Polyamines in *Ecklonia maxima* and their effects on plant growth

By

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"Anyone can count the seeds in an apple, but only
God can count the number of apples in a seed"

~Robert, H. Schuller~

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Regular consultation took place between the student and ourselves throughout the investigation. We advised the student to the best of our ability and approved the final document for submission to the Faculty of Science and Agriculture Higher Degrees Office for examination by the University appointed Examiners.

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ABSTRACT

Kelpak®, a seaweed concentrate (SWC) prepared from the brown seaweed *Ecklonia maxima* (Osbeck) Papenfuss, improves overall plant mass and fruit yield in a variety of crops. The main active principals isolated from Kelpak® are cytokinins and auxins. Although these compounds are partly responsible for the growth promoting effect observed with Kelpak® application, they do not fully account for the complete effect of Kelpak® treatment. For this reason the focus has turned to polyamines (PAs) which are found in all cells of plants, animals and microorganisms, including eukaryotic algae. Polyamines also have growth promoting effects in plants. A study was carried out to investigate the PA levels in *E. maxima* and Kelpak® through a biennial cycle and to investigate if the PAs present in Kelpak® may have an effect on root growth, alleviating nutrient deficiency and the transport and accumulation of PAs in plants.

To determine the amount of PA in the stipes, fronds and SWC prepared from *E. maxima*, samples were collected monthly over a two-year period (June 2009-June 2011). Extracts were benzoylated and quantified using a Varian HPLC. Putrescine concentrations ranged from 15.98-54.46 $\mu g.g^{-1}$, 6.01-40.46 $\mu g.g^{-1}$ and 50.66-220.49 $\mu g.g^{-1}$ DW in the stipe, fronds and SWC, respectively. Spermine concentrations ranged from 1.02-35.44 $\mu g.g^{-1}$, 1.05-26.92 $\mu g.g^{-1}$ and 7.28-118.52 $\mu g.g^{-1}$ DW in the stipe, fronds and SWC, respectively. Spermidine concentrations fell below the detection threshold. This is the first report of PAs being detected in a SWC. The seasonal pattern established for the stipe, frond and SWC followed the same trend over a biennial cycle. Polyamines accumulated in the seaweed tissue during periods of active growth and as a stress response elicited by rough wave action. This PA trend was similar to the cytokinin trend reported by **MOONEY and VAN STADEN (1984b)** for *Sargassum heterophyllum* which suggests that PAs play an important role in the hormone cascade during active growth.

Routine monthly screening of Kelpak® carried out in the Research Centre for Plant Growth and Development indicated that Kelpak® consistently resulted in more rooting

in the mung bean bioassay than the IBA control. The potential root promoting effect of PAs were investigated. Individually applied PAs did not increase rooting in the mung bean bioassay, but a synergistic relationship was observed between Put (10^{-3} M) and IBA (10^{-4} M). When applied together, rooting increased significantly above Put (10^{-3} M) and IBA (10^{-4} M) applied separately. The Put-auxin combination produced a similar number of roots to those treated with Kelpak®. It is possible that the PAs present in Kelpak® have a synergistic effect with auxins present in Kelpak® to promote root development and growth.

Several physiological effects of Kelpak® and PAs on plant growth were investigated in a series of pot trials. Kelpak® significantly improved the growth of P- and K-deficient okra seedlings and masked the detrimental effects exerted by P- and K-deficiency. The application of PAs (10^{-4} M) significantly improved the seedling vigour index (SVI) of okra seedlings subjected to N-deficiency. The statistical difference was attributed to the N-containing growth regulators and polyamines being degraded and metabolized by the okra seedlings. Polyamine application did not alleviate P- and K-deficiency but increased root growth significantly in seedlings receiving an adequate supply of nutrients. It is likely that the additional PAs supported auxin-mediated root growth.

