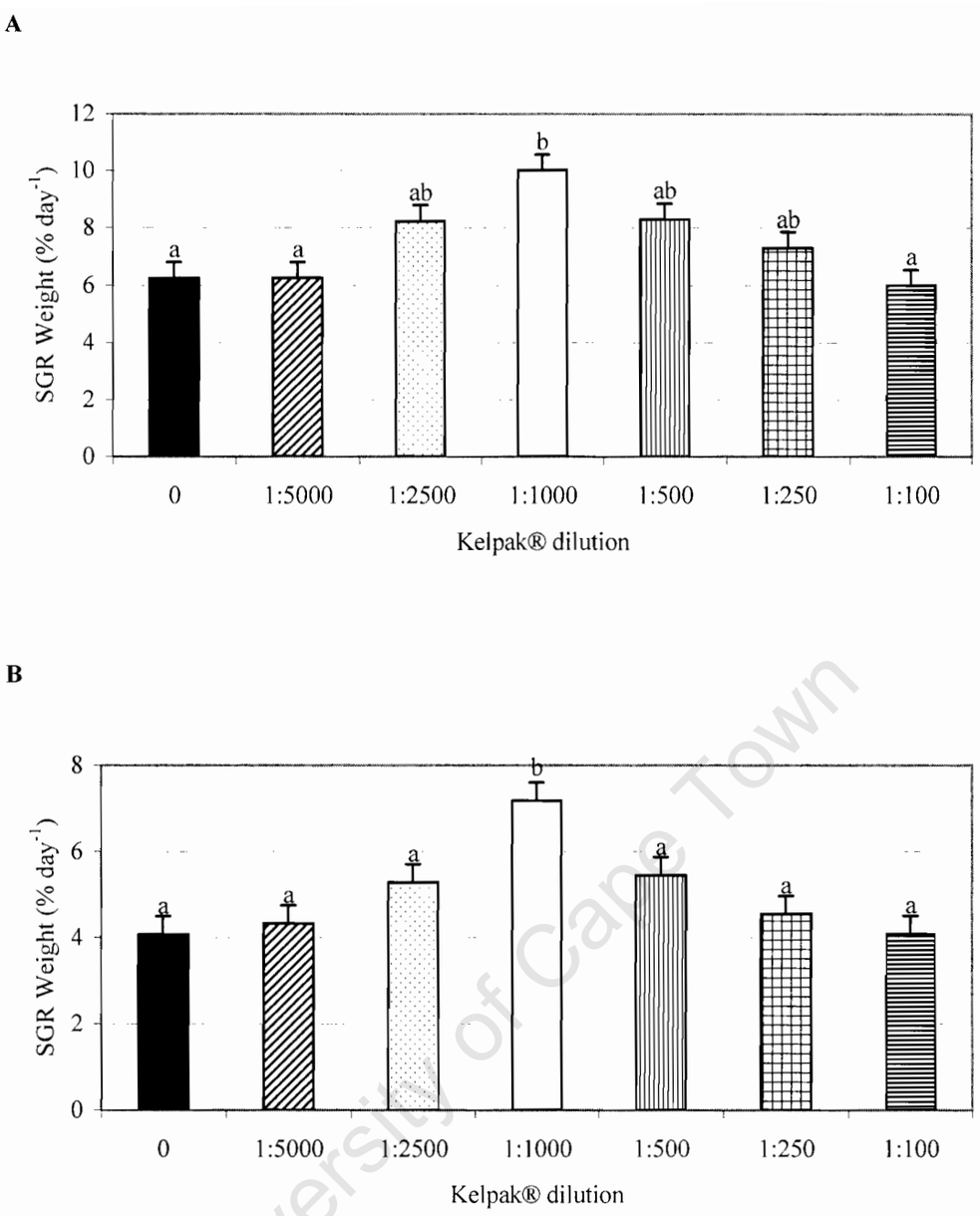


Figure 2.6. The effect of various Kelpak® dilutions on the specific growth rate, SGR (% day^{-1}) determined from fresh mass (mg), of apical segments of *Gracilaria gracilis* with initial length of 15 mm. A. After 7 days in ES culture medium. B. After 15 days in ES culture medium. Vertical lines represent ± one Standard Error, $p = 0.05$. Values of bars marked by a different letter are significantly different according to the LSD post-hoc test ($p < 0.05$).

2.4. DISCUSSION

The ability of commercial seaweed products to promote growth, increase yield and prevent pests and disease in biological agriculture and horticulture has been well documented (Blunden and Wildgoose, 1977; Finnie and van Staden, 1985; Nelson and van Staden, 1986; Featonby-Smith and van Staden, 1984a, 1984b, 1987; Beckett and van Staden, 1990; Stirk and van Staden, 1997, 2004; Stirk *et al*., 2004). Finnie and van Staden (1985) have demonstrated that the water dilution ratio of *Ecklonia maxima* kelp concentrate is an important factor controlling its effectiveness. In land plants, low dilution ratios (1:100 seaweed concentrate dilution in water) were found to have an inhibitory effect upon root growth, whereas higher dilution ratios (1:400, 1:500 and 1:600) had a stimulatory effect. Such seaweed concentrate dilution effects upon plant root growth could be attributed to growth inhibitors in the concentrate that, upon increasing dilution, become less effective than the growth promoting substances. Furthermore, Temple and Bomke (1989) suggested that optimal dilution ratios of the concentrate to land plants might depend on the particular environmental conditions to which plants are subjected. The concentrations of seaweed concentrate Kelpak®, used in this study are within the range of concentrations commonly used in land plant studies. However, in this experiment the seaweeds are permanently in the diluted Kelpak® concentrate whereas in agriculture Kelpak® diluted concentrate is applied intermittently, and either sprayed directly to the leaves or applied to the soil (Verkleij, 1992).

The results of this study show that the addition of seaweed concentrate has the ability to enhance growth of *Gracilaria gracilis* apical segments. Treatment of *Gracilaria gracilis* apical segments with 1:1000 Kelpak® dilution after fifteen days in

ES medium gave the maximum growth compared to the control, independently of the initial length of *Gracilaria gracilis* apical segments. These results contradict the studies obtained by Beckett and van Staden (1990), in which the growth of wheat did not change with low concentrations (1:1000) of Kelpak® compared to control. However, the results obtained in the present study agree with a previous preliminary study on *Gracilaria gracilis* (Leitao, 2001).

Gracilaria gracilis apical segments growing in ES medium with 1:500 Kelpak® dilution had the second highest increase in growth compared to the control. There was an increase in the specific growth rate of apical segments, independently of their initial length, compared to the control. However, apical segments with an initial length of 15 mm had the highest significant increase in growth compared to the control, followed by the apical segments with initial lengths of 10 mm and 5 mm. This is consistent with the report that seaweed concentrate Kelpak® at dilution 1:500 applied regularly, improved the total biomass of *Beta vulgaris* and *Phaseolus vulgaris* (Crouch, 1990). In a study conducted by Nelson and van Staden (1984), root growth was stimulated in greenhouse studies with cucumber plants sprayed weekly with 1:500 seaweed concentrate dilution, leading to 56 % increase in total plant biomass. Furthermore, the treatment tended to increase the phosphorus content in the leaves and to decrease the nitrogen content, leading to the suggestion that the seaweed treatment had induced the uptake of 'unavailable' nutrients by cucumber roots or had improved the efficiency of utilization of 'available' nutrients. Similarly, in a study conducted by Nelson and van Staden (1986), the shoot and root dry mass of wheat increased with the application of 1:500 seaweed concentrate dilution. Interestingly, maximum yield was obtained at sub maximal rates of seaweed concentrate, indicating

that the seaweed did not have a direct effect on growth but acted as a stimulant. The same was observed in this experiment with *Gracilaria gracilis* apical segments.

The 1:2500 Kelpak® dilution also caused a significant increase in the specific growth rate of *Gracilaria gracilis* apical segments, independently of their initial length, compared to the control. These results agree with studies in which Kelpak® concentrate used at a dilution of 1:2500, caused the highest specific growth rate of *Ulva lactuca* L. in a tank experiment on an abalone aquaculture farm (Robertson-Andersson, 2004; Robertson-Andersson *et al*., in press). Furthermore, 1:2500 Kelpak® dilution applied regularly to cucumber plants has enhanced the root growth (Nelson and van Staden, 1984).

Treatment with 1:5000 Kelpak® dilution did not significantly increase the specific growth rate of *Gracilaria gracilis*, which is in agreement with Beckett and van Staden (1990), who showed that the growth of wheat was not stimulated by low concentrations of seaweed concentrate compared to the controls. There was no effect on apical segments of *Gracilaria gracilis* in ES medium with 1:100 Kelpak® dilution. Finnie and van Staden (1985) reported that tomato roots were inhibited with the application of 1:100 seaweed concentrate dilution. Furthermore, this effect was also observed for both *Gracilaria gracilis* and *Ulva lactuca* (Leitao, 2001; Robertson-Andersson, 2004; Robertson-Andersson *et al*., in press).

Santelices and Varela (1995), in their study of *Gracilaria chilensis*, have suggested that intercalary growth was more important than apical growth in contributing to elongation, since thallus length increment was found to be positively

correlated to thallus length, while specific growth rate was inversely correlated to length. According to M. Steentoft (Smit *et al.*, 1997), *Gracilaria gracilis* growth occurs throughout the thallus and not particularly near the apex. Smit and Bolton (1999) have further suggested that growth was significant over the entire thallus; however it was found that the apical region contributes more to the overall elongation than does the proximal part of the thallus. From the present study, when comparing the specific growth rate of *Gracilaria gracilis* apical segments of different initial lengths, the specific growth rates in all the treatments with Kelpak® concentrate is higher for 5 mm apical segments than for those of 10 mm and 15 mm. This suggests that the smaller the apical segments of *Gracilaria gracilis* the more it contributes to the overall elongation. Furthermore, there was an increase in the growth of *Gracilaria gracilis* apical segments when exposed to specific concentrations of the commercial seaweed liquid concentrate Kelpak®. The results of this investigation confirm previous findings on the effects of seaweed concentrate applications on the growth of certain biological agriculture and horticulture crops (Featonby-Smith and van Staden, 1983a, 1983b), and on the growth of seaweeds *Gracilaria gracilis* and *Ulva lactuca* (Leitao, 2001; Robertson-Andersson, 2004; Robertson-Andersson *et al.*, in press).

After 15 days in culture, no branching was observed on *Gracilaria gracilis* apical segments with initial length of 5 mm in any of the treatments. However, there was branching in apical segments with initial length of 10 mm in all the treatments after 15 days in culture, nevertheless not significantly different from the control. Branching also occurred in apical segments with initial length of 15 mm after 15 days in culture in all treatments. It is possible that branching is related to the length of the

apical segments rather than to a particular treatment, since no treatment caused significant branching in the apical segments of *Gracilaria gracilis*.

The reasons for the growth increase obtained with the use of Kelpak® concentrate are not completely understood. It is however, thought that the hormonal content, particularly cytokinin content of the seaweed, plays an important role (Featonby-Smith and van Staden, 1983a, 1983b). The study conducted by Blunden and Wildgoose (1977) demonstrated that aqueous seaweed extract of known cytokinin activity significantly increased the yield of potatoes. Close correlations were also found to exist between the results obtained from the use of synthetic cytokinin, kinin and seaweed extract of equivalent cytokinin activity. Furthermore, Finnie and van Staden (1985) showed that excised tomato roots exposed to low concentration of seaweed concentrate Kelpak® mimicked the effect of low levels of cytokinin. The stimulatory effect of the seaweed was lost if the material was ashed indicating that the regulatory substance is associated with the organic rather than with inorganic fraction. It is known from the literature that seaweeds contain plant growth substances (Bradley, 1991; Stirk *et al*., 2004), and that the additions of plant growth hormones to media increases growth, callus formation of various red algae including *Gracilaria* (Yokoya, 2000; Yokoya *et al*., 1999; 2004). Therefore, plant growth regulators may have been implicated in this study.

In the present study, Kelpak® concentrate was diluted into the ES medium and *Gracilaria* apical segments grown in that solution throughout the experiment. Because additional nutrients are present in the ES medium, and the Kelpak® concentrate was very dilute, it seems less likely that the growth of *Gracilaria* was promoted by

additional nutrients in Kelpak® concentrate, but rather by the plant growth regulators in it.

CHAPTER 3

EFFECTS OF PLANT GROWTH REGULATORS (AUXIN AND CYTOKININ) ON *GRACILARIA GRACILIS* GROWTH IN LABORATORY CULTURE

3.1. INTRODUCTION

Plant growth regulators are substances that influence physiological processes of plants at very low concentrations (Frankenberger and Arshad, 1995). When produced endogenously by plants, they are often referred to as plant hormones. Plant hormones have been viewed as chemical messengers regulating the normal progression of developmental changes, as well as responses to environmental signals (Morgan, 1990). The term plant growth regulator includes a large number of synthetic and naturally occurring compounds. Nickell (1982) defined plant growth regulators as either naturally or synthetic compounds that are applied directly to a target plant to alter its life process or its structure to improve quality, increase yields, or facilitate harvesting. The terms plant growth regulators and plant hormones have been used when referring to auxins, cytokinins, gibberellins, ethylene and abscisic acid (Frankenberger and Arshad, 1995). Despite the fact that plants are capable of synthesizing plant hormones and absorbing plant hormones generated by microorganisms they may also respond to exogenous applications of hormones during certain growth stages and under specific conditions (Frankenberger and Arshad, 1995). Plants may not synthesize enough endogenous plant hormones for optimal growth and development under certain climatic and environmental conditions, and

therefore proper application of plant growth regulators may enhance plant growth. In recent years, some natural products have received attention in agriculture and horticulture (Crouch, 1990; Schmidt, 1990; Senn, 1987, 1991). These naturally derived and hormone-containing products can not only stimulate plant growth and development, but also improve plant resistance to environmental stresses. Additionally these organic substances pose no threat to the environment. These products, a group of plant growth regulators, are also called biostimulants and include seaweed extracts (Schmidt, 1990).

Besides major and trace nutrients, seaweeds and seaweed extracts contain plant growth regulators. The presence of cytokinins in seaweeds and in commercial seaweed products was first demonstrated by Brain *et al*. (1973), and confirmed in many other studies (Blunden and Wildgoose, 1977; Featonby-Smith and van Staden, 1983a, 1983b; Mooney and van Staden, 1984; Zhang *et al*., 1991; Duan *et al*., 1995; Tay *et al*., 1985, 1987). Other plant hormones have also been detected in seaweeds, including gibberellins (Williams *et al*., 1981), auxins (Sumera and Cajipe, 1981), gibberellin-like substances (Bentley-Mowat and Reid, 1969), abscisic acid and indole acetic acid (Kingman and Moore, 1982; Boyer and Dougherty, 1988). The ethylene precursor 1-aminocyclopropane-1-carboxylic acid has also been detected in a seaweed concentrate (Nelson and van Staden, 1984). The concentration of plant hormones in seaweed extract is in line with those generally found in turgid leaves in higher plants (Boyer and Dougherty, 1988; Featonby-Smith and van Staden, 1983a, 1983b, 1984a, 1984b; van Staden and Davey, 1981). The concentrations of cytokinins and auxins in seaweed extracts may be sufficient to produce biological effects on the plants, even at

low rates of application used in practice (Williams *et al.*, 1981; Sanderson and Jameson, 1986).

An extensive literature describes the occurrence and physiological functions of plant growth regulators in land plants. Plants need to be able to control their growth and development and to respond to environmental stimuli. In land plants, the combined effects of plant hormones control these factors. Far less is known about the presence and action of plant growth regulators on seaweeds. Therefore, investigations of the effects of plant growth regulators on algal growth and development are needed not only to understand the physiological basis of algal development but also to improve seaweed cultivation.

Red seaweeds (Rhodophyta) include many economically important species used for agar, carrageenan, human food, and a variety of minor uses. The exploitation and the consequent development of aquaculture of the red seaweed *Gracilaria* has increased around the world, for their agar content and more recently as a food source for abalone *Haliotis midae* L. in aquaculture (Anderson *et al.*, 2003a). There is evidence from Chapter 2, that Kelpak® concentrate enhances the growth of *Gracilaria gracilis* in laboratory culture. Growth, callus induction, and regeneration of different species of red algae have been controlled by plant growth regulators (Bradley and Cheney, 1990; Dawes and Koch, 1991; Dawes *et al.*, 1993; Kaczyna and Megnet, 1993; Yokoya and Handro, 1996; Huang and Fugita, 1997). Furthermore, from the literature there is evidence that cytokinins and auxins are present in Kelpak® concentrate (Crouch, 1990; Stirk and van Staden, 1997). Therefore, in this study I test

the hypothesis that the plant growth regulators (cytokinin and auxin) present in Kelpak® concentrate are responsible for enhanced growth of *Gracilaria gracilis*.

3.2. MATERIALS AND METHODS

3.2.1. Plant Material

The material was collected on the day prior to the start of the experiment. Infertile plants of the red algae *Gracilaria gracilis* were collected from Saldanha Bay on the South African west coast and brought to the University of Cape Town phycology laboratory. In the laboratory, *Gracilaria* was washed with running fresh water and sterile seawater and brushed with a soft brush, to reduce contaminants. The darkest thallus fragments were selected and 10 mm apical segments cut from random plants. The length selected for the apical segments was based on the experiment conducted in Chapter 2, the 10 mm apical segments were more convenient to work with, they had less branching and less contamination of the apical region.

3.2.2. Experimental Design

The concentration of plant growth regulators present in Kelpak® concentrate is similar to those generally found in leaves in higher plants (Verkleij, 1992). In Kelpak® the concentration of cytokinins has been estimated as 0.031 mg / l undiluted extract and the concentration of auxins as 11 mg / l undiluted extract (Verkleij, 1992; Crouch and van Staden, 1992a, 1993). To observe the effects of plant growth regulators on *Gracilaria gracilis* growth, one auxin, indole-3-acetic acid (IAA) and one cytokinin, 6-benzylaminopurine (BA) were used individually and in combination

(IAA:BA). The reason for the chosen auxin and cytokinin in this study is due to their presence in Kelpak®. In addition, research has been done on the effects of the chosen auxin and cytokinin in different seaweeds (Yokoya and Handro, 1996, 1997; Yokoya *et al*., 1999). Each plant growth regulator was tested individually and combined in concentrations equivalent to the diluted Kelpak® concentrate used in Chapter 2, Section 2.2. To mimic the concentrations of plant growth regulators found in undiluted Kelpak® concentrate, the plant growth regulators were diluted in Provasoli Enriched Seawater (ES) medium to make up a stock solution. One-third strength standard Provasoli Enriched Seawater (ES) medium was prepared according to a standard recipe (Appendix 1) (Starr and Zeikos, 1987). The following stock solutions were made:

i. Auxin treatments, mentioned in the text as auxin dilution, 11 mg of indole-3-acetic acid (IAA) was added to one litre of ES medium.
ii. Cytokinin treatments, mentioned in the text as cytokinin dilution, 0.031 mg of 6-benzylaminopurine (BA) was added to one litre of ES medium.
iii. Combined auxin and cytokinin treatments, mentioned in the text as combined auxin and cytokinin dilution, 11 mg indole-3-acetic acid (IAA) and 0.031 mg of 6-benzylaminopurine (BA) was added to one litre of ES medium

The stock solutions were further diluted in ES medium to mimic the amounts of plant growth regulators present in the dilutions of Kelpak® concentrate used in Chapter 2, Section 2.2 The dilutions were: 1:100, 1:250, 1:500, 1:1000, 1:2500, and 1:5000 stock solution added to one third strength ES. The dilutions for the auxin correspond to 1.1 mg/l (1:100), 2.75 mg/l (1:250), 5.5 mg/l (1:500), 11 mg/l (1:1000),

27.5 mg/l (1:2500), and 55 mg/l (1:5000). The dilutions for the cytokinin correspond to 0.0031 mg/l (1:100), 0.0078 mg/l (1:250), 0.016 mg/l (1:500), 0.031 mg/l (1:1000), 0.078 mg/l (1:2500), and 0.155 mg/l (1:5000).

The culture medium was changed every 2 days. Controls without the addition of plant growth regulators were run simultaneously. Each treatment consisted of four replicates with three apical segments of *Gracilaria gracilis* in each, cultured in 200 ml Petri dishes. The culture conditions used in this experiment were the same as those already described for Chapter 2, Section 2.2.2. Observations were made every five days. The experiment was run for 15 days.

3.2.3. Data Analysis

Individual measurements of thallus length (mm) were made using a Vernier calliper and the branches counted every five days. The initial and final biomass (in fresh weight) was determined. The specific growth rate (SGR) was calculated using the following formula:

$$\text{SGR (\% day}^{-1}) = (100 \ln N_t / N_o) / t$$

For the specific growth rate using length, t is time in days, N_o is the initial length (mm) and N_t is the length (mm) at time t. For the specific growth rate using the fresh biomass, t is time in days, N_o is the initial weight (mg) and N_t is the weight (mg) at time t (as used by Engledow and Bolton, 1992).

3.2.4. Statistical Analysis

The effects of different concentrations of plant growth regulators on the growth rate of *Gracilaria gracilis* were statistically analyzed using ANOVA, the single factorial analysis of variance ($p = 0.05$) using the statistical package STATISTICA 7, to test the null hypothesis that the means of the specific growth rate of all tested plant growth regulator concentrations were not significantly different. The least significance difference (LSD) test or planned comparison test was conducted at the 95 % confidence level, to distinguish significantly different results.

3.3. RESULTS

3.3.1. Effect of various dilutions of indole-3-acetic acid (IAA) Auxin on *Gracilaria gracilis* apical segments

The SGR (determined as the average length) after 7 days of apical segments of *Gracilaria gracilis* growing in ES medium with different auxin dilutions is shown in Figure 3.1 A. Apical segments growing in 1:1000 auxin dilution (7.11 % day^{-1}) had significantly higher SGR (in terms of length) compared to the control (6.05 % day^{-1}). There was no significant difference in the SGR (in terms of length) of apical segments between the rest of the treatments and the control. After 15 days in culture (Figure 3.1 B), apical segments of *Gracilaria gracilis* showed significantly higher SGR (in terms of length) in 1:1000 auxin dilution (5.16 % day^{-1}), followed by 1:500 auxin dilution (4.56 % day^{-1}) and 1:2500 auxin dilution (4.47 % day^{-1}) compared to the control (3.71 % day^{-1}). Apical segments growing in 1:5000, 1:250 and 1:100 auxin dilutions were not significantly different from the control and from each other.

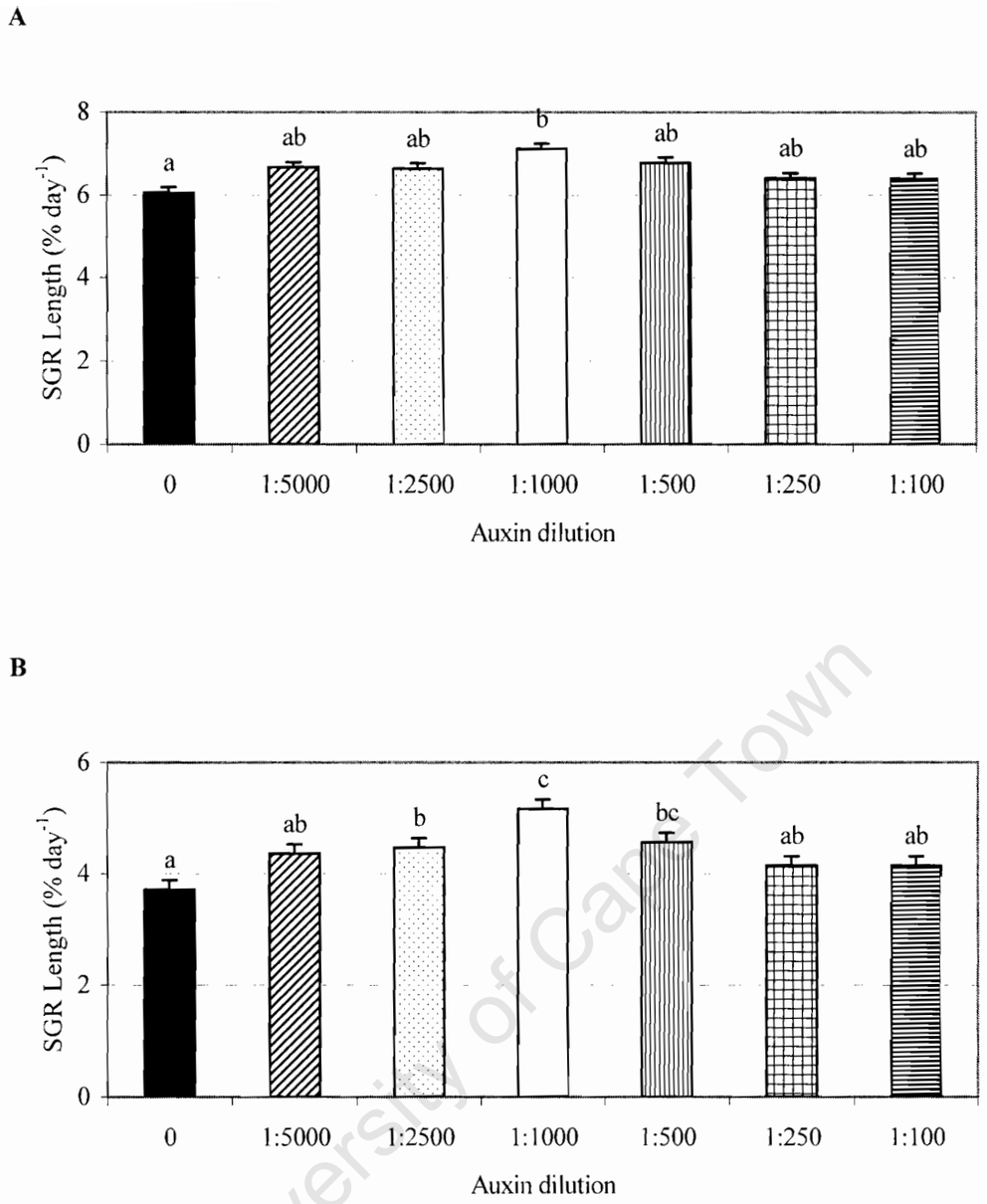


Figure 3.1. The effect of various auxin stock solution (11 mg/l) dilutions on the specific growth rate, SGR (% day^{-1}) determined from average length (mm), of 10 mm apical segments of *Gracilaria gracilis*. A. After 7 days in ES culture medium. B. After 15 days in ES culture medium. Vertical lines represent ± one Standard Error, p = 0.05. Values of bars marked by a different letter are significantly different according to the LSD post-hoc test ($p < 0.05$).

The effect of different auxin dilutions on the average number of branches of *Gracilaria gracilis* apical segments after 15 days in culture is shown in Table 3.1. There was an overall increase in the number of branches with the addition of different auxin dilutions compared to the control. However, branching was only significantly increased on apical segments growing in ES medium with 1:1000 and 1:500 auxin dilutions, compared to the control.

Table 3.1. The effect of various auxin stock solution (11 mg/l) dilutions on the average number of branches on apical segments of *Gracilaria gracilis* with initial length of 10 mm after 15 days in ES medium. Different letters denote significant differences at 5 percent level (one-way ANOVA and LSD post-hoc test ($p < 0.05$)).

Treatment	Average number of branches
0	2.6 ± 2.2 a
1:5000	4.2 ± 3.2 ab
1:2500	4.6 ± 2.8 ab
1:1000	6.1 ± 3.1 b
1:500	5.0 ± 3.6 b
1:250	4.1 ± 2.6 ab
1:100	4.1 ± 1.8 ab

The specific growth rate (in terms of fresh mass) of *Gracilaria gracilis* growing in different auxin dilutions is represented in Figure 3.2. After 7 days (Figure 3.2 A), the different auxin dilutions had little or no effect on the SGR (in terms of fresh mass) of *Gracilaria gracilis* apical segments compared to the control. Apical segments growing in 1:100 auxin dilution had a significantly lower SGR (in terms of

fresh mass) of 15.81 % day^{-1} when compared to apical segments growing in 1:500 auxin dilution of 17.36 % day^{-1}. After 15 days (Figure 3.2 B), apical segments growing in 1:1000 and 1:500 auxin dilutions showed a significantly higher SGR (in terms of fresh mass) of 11.92 % day^{-1} and 11.43 % day^{-1} respectively, compared to the control (10.31 % day^{-1}). Apical segments growing in the other auxin dilutions showed no significant difference in SGR (in terms of fresh mass) when compared to the control or among themselves.