A pot trial with okra plants was conducted to establish if the PAs in Kelpak®, applied as a soil drench or foliar application, are absorbed and translocated in a plant. Plants were also treated with Put, Spm, Spd to establish if PAs can be absorbed and translocated. Once the fruit had matured, plants were harvested and the endogenous PA content quantified by HPLC in the roots, stems and fruits. Applying PAs as a soil drench was not as effective as a foliar spray at increasing the PA content in the different plant parts. Kelpak® treatment (0.4%) did not contribute more PAs in any plant part. Spermidine concentrations were higher, in the various plant parts, than Put or Spm, irrespective of the mode of application. The application of Put, Spd and Spm increased Spd concentrations in the roots. Considering that Spd is the main PA produced in the roots and that exogenously applied PAs are readily converted to

Spd, it seems evident that Spd is the preferred PA for long-distance transport in plants.

The cytokinins and auxins in Kelpak® play an important role in stimulating growth in plants. It is, however, the totality of different compounds in Kelpak® that gives it its unique growth stimulating ability. Polyamines, occurring within the seaweed contribute to this activity, having an active role in root production and thus increased plant growth.

CONFERENCE CONTRIBUTIONS

Oral Presentations

Papenfus, H. B., Stirk, W. A., Finnie, J. F. and van Staden, J. 2010. Seasonal variation of polyamines in the seaweed *Ecklonia maxima*. 12th Annual Research Centre for Plant Growth and Development (RCPGD) Conference, Pietermaritzburg, South Africa.

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TABLE OF CONTENTS

STUDENT DECLARATION i

DECLARATION BY SUPERVISORS ii

FACULTY OF SCIENCE AND AGRICULTURE DECLARATION - PLAGIARISM iii

ACKNOWLEDGEMENTS iv

ABSTRACT v

CONFERENCE CONTRIBUTIONS viii

TABLE OF CONTENTS ix

LIST OF FIGURES xiii

LIST OF TABLES xv

LIST OF ABBREVIATIONS xvii

Chapter 1 - Literature Review 1

1.1. Seaweed species used in agriculture and horticulture 1

1.2. Different physiological effects exerted by seaweed concentrates on the growth and development of plants 5

1.2.1. Effect of seaweed concentrate on rooting, seedling establishment and plant growth 5

1.2.2. Effects of seaweed concentrate on flowering, fruit set and crop yield 8

1.2.3. Effects of seaweed concentrate on nutrient-stressed crops 9

1.3. Growth regulating substances in seaweed concentrates 11

1.3.1. Auxin in plants and seaweeds 11

1.3.2. Cytokinin in plants and seaweeds 12

1.4. Introduction to polyamines 13

1.4.1. Distribution of polyamines 13

1.5. Metabolism of polyamines within plants 14

1.5.1. Biosynthesis 14

1.5.2. Polyamine degradation 16

1.6. Functions exerted by endogenous and exogenously applied polyamines 17

1.6.1. Effect of polyamines on plant growth and development 18

1.6.2. Effect of polyamines on root growth 19

1.6.3. Effect of polyamines on nutrient-deficiency 19

Aims 20

Chapter 2 - Seasonal variation of polyamines in *Ecklonia maxima* (Osbeck) Papenfuss and the seaweed concentrate Kelpak® 21

2.1. Introduction 21

2.2. Materials and Methods 21

2.2.1. Acquisition and preparation of seaweed material and SWC for analysis 21

2.2.2. Preparation and extraction of polyamines from the seaweed concentrate 22

2.2.3. Preparation and extraction of dry seaweed material 24

2.2.4. Preparation of the polyamine standard curves 27

2.2.5. HPLC analysis 27

2.3. Results 28

2.4. Discussion 32

Chapter 3 - Comparison of Kelpak® and polyamines on root growth 41

3.1. Introduction 41

3.2. Materials and Methods 42

3.3. Results 43

3.4. Discussion 46

Chapter 4 - Effect of Kelpak® and polyamines on okra seedlings grown in nutrient deficient conditions 49