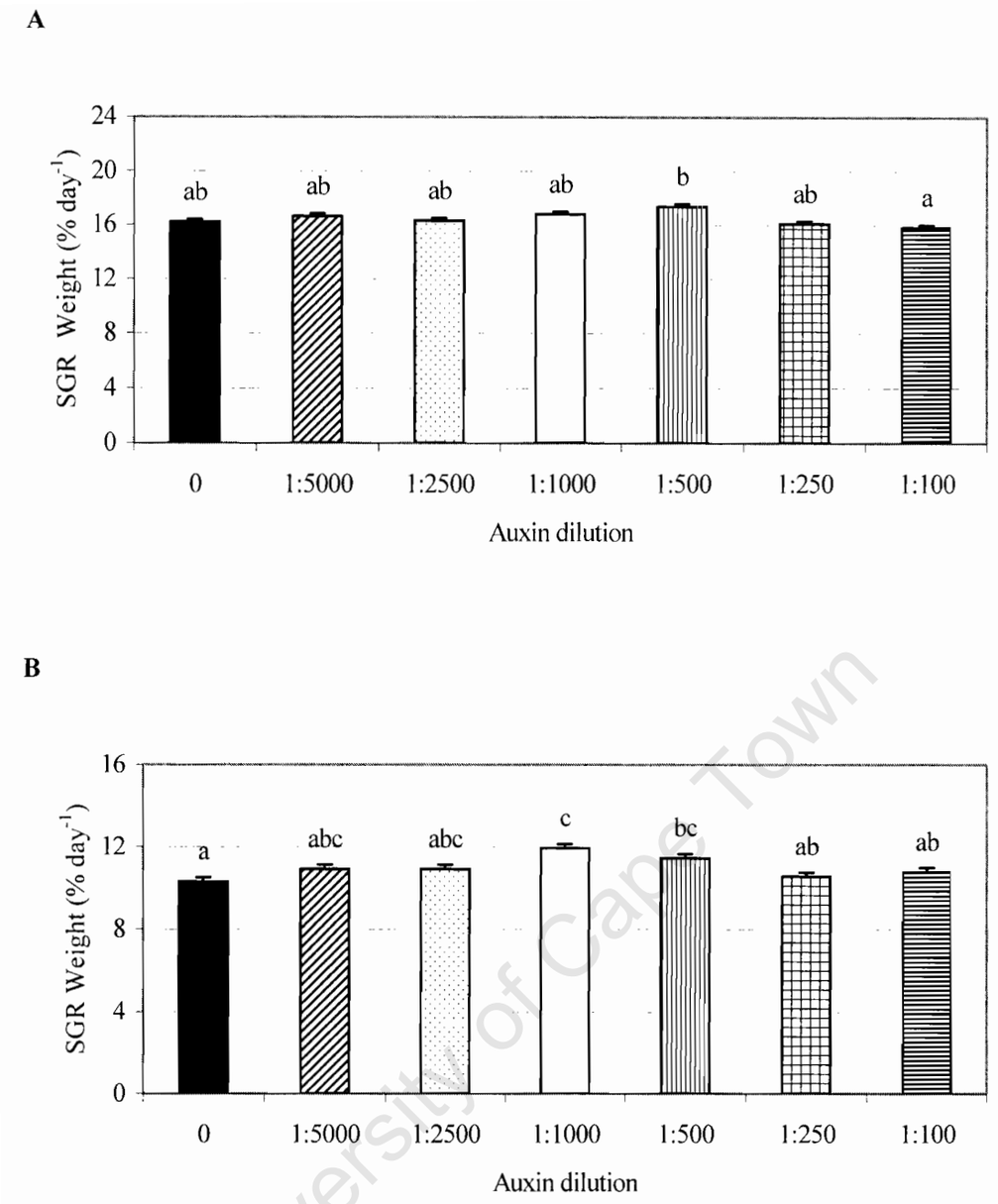


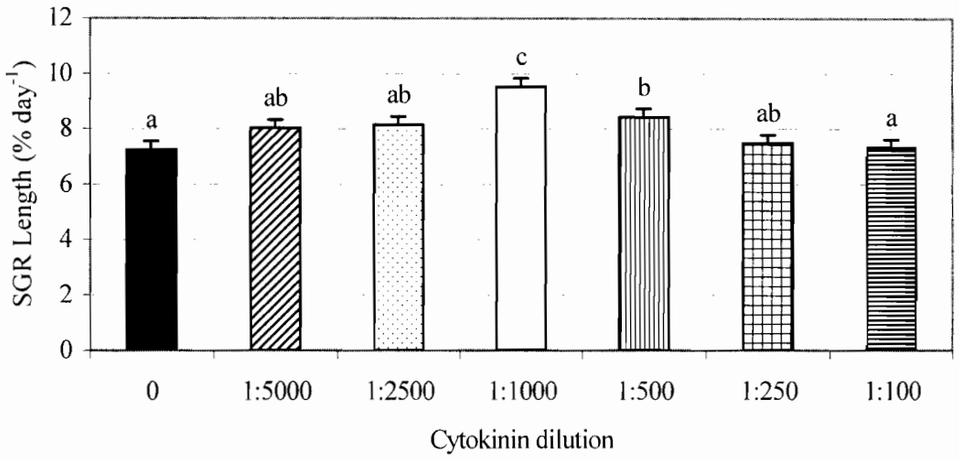
Figure 3.2. The effect of various auxin stock solution (11 mg/l) dilutions on the specific growth rate, SGR (% day^{-1}) determined from fresh mass (mg), of 10 mm apical segments of *Gracilaria gracilis*. A. After 7 days in ES culture medium. B. After 15 days in ES culture medium. Vertical lines represent ± one Standard Error, $p = 0.05$. Values of bars marked by the different letter are significantly different according to the LSD post-hoc test ($p < 0.05$).

3.3.2. Effect of various dilutions of 6-benzylaminopurine (BA) cytokinin on *Gracilaria gracilis* apical segments

Gracilaria gracilis apical segments, after 7 days in culture, showed a significantly higher SGR (in terms of length) in 1:1000 cytokinin dilution (9.53 % day^{-1}) compared to the control (7.24 % day^{-1}) and the other treatments (Figure 3.3 A). Apical segments in 1:500 cytokinin dilution had significantly higher SGR (in terms of length) of 8.42 % day^{-1} compared to the control (7.24 % day^{-1}) and 1:100 cytokinin dilution (7.31 % day^{-1}). Apical segments in the other cytokinin dilutions showed no significant difference in SGR (in terms of length) when compared to the control. After 15 days in culture (Figure 3.3 B), apical segments growing in 1:1000 cytokinin dilution (6.27 % day^{-1}) had a significantly higher SGR (in terms of length) compared to the control (4.78 % day^{-1}) and the other cytokinin dilutions tested.

The average number of branches of *Gracilaria gracilis* apical segments after 15 days in culture was slightly increased with the addition of different cytokinin dilutions (Table 3.2). Apical segments growing in ES medium with 1:1000 cytokinin dilution had significantly more branching compared to the control and 1:5000, 1:250 and 1:100 cytokinin dilutions. There was no significant change in the branching of *Gracilaria gracilis* in the other cytokinin dilutions tested compared to the control.

A



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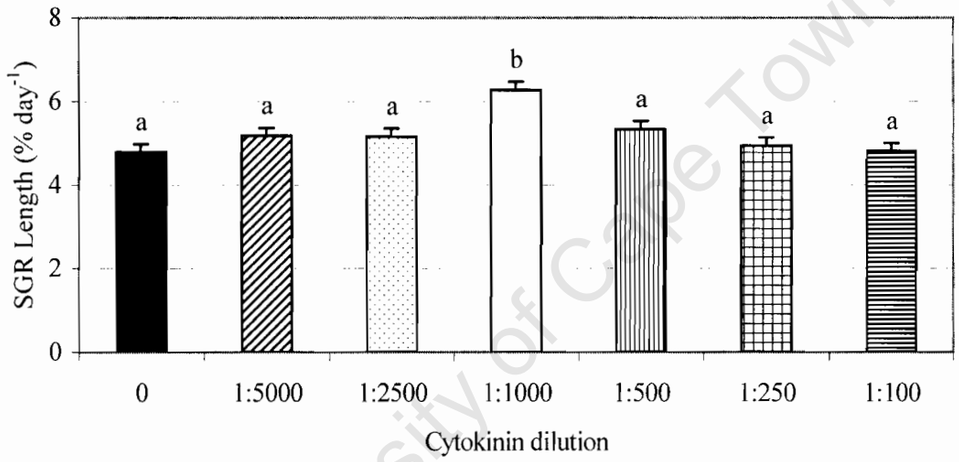


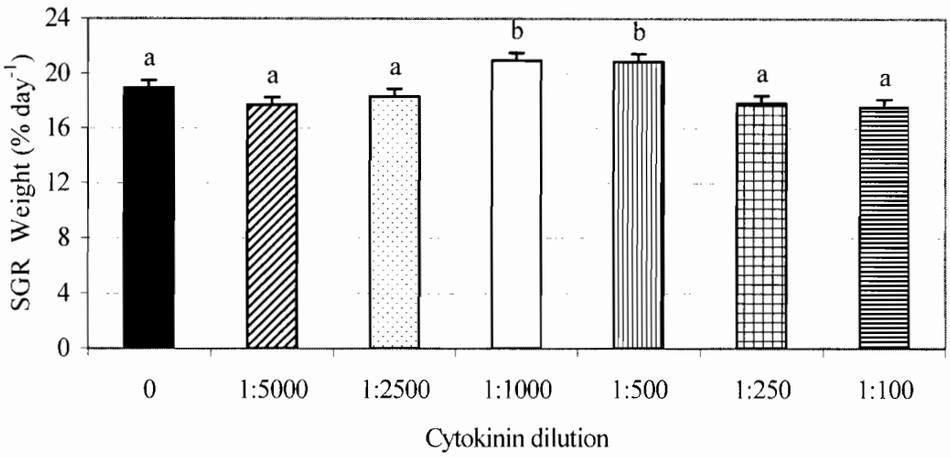
Figure 3.3. The effect of various dilutions of cytokinin stock solution (0.031 mg/l) on the specific growth rate, SGR (% day^{-1}) determined from average length (mm), of apical segments of *Gracilaria gracilis* with initial length of 10 mm. A. After 7 days in ES culture medium. B. After 15 days in ES culture medium. Vertical lines represent ± one Standard Error, p = 0.05. Values of bars marked by a different letter are significantly different according to the LSD post-hoc test ($p < 0.05$).

Table 3.2. The effect of various dilutions of cytokinin stock solution (0.031 mg/l) on the average number of branches on apical segments of *Gracilaria gracilis* with initial length of 10 mm after 15 days in ES medium. Different letters denote significant differences at 5 percent level (one-way ANOVA and LSD post-hoc test ($p < 0.05$)).

Treatment	Average number of branches
0	5.9 ± 3.9 a
1:5000	7.3 ± 4.4 a
1:2500	8.2 ± 4.1 ab
1:1000	10.3 ± 2.6 b
1:500	8.4 ± 2.8 ab
1:250	6.5 ± 2.2 a
1:100	6.5 ± 2.0 a

The SGR (in terms of fresh mass) of *Gracilaria gracilis* apical segments after 7 days in culture (Figure 3.4 A), showed a significant increase in 1:1000 and 1:500 cytokinin dilutions of 20.96 % day^{-1} and 20.88 % day^{-1} respectively, when compared to the control (18.93 % day^{-1}) and the other cytokinin dilutions tested. Apical segments in 1:5000, 1:2500, 1:250 and 1:100 cytokinin dilutions showed no significant difference in the SGR (in terms of fresh mass) compared to the control or among themselves. After 15 days, the SGR (in terms of fresh mass) of apical segments (Figure 3.4 B) was significantly higher in 1:1000 cytokinin dilution (14.66 % day^{-1}), followed by 1:500 cytokinin dilution (14.39 % day^{-1}) compared to the control (12.38 % day^{-1}), 1:250 and 1:100 cytokinin dilutions. The other cytokinin dilutions tested showed no significant difference in the SGR (in terms of fresh mass) of apical segments compared to the control.

A



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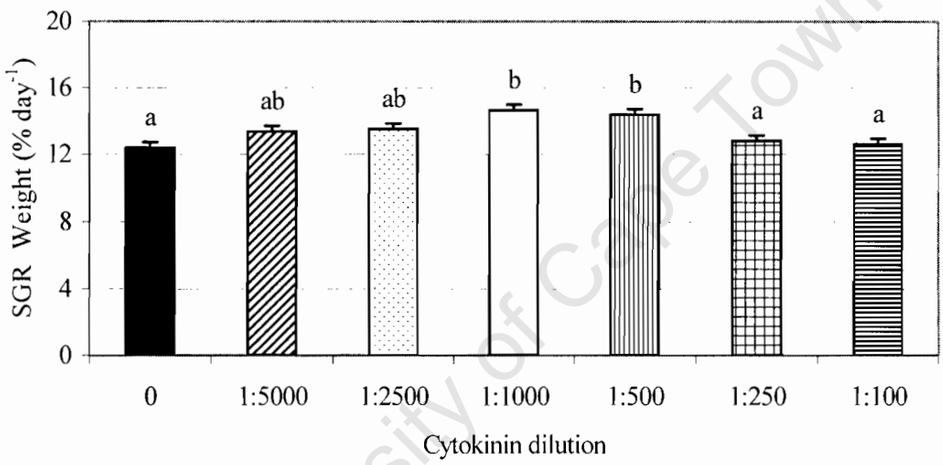


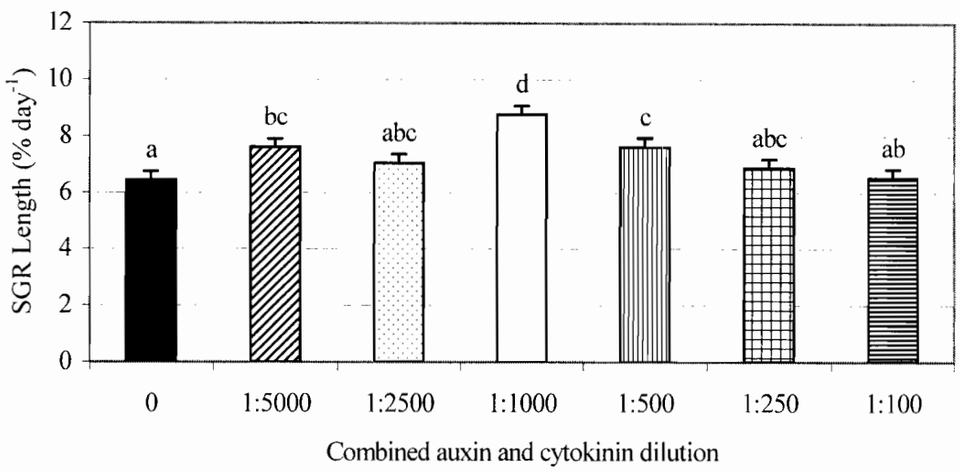
Figure 3.4. The effect of various dilutions of cytokinin stock solution (0.031 mg/l) on the specific growth rate, SGR (% day^{-1}) determined from fresh mass (mg), of apical segments of *Gracilaria gracilis* with initial length of 10 mm. A. After 7 days in ES culture medium. B. After 15 days in ES culture medium. Vertical lines represent ± one Standard Error, p = 0.05. Values of bars marked by a different letter are significantly different according to the LSD post-hoc test ($p < 0.05$).

3.3.3. Effects of various dilutions of combined indole-3-acetic acid (IAA) auxin and 6-benzylaminopurine (BA) cytokinin on *Gracilaria gracilis* apical segments

Figure 3.5 A shows the effect of various dilutions of combined auxin and cytokinin on the SGR (in terms of average length) of apical segments of *Gracilaria gracilis* after 7 days in ES culture medium. Apical segments had a significantly higher SGR (in terms of length) in 1:1000 combined auxin and cytokinin dilution (8.77 % day^{-1}) compared to the control (6.44 % day^{-1}) and the other treatments. The SGR (in terms of length) of apical segments was also significantly higher in 1:500 combined auxin and cytokinin dilution compared to the control and 1:100 combined auxin and cytokinin dilution. Furthermore, apical segments in 1:5000 combined auxin and cytokinin dilution had a significantly higher SGR (in terms of length) compared to the control. All the other combined auxin and cytokinin dilutions had no significant effect on the SGR (length) of apical segments.

After 15 days (Figure 3.5 B) in ES medium with 1:1000 combined auxin and cytokinin dilution (5.8 % day^{-1}), the SGR (in terms of length) of apical segments was significantly higher compared to the control (4.89% day^{-1}), 1:2500 and 1:5000 combined auxin and cytokinin dilutions (4.95 % day^{-1} and 4.89 % day^{-1}, respectively). No significant difference was observed in the SGR (in terms of length) of apical segments growing in the rest of the treatments tested compare to the control.

A



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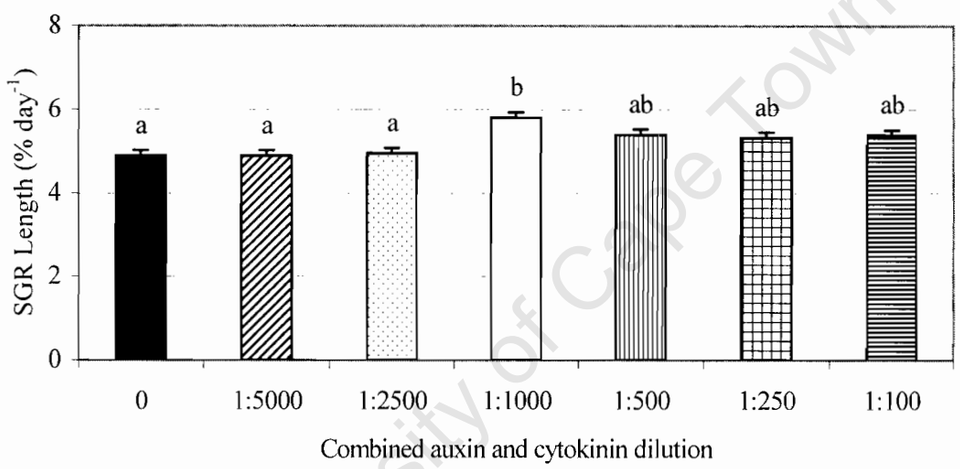


Figure 3.5. The effect of various dilutions of combined auxin and cytokinin stock solution (11 mg/l and 0.031 mg/l, respectively) on the specific growth rate, SGR (% day^{-1}) determined from average length (mm), of apical segments of *Gracilaria gracilis* with initial length of 10 mm. A. After 7 days in ES culture medium. B. After 15 days in ES culture medium. Vertical lines represent ± one Standard error, p = 0.05. Values of bars marked by the different letter are significantly different according to the LSD post-hoc test ($p < 0.05$).

Table 3.3 illustrates the effects of various dilutions of combined auxin and cytokinin on the branching of *Gracilaria gracilis* apical segments after 15 days in culture. There was a significant increase in branching of *Gracilaria gracilis* apical segments grown in ES medium with 1:1000 and 1:500 combined auxin and cytokinin dilutions, followed by 1:250 and 1:100 combined auxin and cytokinin dilutions, compared to the control. Apical segments in ES medium with 1:5000 and 1:2500 combined auxin and cytokinin dilutions showed no significant difference in branching compared to the control.

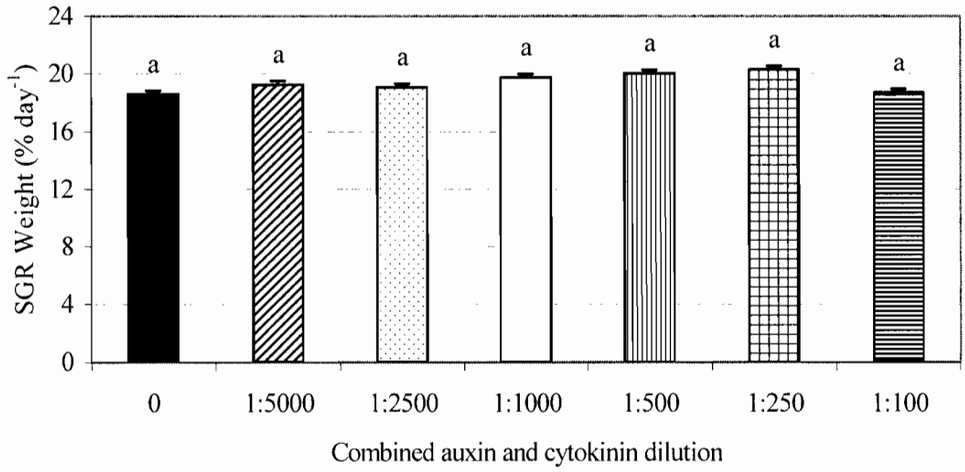
Table 3.3. The effect of various dilutions of combined auxin and cytokinin stock solution (11 mg/l and 0.031 mg/l, respectively) on the average number of branches on apical segments of *Gracilaria gracilis* with initial length of 10 mm after 15 days in ES medium. Different letters denote significant differences at 5 percent level (one-way ANOVA and LSD post-hoc test ($p < 0.05$)).

Treatment	**Average number of branches**
0	5.9 ± 2.9 a
1:5000	6.9 ± 3.1 ab
1:2500	8.8 ± 4.2 abc
1:1000	10.1 ± 3.3 c
1:500	10.0 ± 3.7 c
1:250	9.3 ± 4.4 bc
1:100	8.9 ± 3.8 bc

The SGR (in terms of fresh mass) of apical segments of *Gracilaria gracilis* growing in ES medium with combined auxin and cytokinin dilutions after 7 days is represented in Figure 3.6 A. There was no significant difference in the SGR (in terms

of fresh mass) of *Gracilaria gracilis* growing in different combined auxin and cytokinin dilutions compared to the control or among them. After 15 days (Figure 3.6 B), apical segments growing in ES medium with 1:1000, 1:500 and 1:250 combined auxin and cytokinin dilutions had significantly higher SGR (in terms of fresh mass) of 15.07 % day^{-1}, 14.68 % day^{-1} and 14.23 % day^{-1} respectively, when compared to the control (12.89 % day^{-1}) and to the 1:2500 combined auxin and cytokinin dilution (13.88 % day^{-1}). There was no significant increase in the SGR (in terms of fresh mass) of apical segments growing in 1:5000, 1:2500 and 1:100 combined auxin and cytokinin dilutions compared to the control.

A



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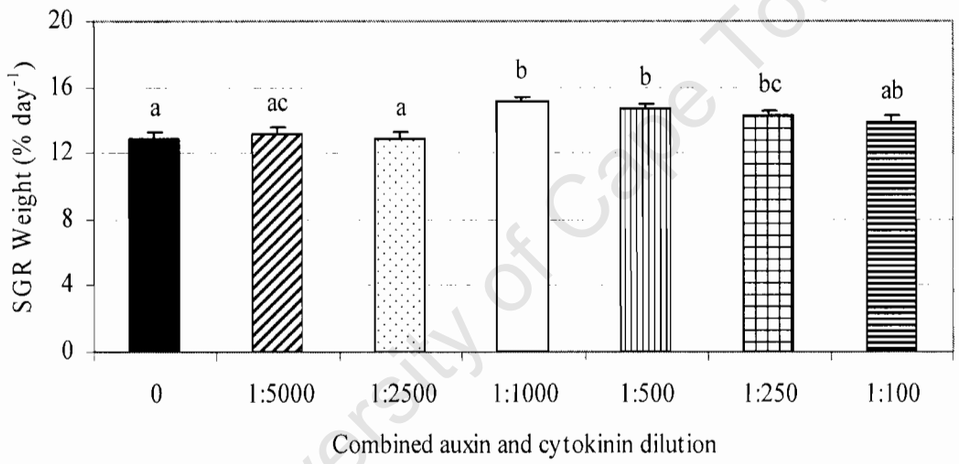

Figure 3.6. The effect of various dilutions of combined auxin and cytokinin stock solution (11 mg/l and 0.031 mg/l, respectively) on the specific growth rate, SGR (% day^{-1}) determined from fresh mass (mg), of apical segments of *Gracilaria gracilis* with initial length of 10 mm. A. After 7 days in ES culture medium. B. After 15 days in ES culture medium. Vertical lines represent ± one Standard Error, $p = 0.05$. Values of bars marked by a different letter are significantly different according to the LSD post-hoc test ($p < 0.05$).

3.4. DISCUSSION

Plant growth regulators, specialized chemical substances produced by plants, are the main internal factors controlling growth and development of plants. Growth, callus induction and regeneration of different species of red algae have been shown to be controlled by plant growth regulators (Bradley and Cheney, 1990; Dawes and Koch, 1991; Dawes *et al*., 1993; Kaczyna and Megnet, 1993; Yokoya and Handro, 1996, Huang and Fugita, 1997). The results of this study showed that plant growth regulators, the auxin indole-3-acetic acid (IAA) and the cytokinin 6-benzylaminopurine (BA), that are active in land plants, are also capable of stimulating growth of the marine red alga *Gracilaria gracilis*. These results could be related to the regulatory roles of auxin and cytokinin on cell division and enlargement in higher plants (Evans, 1984) as well as in various red algae (Bradley and Cheney, 1990; Yokoya and Handro, 1996, 1997; Yokoya, 2000).

It has been suggested that the beneficial responses obtained with the use of seaweed concentrate extracts are similar to those observed when plant growth regulators, particularly auxins and cytokinins, are applied to plants (Booth, 1966). Blunden and Wildgoose (1977) demonstrated close correlations between the results obtained from the use of a plant growth regulator and commercial seaweed extracts of equivalent cytokinin activities in field trials conducted on potatoes. They suggested that the effects of the seaweed extracts were due to their cytokinin content. Similar results were obtained when the shelf life of citrus fruits was increased after post-harvest immersion of the fruit in plant growth regulator and seaweed extract of known cytokinin activity (Blunden *et al*., 1978). Plant growth regulators have been identified in the seaweed concentrate Kelpak®. In the present investigation, the concentrations

of the plant growth regulators (auxin and cytokinin) mimic the concentrations present in Kelpak® seaweed concentrate at various dilutions as used in Chapter 2. Auxin (IAA) and cytokinin (BA) either tested singly or in combination over a range of concentrations, promoted the growth and the elongation of apical segments of *Gracilaria gracilis*. The results obtained are in agreement with the effects of these plant growth regulators on vascular plants (Nogle and Fritz, 1976). In addition, Yokoya and Handro (1996) reported that auxins and cytokinins promote the growth of intercalary and apical segments of *Grateloupia dichotoma*. Dawes and Koch (1991) have also reported that auxins and cytokinins induced the highest callus growth in the red alga *Kappaphycus alvarezii* (Doty) Doty. Other observations on the stimulation of growth in species of red algae when treated with plant growth regulators were reported by Bradley and Cheney (1990), Huang and Fugita (1997) and Yokoya *et al.* (1999, 2004). Furthermore, the results obtained in the present study are in agreement with results from Chapter 2 that the addition of Kelpak® concentrate enhances growth of *Gracilaria gracilis* apical segments.

Auxins are able to induce cell division and growth even in the more recalcitrant plant tissue (Evans *et al.*, 1983). From this study the addition of the auxin (IAA), irrespective of the concentration, caused an increase in growth of *Gracilaria gracilis*. However, the 1:1000 and 1:500 auxin dilutions caused a significant increase in growth of *Gracilaria gracilis* apical segments after 15 days in culture compared to the control that had no addition of the auxin. Yokoya *et al.* (2004) also demonstrated increased growth in *Gracilaria tenuistipitata* and *Gracilaria perplexa* with added plant growth regulators, with apical callus formation stimulated by the auxin IAA. Furthermore, the results coincide with the study conducted by Yokoya (2000), which

illustrated that the auxin IAA had stimulated effect on the regeneration of apical segments of *Gracilariopsis tenuifrons* (Bird *et* Oliveira) Fredericq *et* Hommersand. Furthermore, IAA promoted growth processes such as the elongation of upright axes originating from direct regeneration in axenic culture of *Gracilariopsis tenuifrons* (Yokoya, 2000). There is evidence that auxin concentrations vary within the tissues of algae, as well as throughout their life cycle (Jameson, 1993). These phenomena are all characteristics of processes governed by growth regulators in higher plants. However, the red, green and brown algae appear to respond differently to IAA (Jameson, 1993). Hanisak (1979) noted that IAA had a markedly positive effect on growth of unialgal cultures of *Codium fragile*, but also observed that several synthetic auxins had no effect, from which he concluded that the mechanism of auxin action on the growth of algae is different from that of higher plants. However, according to recent work by Jameson (1993), the green algae, the supposed ancestors of the higher plants, are more responsive to IAA then the brown or red algae.

The cytokinin BA had a positive effect on the growth of *Gracilaria gracilis* in this study. Treatment with 1:1000 cytokinin dilution caused the highest growth rate of *Gracilaria gracilis* apical segments in this experiment. This observation is in agreement with the previous study on Chapter 2, that Kelpak® caused the highest growth of *Gracilaria* at 1:1000 dilution. Stimulatory effects of cytokinins on regeneration processes were also observed in *Grateloupia dichotoma* (Yokoya and Handro, 1996) and *Agardhiella subulata* C. Agardh (Bradley and Cheney, 1990). In this case the effect of cytokinins might be related to cell division, by increasing the level of protein synthesis and metabolic activity affecting the process of cell differentiation (Jameson, 1993). These results could be related to the stimulatory role

played by cytokinins on cell division and differentiation in tissue cultures as shown in higher plants (Evans, 1984) as well as in mosses (Boop and Erichsen, 1981). In addition, the observations from this study agree with those described by Nehlsen (1978), who reported that one of the most pronounced effect of cytokinins was the induction of buds on mosses. High concentrations of cytokinins, (1:250 and 1:100 cytokinin dilutions) had no significant effect on growth of apical segments of *Gracilaria gracilis*. Yokoya (2000) described that the cytokinin BA at higher concentration (5 mg l^{-1}) then the present study had a strong stimulatory effect on the growth of apical and intercalary segments of *Gracilariopsis tenuifrons*. However, Bradley and Cheney (1990) observed that even higher concentrations of cytokinins (10 mg l^{-1}) inhibited regeneration of plants of the red alga *Agardhiella subulata*.

From the present study, the treatments with the combination of auxin and cytokinin dilutions mimicked the exact dilutions of Kelpak® concentrate used in Chapter 2. *Gracilaria gracilis* apical segments growth was highly stimulated by the 1:1000 dilution treatment with the combined use of auxin and cytokinin, showing the highest specific growth rate. The 1:500 combined auxin and cytokinin dilution also caused a significant increase on the growth of *Gracilaria gracilis*. Combined auxins and cytokinins had a regulatory role in the growth and morphogenesis *of Gracilaria tenuistipitata* and *Gracilaria perplexa* (Yokoya *et al*., 2004). The observations from the present study agree with those described by Yokoya and Handro (1996), who described the process of plant regeneration in *Grateloupia dichotoma* was stimulated mainly by treatments with auxin IAA combined with cytokinin BA. Bradley and Cheney (1990) specifically addressed the phenomenon of plant growth regulator interaction when assessing whether auxins and cytokinins, singly or in combination,

stimulated cell division in tissue culture of the marine red algae *Agardhiella subulata*. They found that the combination of auxin and cytokinin was the most effective treatment. Skoog and Miller (1957) also concluded from their experiments that growth might depend more on the quantitative interactions between the plant hormones than on the qualitative action of each of the plant growth regulator acting alone. Relatively high concentrations of combined auxin and cytokinin (1:250 and 1:100 dilutions) did not cause inhibition in growth of *Gracilaria gracilis*. This contradicts the observation made in Chapter 2 that Kelpak® concentrate at 1:100 dilution caused growth inhibition of apical segments of *Gracilaria gracilis*. Furthermore, according to Jameson (1993) plant growth regulators are promotory at low concentrations and inhibitory at high concentrations.

Tay *et al*. (1987) observed that the levels of cytokinin in the seaweed concentrate do not appear to be sufficiently high to suggest that these are the only compounds responsible for the observed beneficial responses of seaweed products on plants, especially in view of the high dilutions of seaweed concentrate used in practice. The wide range of physiological responses elicited by seaweed concentrates also suggests that growth regulators other than cytokinins are involved. The role of auxin in plant growth and development has been attributed to its cell extension properties (Evans, 1973) and also to its ability to enhance RNA and protein synthesis (Key, 1969). Therefore, this group of plant growth regulators may also be instrumental in improving plant yield by affecting the growth processes. According to Brain *et al*. (1973), the cytokinin content of seaweed concentrate is lost when applied to the soil in agriculture. This was not the case in this experiment as *Gracilaria gracilis* apical segments were immersed in the solution during the experimental

period. Nonetheless, from the study conducted it seems reasonable to conclude that the cytokinin and auxin present in the seaweed concentrate Kelpak® are responsible for the increased growth and branching of *Gracilaria gracilis* apical segments.

Gracilaria gracilis apical segments growing with the combined auxin and cytokinin treatments had higher growth rates when compared to apical segments growing with single auxin treatments. Furthermore, *Gracilaria gracilis* apical segments had higher growth rates with cytokinin treatments used singly than with auxin treatments used singly. However, when comparing apical segments growing with combined auxin and cytokinin treatments with apical segments with single cytokinin treatments, the growth rates were very similar. Apical segments growing with the combined auxin and cytokinin at dilutions of 1:1000 and 1:500 had higher specific growth rates than apical segments growing with 1:1000 and 1:500 cytokinin dilutions.

In conclusion, this study indicates that plant growth regulators do have a role in controlling growth in *Gracilaria gracilis*, moreover some effects are comparable with those observed in vascular plants and different species of red algae (Bradley and Cheney, 1990; Kaczyna and Megnet, 1993; Yokoya and Handro, 1996; Huang and Fugita, 1997; Yokoya *et al*., 1999). Furthermore, the results obtained in this study agree with those results obtained in Chapter 2, which suggest that the growth of *Gracilaria gracilis* with the addition of Kelpak® seaweed concentrate was due to the auxin and cytokinin content present in the seaweed extract. It is recommended that plant growth regulators should be used in combination to enhance growth of seaweeds. However, considerable additional work is required to elucidate the

physiological responses of this and other species of seaweed to different types and concentrations of plant growth regulators. This study also shows the importance that plant growth regulators could have in aquaculture of economic red algae.

CHAPTER 4

EFFECTS OF SEAWEED CONCENTRATE KELPAK® ON *GRACILARIA GRACILIS* GROWTH ON A PILOT COMMERCIAL SCALE

4.1. INTRODUCTION

The seaweed industry provides a wide variety of products that have an estimated total annual production value of US $5.5 to 6 billion (FAO, 2004). Food products for human consumption contribute about US $5 billion to this figure (FAO, 2004). Substances that are extracted from seaweeds, the hydrocolloids, account for a large part of the remaining billion dollars, while smaller, miscellaneous uses, such as fertilizers and animal feed additives, make up the rest. The industry uses 7.5 to 8 million tones of wet seaweed annually (FAO, 2004), harvested either from naturally growing or from cultivated seaweed. The mariculture of seaweed has expanded rapidly as demand has outstripped the supply available from natural resources. Commercial harvesting occurs in about 35 countries, spread between the northern and southern hemispheres, in waters ranging from cold, through temperate, to tropical (FAO, 2004). Seaweeds can be cultivated intensively in tanks or ponds if water movement is provided to mimic that of natural environments (Friedlander and Levy, 1995). In order to maintain high growth rates, such seaweeds also need to be supplied with high concentrations of inorganic nitrogen, phosphorous and carbon and maintain pH close to natural seawater pH values (Israel *et al.*, 1999; Israel *et al.*, in press).

Rising global demand for seafood and declining catches from capture fisheries contributed to the doubling of aquaculture production in the last decade (Neori *et al.*, 2004). Aquaculture development has however, been associated with different types of adverse impacts on the environment. One of the main environmental issues is the direct discharge of significant nutrient loads into coastal waters (Chopin *et al.*, 2001). The resulting environmental impacts and rising feed costs have hamper further growth of aquaculture farms. Therefore, integrated aquaculture has been suggested for increased production and sustainability within aquaculture (Folke and Kautsky, 1992; Neori *et al.*, 2004). Such aquaculture systems are co-cultivations composed of different organisms utilizing each other's wastes. This has the potential to reduce the dependency on external ecosystems for food and energy, and reduces negative environmental impacts from wastes release. Seaweeds are nutrient assimilating photoautotrophic plants that use solar energy to turn nutrient rich effluents into profitable resources. Therefore, seaweeds have been incorporated in aquaculture systems; they provide biofiltration due to their high capacity for nutrient uptake and are themselves valuable products. Seaweed integrated land-based systems have proved successful, both from a technical and economic perspective (Neori *et al.*, 2004).

Nitrogen is the nutrient most frequently reported to limit growth of seaweeds in natural ecosystems (Hanisak, 1990). Macroalgae have physiological mechanisms to acquire, utilize, and store various forms of nitrogen in environments that have tremendous spatial and temporal variations in concentrations of nitrogen. The successful cultivation of seaweeds requires the knowledge of nitrogen nutrition of seaweeds (Hanisak, 1990). *Gracilaria* has the capacity to take up and store nitrogen in

excess of immediate requirements, and use it to sustain growth during subsequent periods of nutrient deficiency (Smit *et al*., 1997; Navarro-Angulo and Robledo, 1999). Storage can be in the form of inorganic nitrogen and or metabolites such as proteins and pigments (Navarro-Angulo and Robledo, 1999). This capacity has been utilized in the cultivation of seaweeds to minimize the growth of epiphytes and provide the physiological basis for pulse feeding.

Gracilaria vegetative reproduction and high biofiltering capacity (Buschmann *et al*., 1994; 1996) makes it a suitable candidate for integrated aquaculture. A case study conducted by Buschmann *et al*. (1996) used *Gracilaria* for removing dissolved nutrients from an outdoor intensive fish tank. *Gracilaria* was able to remove 50 % of the dissolved ammonia in winter and 90 to 95 % in spring. Troell *et al*. (1997) conducted a study in Chile, on the cultivation of *Gracilaria* on rope cultures with coastal salmon cage farm. The results showed that *Gracilaria* cultivated at 10 m from the fish cages had up to 40 % higher growth rate (specific growth rate 7 % d^{-1}) than at 150 m and 1 km distance from the fish cages. The conclusion from the study was that both economic and environmental advantages could be achieved by integrating algal cultivation with fish farming in open sea systems.

Tank systems are the most productive among all the tried *Gracilaria* cultivation techniques, but they are expensive to operate, as they require high-energy input and capital investment (Oliveira *et al*., 2000). *Gracilaria* maximal yields are possible only when nutrient conditions do not limit growth. Previous research has suggested that continuous nutrient enrichments would maximize growth (Hanisak, 1990). However, constant high nutrient availability is usually not natural for most

seaweed and is unnecessary for their cultivation. Given their high nitrogen uptake rates, the continuous enrichment of high concentrations of nitrogen quickly saturates the nitrogen requirement for growth. Furthermore, fertilization is not only wasteful and uneconomical, but also provides nutrients for opportunistic epiphytes that are often considered the most serious threat in maintaining seaweed cultures (Hanisak, 1990). Thus, the optimal management of nutrients in a seaweed cultivation system includes the application of enough fertilizer to sustain maximal yields, but without the substantial excess that would contribute to epiphyte problems and or favourable economics (Hanisak, 1987). Normal seawater may not contain sufficient nutrients to grow *Gracilaria* on a commercial scale to ensure high and sustainable production. Increasing the exchange rate in the culture tanks is an expensive factor in tank cultivation systems (Huguenin, 1976), however a direct nutrient replenishment, addition of fertilizer and use of wastewater is the most cost-effective way of culturing species since it requires lower turn over rates thus reducing the operational costs in the farm.

There is a large international market for the abalone *Haliotis midae*. Due to the global demand and depletion of natural populations of abalone due to overfishing and poaching, they are now being farmed. Abalone are grown successfully in farms along the west coast of South Africa and generates good export earnings owing to their commercial value. Food preferences differ among abalone species around the world, depending on habitat and food availability (Dunstan *et al*., 1996). In the wild, abalone consumes different macroalgal species, obtaining their required nutrients from a combination of species. A suitable diet and the consequent increase in the growth rate are important in the success of abalone aquaculture (Capinpin *et al*., 1999). As the

South African abalone (*Haliotis midae*) feed on *Ecklonia maxima* fronds and daily consume 7-10 % of their own body mass (Rotmann, 1999), there is now a large demand for harvested fronds. As these abalone farms reach full production, the demand for kelp will increase and other potential seaweeds such as local *Gracilaria*, *Porphyra* and *Ulva* species should be investigated for their feed quality and potential for aquaculture (Griffin *et al*., 1999; Anderson *et al*., 2003a). *Gracilaria* species have been reported to promote high growth and survival when fed to different species of abalone such as *Haliotis asinina* and *Haliotis tuberculata coccinea* (Bautista-Teruel and Millamena, 1999; Capinpin *et al*., 1999; Viera *et al*., *in press*). *Gracilaria gracilis* has been cultivated in tanks at an on-shore abalone farm situated near Port Elizabeth since July 1993, where it is used as the principal food for abalone (Smit *et al*., 2003).

Marine algae have been used as manures and fertilizers for crops for centuries (Crouch, 1990). Recently, the use of seaweed extracts in agriculture, as plant growth supplements, is increasing (Stirk and van Staden, 1997). It is thought that plant growth regulators may be one of the active constituents in seaweed products (Crouch and van Staden, 1993). Far less is known about effect of seaweeds products and plant growth regulators on the growth of seaweeds. Commercial liquid fertilizers that contain plant growth regulators have been used in tanks to grow *Gracilaria* in Molokai, Hawaii (Glenn *et al*., 1996). A preliminary study conducted by Leitao (2001) using Kelpak® concentrate, a seaweed extract, to grow *Gracilaria gracilis* apical segments in the laboratory suggested an increase in apical growth as well as increased branching. Furthermore, according to a study conducted by Robertson-Andersson (2004) in an abalone farm, the addition of Kelpak® concentrate increased the growth rate of *Ulva lactuca*. Therefore, the main objective of this experiment was

to investigate the feasibility of using nutrient-enriched seawater (abalone effluent) in combination with different Kelpak® concentrations to grow *Gracilaria gracilis* in culture tanks in a pilot-scale aquaculture system on an abalone farm.

4.2. MATERIALS AND METHODS

4.2.1. Study Site

Jacobsbaai Sea Products Pty. (JSP) is a land-based intensive mariculture operation situated on the point of Jacobs Bay, along the South African West Coast approximately 120 km north of Cape Town (Figure 4.1). Established in the early 1990's the farm cultivates mainly abalone (*Haliotis midae*) and turbot (*Scopthalmus maximus*). The farm currently stocks approximately 2.7 million abalone individuals (approximately 76.8 tons) that range from spats to 6 year-old animals (Robertson-Andersson, 2004). Approximately, 77 tons of fresh kelp (*Ecklonia maxima* and *Laminaria pallida* Greville) is used each month as abalone feed, which eat approximately 5 % to 7 % body weight per day. *Ulva lactuca* is also cultivated at the farm but only on an experimental scale. Small-scale *Gracilaria gracilis* cultivation has been attempted in the dams and oyster raceways. However, the cultivation project failed partially due to insufficient water flow in both the raceways and the dam (Miller, 2001) that caused sediments to settle on the thalli, smothering the algae.

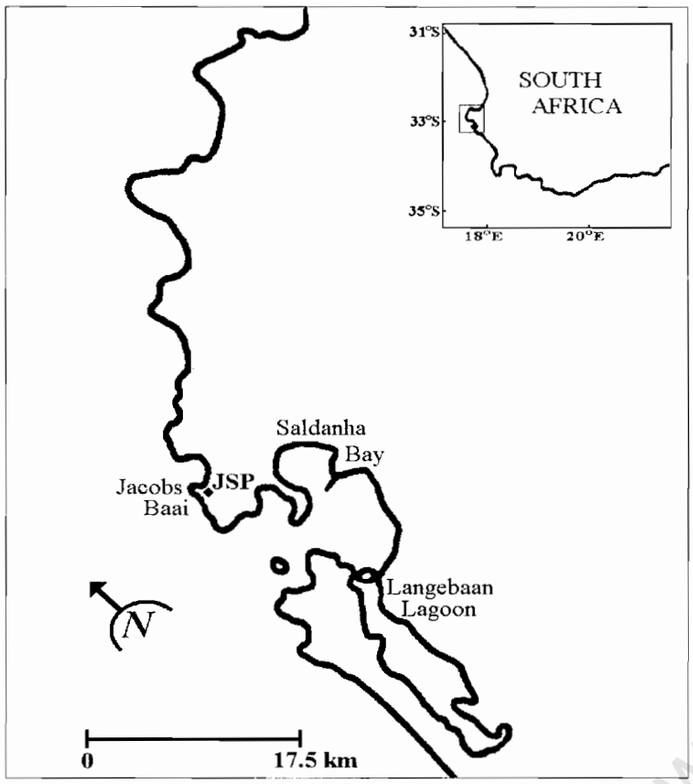

Figure 4.1. Map showing Jacobsbaai Sea Products Pty. (JSP). (Adapted from Anderson *et al.*, 2003b).

In a polyculture system, algae can function both as a biofilter of effluent water or and as a food source for the animals. Therefore, it would be beneficial for JSP to cultivate algae, firstly to absorb nutrients in wastewater in case the farm has to re-circulate its water during a harmful algal bloom. Harmful algal blooms occur frequently in the Western Cape and are known to cause large abalone mortalities (Mathews and Pitcher, 1996; Pitcher, 1999). Secondly, cultivation would provide an alternative food supply. The abalone are fed on kelp that is harvested daily from the nearby shore, or during storms from further away, and hence on-farm food production could save money.

Figure 4.2 is a diagram depicting the layout of JSP (Morgan, 2000). JSP consists of four primary components, the settling and holding dams (A, B, and C); the abalone tanks (D); the turbot tanks (E) and the (inactive) oyster raceways (F). Seawater is pumped directly into the top settling reservoir dam (A) and from there it is gravity fed either to the turbot tanks (E) or to the bottom holding dams (B and C). Water can also be pumped directly into the holding dams where it is consequently heated by solar radiation. This is done to raise the temperature of the seawater during summer, as upwelling of cold water occurs on the West Coast. Low water temperatures slow the growth rate of abalone. Water turnover rate for the top-settling reservoir is 5.6 volumes d^{-1} and 4.5 volumes d^{-1} for the two bottom dams. From the dams, the water is pumped into the mixing tank (G) where it can be distributed to the abalone tanks, turbot tanks, and oyster raceways or returned back to the dams. Effluent water is chanelled up to the sump (H) where the particulates are settled out, and the remaining wastewater is returned to the sea. The re-circulation consists of half fresh seawater and half wastewater.

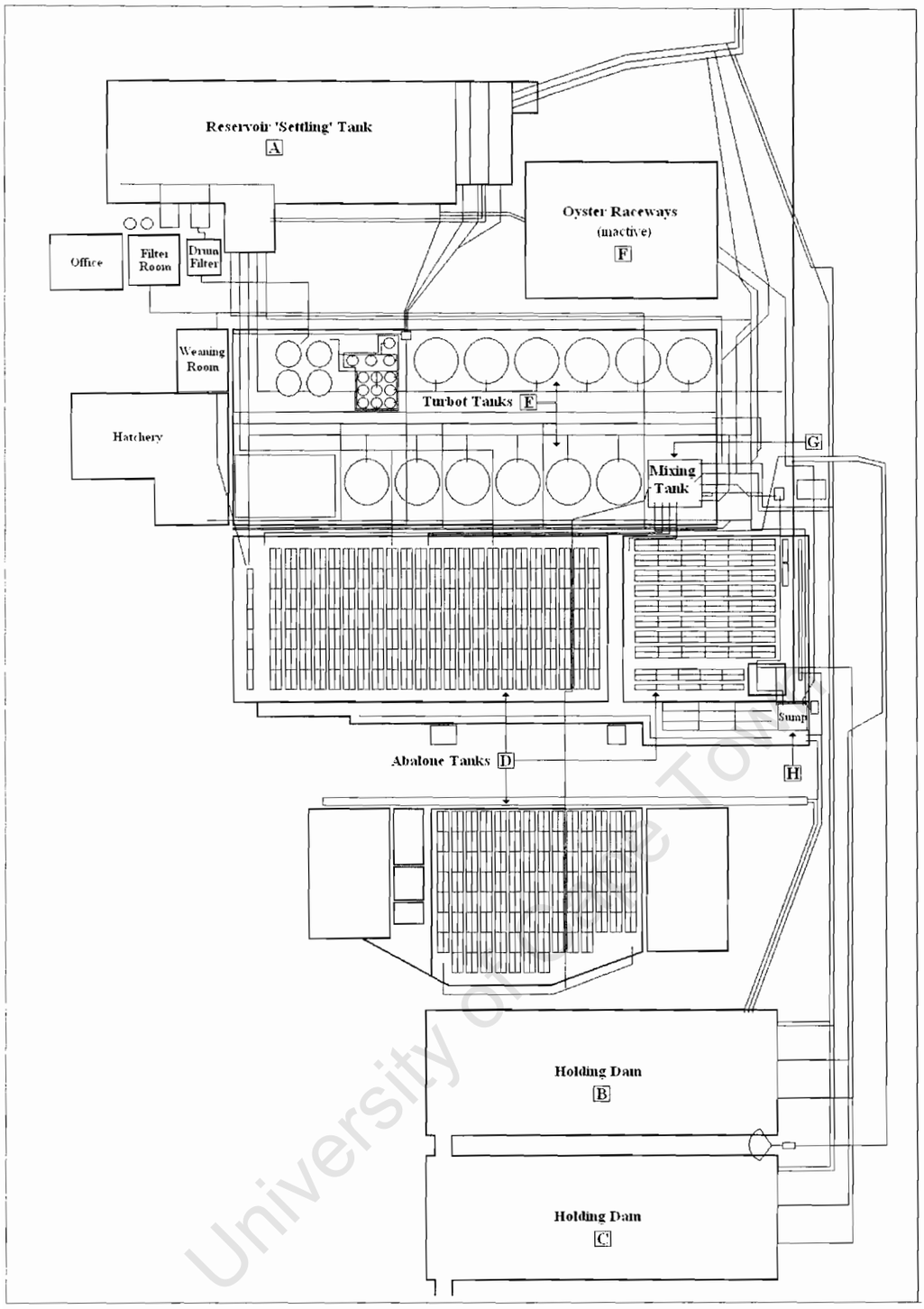

Figure 4.2. Diagram depicting the layout of JSP. (Adapted from Morgan, 2000).

4.2.2. Tank Design

Small, rectangular tanks made from speckled, light grey polyethylene were used. The tank had an effective volume of approximately 100 l, and the inside dimensions were 0.60 m x 0.40 m x 0.40 m. Figure 4.3 shows the longitudinal view of the tank.

Figure 4.3. Longitudinal view of the tanks at JSP.

The tanks were supplied with air via a Howard and Donkin channel blower in a 100 mm diameter and 2 mm thick PVC pipe. The pipes were perforated by drilling 2 mm diameter holes at 90 mm intervals along their length. To compensate for the decreased air pressure towards the ends of the legs, two intermediate holes were drilled between the last two holes, hence ensuring uniform air supply. The aeration system was made of a 'U' shaped frame comprising two 544 mm long, 20 mm diameter pipes closed at the terminal ends by a stopper. Each pipe was joined to a 145 mm long and 20 mm diameter pipe, and further joined by a 20 mm 'tie'. The whole

system was connected to the main air supply 63 mm diameter pipe. PVC solvent was used to permanently join the pipes to avoid air leakage. To ensure that the pipes would not float and would remain attached, ensuring a uniform supply of air, the frames were cemented to the base of the tanks using silicone sealer.

Water was fed into the tanks from the main water system using a 20 mm PVC pipe. The water was supplied directly to the tanks from the main pipe above, with the supplying pipe facing downwards. The output system consisted of a 32 mm drilled hole, at about 80 mm from the top of the tank at the end of the tank opposite to the air supply. To ensure that no thalli was washed out, a 200 mm diameter common kitchen colander was placed over the output pipe and was held in place using silicone and two cable ties. A 100 mm x 32 mm running nipple was connected through the hole with nuts and gasket to tighten it, chanelling the output water to the sump (Robertson-Andersson, 2004).

4.2.3. Water Source

The water was gravity fed from the reservoir to a shallow dam. At the shallow dam, the water is heated by solar radiation for eight hours before being distributed to the abalone culture tanks. The effluent water from these tanks was then chanelled into a sump to allow for settling of particles. It is then pumped from the sump to a second 1000 l closed tank and from there it is gravity fed to the second set of seaweed tanks.

4.2.4. Plant Material

The stocking *Gracilaria gracilis* material was collected in Saldanha Bay by divers at a depth of approximately three meters. The material was first cleaned and the

epiphytes removed by hand, weighed to the required stocking capacity (470 g m^{-2}) and then placed in running abalone effluent water. Culture conditions were uniform and the culture tanks were monitored daily by the farm personnel.

4.2.5. Seaweed Concentrate

The commercially available seaweed concentrate used in this study was Kelpak ®, previously described in Chapter 2, Section 2.2.3.

4.2.6. Experimental Design

The tank system at JSP was set up to run 21 tanks on abalone effluent (Figure 4.4). During the first week of the experiment, *Gracilaria gracilis* was placed in tanks under abalone effluent only for the seaweed to acclimatize to the culture conditions, after which the seaweed was harvested and the tanks restocked to 470 g m^{-2} using the harvested material and the treatments applied. According to Robertson-Andersson (2004), *Ulva* had higher significant growth rates on the second run of the experiment, implying that the seaweed requires time to acclimatize to the experimental conditions before the effects could be monitored.

The treatments used in the experiment were the same as used in Chapter 2, section 2.2.2, however instead of ES medium, abalone effluent water was used. Therefore, the treatments were as follow: control (no Kelpak®), 1:100, 1:250, 1:500, 1:1000, 1:2500, and 1:5000 Kelpak® concentrate added to abalone effluent. Each treatment had three replicates. Kelpak® concentrate was added to the tanks once every two weeks. After the addition of the Kelpak® concentrate, the water was not

changed for 20 hours after which a normal water exchange resumed. Water volume exchange rate of all the tanks was 20 times a day for the duration of the experiment.

Figure 4.4. Culture system set up of tanks at JSP.

Once the treatments started, the farm was visited every week for one month to harvest the material, clean the tanks and record growth data. *Gracilaria* was harvested from the tanks by collecting all the material by hand into net bags, draining excess water by hanging the bags until dripping ceased (taking about 3 to 4 minutes) and weighing it using a spring balance. During harvesting, the tanks were cleaned and re-supplied with water and air. The tanks were restocked with the standard initial stocking weight (470 g m^{-2}).

Branches of *Gracilaria* from each individual tank were collected and placed in small labelled plastic bags filled with water from the respective tank. In the laboratory, each sample was rinsed with distilled water. Twenty individual branches

of *Gracilaria* from each tank were selected and measured from the tip and cut to 20 cm. The number of branches was counted and the wet weight (in grams) recorded. Furthermore, *Gracilaria* individual samples from each tank were weighed to 100 g then labelled and transferred into foil trays and oven dried at 70 ^{o}C for about 72 hours. The dried material was then re-weighed and the dry weight recorded.

4.2.7. Data Analysis

The initial and final biomass (in fresh weight) was determined, from which the specific growth rate (SGR) could be calculated using the following formula:

$$SGR\ (\%\ day^{-1}) = (100 \ln N_t / N_o) / t$$

Where t is time in days, N_o is the initial weight (g) and N_t is the weight (g) at time t (as used by Engledow and Bolton, 1992).

The yield (Y) could be calculated using the following formula:

$$Y\ (g\ wwt\ m^{-2}\ d^{-1}) = [(W_t - W_0] / t] / SA$$

Where t is time in days, W_0 is the initial wet weight (g) and W_t is the wet weight (g) at time t and SA is the surface area of the tank in m^2 (Evans, 1972).

4.2.8. Statistical Analysis

The effects of different Kelpak® dilutions on the growth rate of *Gracilaria gracilis* was analysed using ANOVA, the single factorial analysis of variance (p =

0.05) using the statistical package STATISTICA 7, to test the null hypothesis that the means of the specific growth rate of all tested Kelpak® dilutions were not significantly different. The least significance difference (LSD) test or planned comparison test was conducted at the 95 % confidence level, to distinguish significantly different results.

4.3. RESULTS

At the start of the experiment *Gracilaria gracilis* appeared dark red indicating that the seaweed was very healthy. After 15 days in culture, *Gracilaria gracilis* still had a dark red colour. However, after 30 days, the experiment had to be discontinued as *Gracilaria gracilis* had a pale yellow colour.

The yield of *Gracilaria gracilis* growing in abalone effluent with different dilutions of Kelpak® concentrate is represented in Figure 4.5. After 15 days (Figure 4.5A), *Gracilaria* growing in 1:100 and 1:250 Kelpak® dilution had a significant lower yield (6.24 and 7.54 g wwt m^{-2} d^{-1}, respectively) compared to the control (15.24 g wwt m^{-2} d^{-1}). There was an overall decrease in the yield of *Gracilaria gracilis* after 30 days (Figure 4.5B). However, there was no significant difference in the yield of *Gracilaria gracilis* in the different Kelpak® concentrate dilutions compared to the control. Furthermore, there was no significant difference between the treatments.

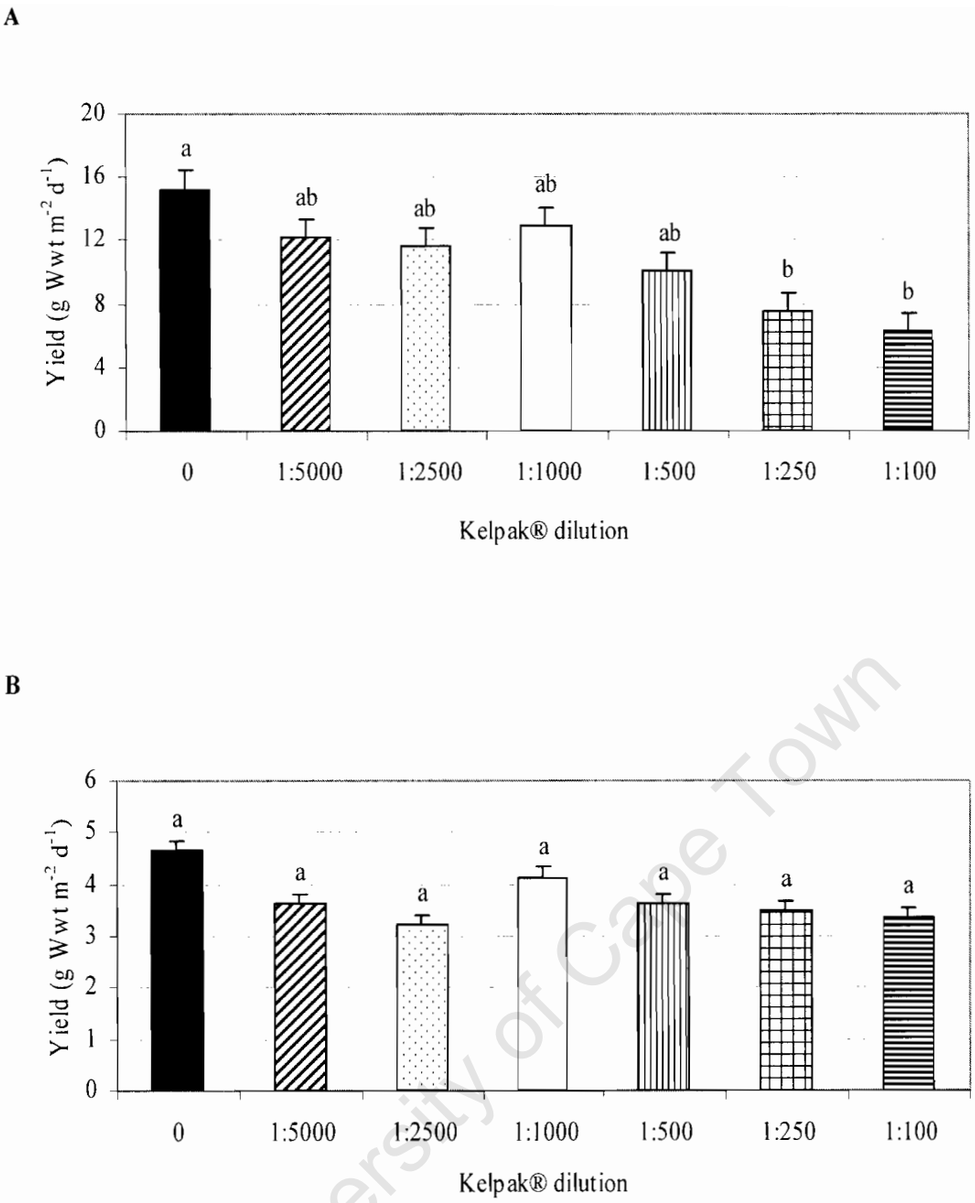

Figure 4.5. The effect of various Kelpak® concentrate dilutions on the yield (g Wwt m^{-2} d^{-1}) of *Gracilaria gracilis* cultivated in abalone effluent on a pilot commercial scale. A. After 15 days. B. After 30 days. Vertical lines represent ± one Standard Error, p = 0.05. Values of bars marked by different letters indicate significantly difference according to LSD post-hoc test ($p < 0.05$).

All dilutions of Kelpak® concentrate added to the abalone effluent inhibited *Gracilaria gracilis* specific growth rate after 15 days (Figure 4.6A). *Gracilaria gracilis* specific growth rate was significantly inhibited with 1:100 and 1:250 Kelpak® dilution (4.51 % day^{-1} and 4.02 % day^{-1} respectively) compared to the control (7.44 % day^{-1}). There was no significant difference between the control and the rest of the treatments, or among those treatments. After 30 days (Figure 4.6B), *Gracilaria gracilis* cultured in different Kelpak® dilutions showed no significant difference in the specific growth rate among control and all treatments.

Branching of *Gracilaria gracilis* was statistically similar in the control and all the Kelpak® dilutions (Table 4.1) after 30 days.

Table 4.1. The effect of various Kelpak® concentrate dilutions on the average branches of the 20 cm main branch of *Gracilaria gracilis* cultivated in abalone effluent on a pilot commercial scale after 30 days. Data are the means (± SD). The same letter denotes no significant differences at 5 percent level (one-way ANOVA and LSD post-hoc test ($p < 0.05$)).

Kelpak® dilution	**Average number of branches**
0	83.5 ± 18.7 a
1:5000	81.9 ± 17.1 a
1:2500	78.7 ± 18.4 a
1:1000	81.8 ± 18.7 a
1:500	80.9 ± 22.6 a
1:250	79.0 ± 19.5 a
1:100	78.8 ± 19.3 a

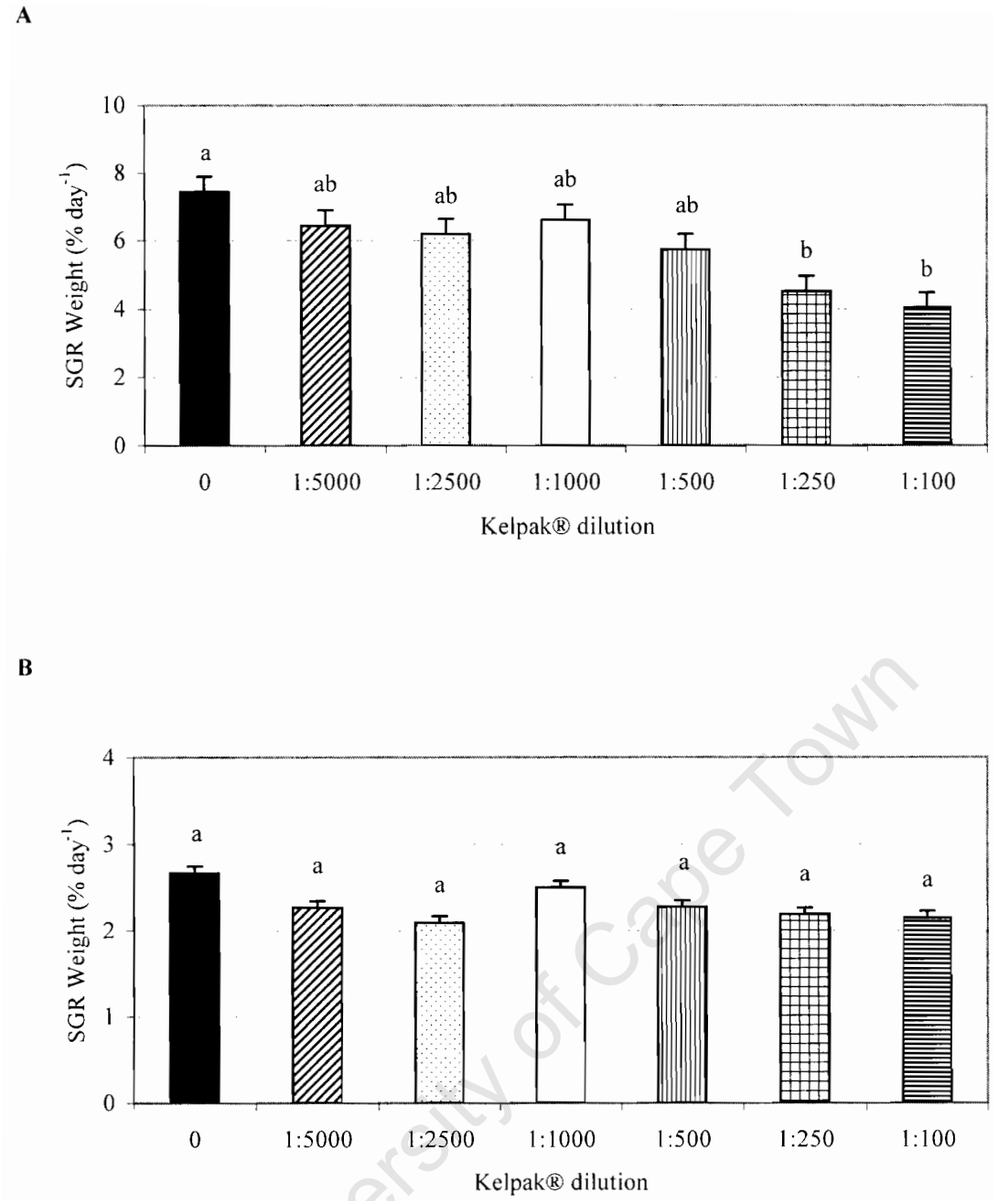

Figure 4.6. The effect of various Kelpak® concentrate dilutions on the specific growth rate, SGR (% day^{-1}) determined from fresh mass (g), of *Gracilaria gracilis* cultivated in abalone effluent on a pilot commercial scale. A. After 15 days. B. After 30 days. Vertical lines represent ± one Standard Error, $p = 0.05$. Values of bars marked by the different letters are significantly different according to the LSD post-hoc test ($p < 0.05$).

After 30 days, there were no significant differences between weights of the apical segments of *Gracilaria gracilis* in all the Kelpak® concentrate dilutions and the control or between treatments (Figure 4.7).

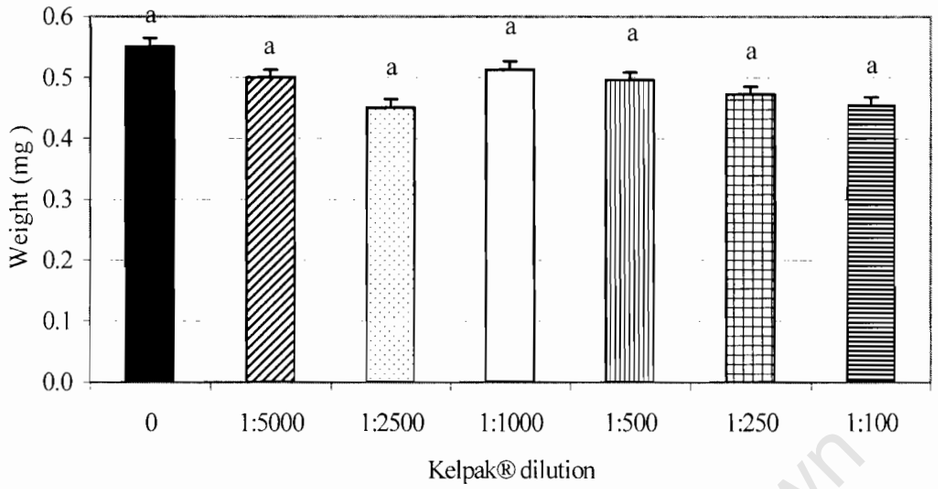

Figure 4.7. The effect of various Kelpak® concentrate dilutions on the weight (mg) of 20 cm apical segments of *Gracilaria gracilis*, cultivated in abalone effluent on a pilot commercial scale after 30 days. Vertical lines represent ± one Standard Error, p = 0.05. Values of bars marked by the different letters are significantly different according to the LSD post-hoc test ($p < 0.05$).

4.4. DISCUSSION

Maximal yields are only possible when nutrient conditions do not limit growth. Hanisak (1990) has suggested that nitrogen is the nutrient most frequently reported to limit the growth of seaweeds. Past studies have found that levels of pigment proteins are often closely correlated with the nitrogen content (Lapointe and Ryther, 1979). As pigment protein such as phycoerythrin in red algae is largely responsible for determining the colour of seaweeds, changes in the concentration of

these pigments according to N availability cause lightening or darkening of the seaweed. Hence, there is a relationship between the colour and the health of the seaweed. *Gracilaria gracilis* at the start of the experiment appeared dark red indicating that it was very healthy. However, after 30 days, *Gracilaria gracilis* had a yellow colour, and the experiment had to be discontinued. This is showed that despite being grown in abalone wastewater, the thalli had become nutrient-starved. Under these conditions, it is not surprising that Kelpak® concentrate would have had little or no effect, since the condition of the plants indicated that nitrogen was limiting growth very severely.

For a commercial seaweed farm to be economically viable cultivation must be continuous and the system easily and cheaply operated (Smit *et al*., 1997). According to Smit *et al*., (1997), the most cost-effective form of seaweed tank cultivation is using mariculture effluent water, where seawater is readily available and no addition of fertilizers is required. It is possible however, that the ambient nutrient levels in the effluent water are too low to sustain high seaweed densities required for biomass production, in which case growth promoters or external nutrient supplies are needed. In biological agriculture and horticulture diluted extracts of seaweeds are applied to promote growth, prevent pests and diseases and improve quality of the products (Finnie and van Staden, 1985). However, the effect of diluted extracts of seaweed on seaweed mariculture or aquaculture is still uncertain. Kelpak® concentrate is not a fertilizer but it is additional to normal nutrient requirements.

Treatment with 1:5000 Kelpak® concentrate dilution did not significantly increase the specific growth rate or the yield of *Gracilaria gracilis*. This is in

agreement with a preliminary study conducted by Leitao (2001), which showed the same effect for *Gracilaria gracilis* growing in the laboratory. Furthermore, *Ulva* growing in 1:5000 Kelpak® concentrate dilution with both turbot effluent and abalone effluent, showed no significant increase in the specific growth rate (Robertson-Andersson, 2004; Robertson-Andersson *et al*., in press). This is in concurrence with Beckett and van Staden (1990), who demonstrated that the growth of wheat was not stimulated by low concentrations of Kelpak® compared to the control.

The growth of *Gracilaria gracilis* was not significantly increased with 1:2500 Kelpak® concentrate dilution. This result contradicts with the study on Chapter 2. Additionally, the results contrast with the study conducted by Robertson-Andersson (2004), in which 1:2500 Kelpak® concentrate dilution caused the highest specific growth rate increase of *Ulva* in tank experiments. Furthermore, the results disagree with studies in which Kelpak® concentrate used at 1:2500 and applied regularly improved the total biomass of *Beta vulgaris* and *Phaseolus vulgaris* (Crouch, 1990).

The Kelpak® concentrate dilution of 1:1000 and 1:500 had no significant effect on the growth of *Gracilaria gracilis*. These results contradict those obtained in Chapter 2, where *Gracilaria* had the highest growth rate with 1:1000 Kelpak® concentrate dilution, followed by 1:500 Kelpak® concentrate dilution. Furthermore, they contradict the results obtained in a preliminary study on apical segments of *Gracilaria gracilis* in the laboratory (Leitao, 2001). This could be because in the pilot scale experiment the addition of Kelpak® concentrate was a once-off pulse addition, while in the laboratory, the *Gracilaria* is constantly exposed to the Kelpak® concentrate in the medium.

A pronounced inhibitory effect on the yield and specific growth rate was observed after 15 days, using a 1:100 Kelpak® concentrate dilution. This effect was also observed in Chapter 2, where *Gracilaria gracilis* apical segments were inhibited by 1:100 Kelpak® dilution. Furthermore, the inhibitory effect using 1:100 Kelpak® dilution was observed for *Gracilaria* and *Ulva* in the laboratory and in the aquaculture farm (Leitao, 2001; Robertson-Andersson, 2004; Robertson-Andersson *et al*., in press). A study conducted by Finnie and van Staden (1985) reported inhibition of tomato roots at this concentration. The yield and the specific growth rate of *Gracilaria gracilis*, after 30 days in culture, did not increase with the application of any of the Kelpak® dilutions. Furthermore, there was no significant difference in the yield and the specific growth rate of *Gracilaria gracilis* with application of different Kelpak® dilutions compared to the control after 30 days.

Gracilaria growth rate in land-based systems is a function of the combination of various factors, including temperature, light, salinity, pH, nutrients, water exchange, aeration, stocking biomass, and epiphytes. To obtain the desired high levels of growth rate in *Gracilaria* cultures, there should be a continuous management of all these factors. The interaction of these factors, their interdependences and relationship with the target species is of vital importance. The failure to management of one of these factors can lead to poor growth rate because of the interdependence of these factors. One of the problems encountered with in this study was the presence of epiphytes, even though they were removed from the tanks every week. The grazer *Paridotea reticulata*, and epiphytic algae such as *Ulva* and *Ceramium,* were found in the tanks. Epiphytic algae are opportunistic and are reported to show high rates of

nitrogen uptake and photosynthesis, resulting in their high growth rates (Friedlander and Gonen, 1996). *Ulva* is known to affect *Gracilaria* growth by its ability to produce allelopathic compounds that lead to retarded growth of the seaweed beyond simple competition for light and nutrients (Friedlander and Gonen, 1996). Another problem encountered was in the aeration. A certain degree of water movement is necessary in the culture tanks, to keep the seaweeds moving in the tanks to expose them to light as well as to improve nutrient and gas exchange. Even though there was water movement in the tanks, provided by the release of compressed air from a perforated pipe along the bottom of the tank, the seaweed thalli was at times entangled in the aeration pipe. Therefore, without the movement in the tank, the *Gracilaria* thalli were sometimes subject to self-shading, leading to unequal light and temperature exposure that might have had a detrimental effect on photosynthesis. These problems could have contributed to the poor growth rate of *Gracilaria gracilis* in this study.

In addition, the chemistry of the abalone effluent water at the time of the experiment was not known. The condition of the plants indicates that it was likely that the abalone effluent water had insufficient concentrations of nutrients, especially of nitrogen, which could have affected the growth rate of *Gracilaria gracilis*, since nitrogen is the nutrient most frequently reported to limit the growth of seaweeds (Hanisak, 1990). It has been suggested that lightening in the colour of *Gracilaria* is an indication of lack of available nitrogen, which has necessitated the utilization of pigment proteins (phycoerythrins) as a nitrogen source (Lapointe and Ryther, 1979). The relationship between thallus colour and nitrogen content can be documented using a standard colour guide. If a clearly observable relationship is found to occur, colour can be used to rapidly assess the nutrient status of seaweeds in cultivation. The

specific reasons for the poor growth rates of *Gracilaria gracilis* in tanks on the abalone farm are not clear. Further experiments need to done using applications of fertiliser.

CHAPTER 5

GENERAL DISCUSSION

Cultivation of seaweeds is increasing very rapidly, as natural stocks are unable to supply demand (Santelices and Doty, 1989). The cultivation of the red seaweed, *Gracilaria gracilis* is biologically and technically feasible in land-based culture systems on the west coast of South Africa. However, it is very important as suggested by Santelices (1999) to have a good knowledge about the factors that increase production and the interactions among them, both the local environmental conditions and the culture conditions. *Gracilaria* maximal yields are possible only when nutrient conditions do not limit growth. The most cost-effective way of culturing species and having optimal management of nutrients in a seaweed cultivation system includes the application of enough fertilizer and/or use of wastewater. Because of the many adverse effects of synthetic fertilizers upon the environment, there is a need for natural sources of fertilizers. Therefore, considerable attention is being focused on the development of new biodegradable products that enhance plant growth and improve yield characteristics.

A review of the literature indicated that seaweed concentrates have been applied to many different agricultural plants in controlled experiments in order to increase overall growth and yield. Examinations of these studies reveal that there are several schools of thought related to the mode of action of seaweed extracts on plant

growth. Moreover, in biological agriculture and horticulture, seaweed concentrates are applied to promote growth, prevent pests and disease and improve the quality of products. Early research attributed improved plant growth to either soil conditioning properties of the seaweed or to an enhancement of nutrient uptake by the plants. Furthermore, the presence of endogenous trace elements in seaweeds was also thought to explain some of the beneficial effects obtained through the application of seaweed concentrate. However, these constituents alone cannot explain the beneficial growth responses resulting from the dilute amounts of seaweed concentrate administered to plants. Recent research has shown the occurrence, in seaweed concentrate products, of certain plant growth regulators, and at present, the observed beneficial effects are attributed to these constituents. Nevertheless, the role of seaweed concentrates in promoting growth of commercially important seaweeds, such as *Gracilaria gracilis*, in mariculture is still unclear.

The aim of this project was to analyze the effects of the commercial seaweed concentrate, Kelpak®, on the growth of the commercial important seaweed *Gracilaria gracilis*. In the laboratory under controlled conditions, *Gracilaria gracilis* apical segments were grown in the laboratory in Provasoli Enriched Seawater medium with the addition of different dilutions of Kelpak® concentrate. Apart from examining the effects of the commercial seaweed concentrate on apical segments, the possible involvement of plant growth regulators, especially auxin and cytokinin, in bringing about some of the responses of plants to the seaweed treatments, motivated a study on the effects of plant growth regulators, used singly or combined, on the growth of *Gracilaria gracilis* apical segments. Furthermore, a study was conducted to assess the feasibility of using nutrient-enriched seawater (abalone effluent) in combination with

Kelpak® seaweed concentrate to grow *Gracilaria gracilis* in culture tanks in a pilot scale aquaculture system. It is hoped that the results of this study will aid in the understanding of the effects of the commercial seaweed concentrate in algae growth and development with a view to the potential in mariculture.

The study conducted on the effect of Kelpak® concentrate on growth when applied to *Gracilaria gracilis* apical segments found that the seaweed concentrate at a dilution of 1:1000 and 1:500 improved the overall growth of treated apical segments significantly. These results are similar to those obtained in a preliminary study conducted under controlled conditions, where *Gracilaria gracilis* apical segments grown in ES medium with 1:1000 and 1:500 Kelpak® dilutions had a significant increase in the overall growth (Leitao, 2001). Furthermore, the results are in agreement with the results obtained for French bean plants (Featonby-Smith and van Staden, 1983b), Swiss chard plants (Crouch, 1990) and cucumber plants (Nelson and van Staden, 1984) with the addition of seaweed concentrate at similar dilutions.

Algae respond to plant growth regulators, whether these plant growth regulators originate from epiphytic bacteria, free living marine bacteria or are biosynthesized by the algae itself (Jameson, 1993). The demonstration of a response to or the occurrence of, a plant growth regulator in any algae is by no means indicative of a functional role of the plant growth regulator in algal metabolism (Boop, 1990). However, the potential for chemical regulation of development is present in many algae (Evans and Trewavas, 1991). The classic example is that of the regulation of apical dominance in *Fucus* and *Ascophyllum* (Moss, 1965, 1970), where removal of the apical cell region resulted in regeneration of new branches. In higher

plants, apical dominance is associated with the release of auxin from the apical tip and in these seaweeds auxins were seen, in some cases, to restore apical dominance in segments with the apical cell region removed, while cytokinins promoted branching in segments with apical cell intact (Moss, 1965). As in higher plants, a functional role for auxin and cytokinin can be suggested (Jameson, 1993).

The study conducted to determine if the enhanced growth of *Gracilaria gracilis* apical segments with the application of Kelpak® concentrate was probably due to the plant growth regulators present in Kelpak® concentrate indicated that certain dilutions of auxins and cytokinins used singly or in combination were capable of enhancing growth. Auxin applied singly at a dilution of 1:1000 and 1:500 significantly improved growth of *Gracilaria gracilis* apical segments. Cytokinin tested singly at a dilution of 1:1000 caused a significant increase in growth of *Gracilaria gracilis* apical segments. The combined auxin and cytokinin significantly increased growth of *Gracilaria gracilis* apical segments when applied at 1:1000 and 1:500 dilutions. Associated with the growth increase was a significant increase in branching on the apical segments of *Gracilaria gracilis* with the application of both auxin and cytokinin singly or in combination at 1:1000 dilution. Although close correlations have been found to exist between the results obtained from the use of auxin and cytokinin, either singly or combined, and the seaweed concentrate Kelpak® on the growth of *Gracilaria gracilis* apical segments, there remains no direct evidence to suggest that this group of plant growth regulators are solely responsible for the improved results obtained with the use of the seaweed concentrate. It does however, seem probable that the beneficial results obtained, and the cytokinin and auxin content of the seaweed extract are related.

The final stage of this study examined the effect of seaweed concentrate Kelpak®, on the growth of *Gracilaria gracilis* in a pilot scale aquaculture farm. For commercial seaweed farms to be economically viable cultivation must be continuous and the system easily and cheaply operated (Smit *et al*., 1997). Kelpak® concentrate used was additional to normal nutrient requirements supplied by the abalone effluent water. According to the study conducted by Robertson-Andersson (2004), the use of abalone and turbot effluent water with additions of both fertilizer and Kelpak® concentrate significantly increased the specific growth rate of *Ulva* compared to the effluent or seawater controls alone. However, the results obtained in the present study did not show a significant increase in the growth of *Gracilaria gracilis* with the application of different dilutions of Kelpak® concentrate compared to the control. Furthermore, the experiment had to be discontinued after 30 days, as *Gracilaria gracilis* turned pale and yellow, probably indicating that *Gracilaria* pigment proteins had been used as a nitrogen source by the plant. The specific reasons for the poor growth rates of *Gracilaria gracilis* in tanks on the abalone farm are not clear.

In the laboratory experiments, *Gracilaria gracilis* apical segments were longer with the addition of Kelpak® concentrate at different dilutions, especially with 1:1000 and 1:500 Kelpak® concentrate dilutions compared to the addition of plant growth regulators. However when the specific growth rates were compared in terms of fresh mass, the addition of plant growth regulators caused a higher specific growth rate of *Gracilaria gracilis* apical segments, especially with 1:1000 dilution, compared to the addition of Kelpak® concentrate. Both the addition of Kelpak® concentrate and plant growth regulators caused an increase in the specific growth rate of *Gracilaria gracilis* apical segments in laboratory cultures, but in the experiment conducted on an abalone

farm *Gracilaria gracilis* specific growth rate did not increase with the addition of Kelpak® concentrate. It is not clear why the results from the experiment in the pilot commercial scale abalone farm were not similar to the results from the laboratory experiments. The main difference between the experiments conducted on the pilot commercial scale abalone farm and the laboratory is that in the pilot commercial scale abalone farm experiment used wastewater instead of ES medium. Therefore, there is a possibility that the wastewater had lower nutrients, especially nitrogen, then the ES medium affecting the growth rate of *Gracilaria gracilis*. The results from the laboratory experiments were consistent, so perhaps what is necessary in the pilot commercial scale abalone farm experiment is a more careful and detailed set of experiments, using wastewater and addition of fertiliser to grow *Gracilaria gracilis*.

This study was initiated to answer some unsolved questions related with seaweed research. However, in attempting to answer these, many more were generated. The experiments outlined in this study indicate that further work should be attempted on the understanding of growth of commercial economic seaweeds with the application of seaweed concentrates. Of prime importance is the need to examine the effect of seaweed concentrates and plant growth regulators on the yield and physical properties of agar of the agarophytes, especially of *Gracilaria gracilis*. The physical properties of the agar should include, in particular, the gel strength, the gelling temperature and the chemical properties as it determines its commercial value. Other areas of research include identification of the components responsible for growth in seaweed concentrates, as well as the effects of plant growth regulators on seaweeds throughout their life history. Even though the results obtained under controlled conditions could not be extrapolated to the pilot scale commercial conditions (abalone

farm), such experiments are relatively cheap, fast and supply valuable clues on responses to environmental parameters that can help when sites for mariculture are being sought. As more information about the composition, biological activity and nutrient content as well as effects on seaweeds, of different seaweed concentrates becomes available, these products will probably be used to a greater extent in mariculture. Their relevance in mariculture is emphasized by their relative cheapness and because they are natural biodegradable products.

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APPENDIX A: SOLUTION

ES ENRICHED SEAWATER MEDIUM (PROVASOLI, 1963)

FE-SOLUTION

Dissolve 351 mg of $Fe(NH_4)_2(SO_4)_2.6H_2O$ and 300 mg of Na_2EDTA in 500 ml of glass-distilled water.

PII METAL SOLUTION

Na_2EDTA	100 mg
H_3BO_3	114 mg
$FeCl_3.6H_2O$	4.9 mg
$MnSO_4.H_2O$	16.4 mg
$ZnSO_4.7H_2O$	2.2 mg
$CoSO_4.7H_2O$	0.48 mg

Dissolve all materials in 100 ml glass-distilled water.

ES-ENRICHED SOLUTION

$NaNO_3$	350 mg
Na_2glycerophosphate.$5H_2O$	50 mg
Fe-solution	25 ml
PII-metals	25 ml
Vitamin B_{12}	1 ml
Thiamine	1 ml
Biotin	1 ml
Tris Buffer (Sigma)	500 mg

Dissolve all materials in 100 ml glass distilled water. Adjust the pH to 7.8. Autoclave. Store at 10 ^{o}C. For a 1/3 ES-enriched solution, combine 6 ml ES-enriched solution and 1000 ml autoclaved seawater.