4.1. Introduction 49

4.2. Materials and Methods 50

4.3. Results 52

4.4. Discussion 56

Chapter 5 - Uptake and transport of polyamines by okra plants 60

5.1. Introduction 60

5.2. Materials and Methods 61

5.2.1. Growth conditions of the plant material 61

5.2.2. HPLC analysis of okra plant material for polyamine content 61

5.3. Results 62

5.4. Discussion 65

Chapter 6 - General Conclusions 67

References 72

Appendix 87

LIST OF FIGURES

Chapter 1

Figure 1. 1. Harvesting and processing *Ecklonia maxima*. a-Kommetjie, harvest site, b-loading seaweed on boat, c-separating fronds and stipes, d-cutting seaweed into smaller pieces, e-washing seaweed and f-mincing seaweed material. 4

Figure 1. 2. Schematic representation of the biosynthesis of polyamines within plants. Adapted from **(KUSANO *et al.*, 2008)**..... 15

Figure 1. 3. Schematic representation of the degradation of polyamines by amine oxidases. Adapted from **(KUSANO *et al.*, 2008)**. 16

Chapter 2

Figure 2. 1. Chemical reaction for the benzoylation of polyamines. 23

Figure 2. 2. Polyamine extraction procedure for the seaweed material and seaweed concentrate. 26

Figure 2. 3. Polyamine standards of putrescine (29.81 µg), spermidine (12.12 µg) and spermine (50.16 µg) eluting at 19.6, 28.0 and 30.1 min, respectively. 29

Figure 2. 4. HPLC sample of Kelpak® (harvested on 18 April 2011) showing putrescine (19.6 min) and spermine (30.1 min) (indicated with black arrows). No peak was detected for spermidine. 29

Figure 2. 5. Seasonal variation of the polyamines putrescine and spermine in the stipes, fronds and seaweed concentrate prepared from *E. maxima* over a two-year-cycle. Results are shown as mean ± SE (n=3)..... 30

Figure 2. 6. Mean seawater temperatures at Kommetjie during 2007, the harvest site of *E. maxima* **(Courtesy of (LOURENS, 2011)/SOUTH AFRICAN WEATHER SERVICE)** and the seasonal variation of the polyamines putrescine and spermine in the seaweed concentrate prepared from *E. maxima* through an annual cycle. 37

Chapter 3

Figure 3. 1. The effect of polyamines, IBA, combinations of IBA and polyamines and Kelpak® on rooting in the mung bean rooting bioassay. Treatments were as follows: a-Spermidine, b-Spermidine + 10^{-4} M IBA, c-Spermine, d-Spermine + 10^{-4} M IBA, e-Putrescine, f-Putrescine +10^{-4} M IBA, g-IBA and h-Kelpak®. 45

Figure 3. 2. Comparison between the best rooting values found for the polyamine treatments, IBA, combinations of polyamines and 10^{-4} M IBA and Kelpak®. Significant differences between values within each treatment are annotated by the letters a, b and c at $P<0.05$, calculated with Duncan's Multiple Range Test (n=60). 46

Chapter 4

Figure 4. 1. The effect of 0.4% Kelpak® and 10^{-4} M PAs on okra, *Abelmoschus esculentus*, seedlings in relieving nutrient deficiencies (-N, -P and -K) after 8 weeks growth. Scale bars = 100 mm. 52

Figure 4. 2. Effect of 0.4% Kelpak® and 10^{-4} M polyamines on the Seedling Vigour Index (SVI) of okra, *Abelmoschus esculentus*, seedlings deprived of nitrogen (-N), phosphorous (-P) and potassium (-K). Significant differences between values within each treatment is indicated by the letters a and b at $P<0.05$, calculated with the Duncan's Multiple Range Test (n=11). 55

LIST OF TABLES

Chapter 1

Table 1. 1. Yield increases for various crops treated with Kelpak® (experiments conducted by Kelpak® (Pty) Ltd.)..... 7

Chapter 2

Table 2.1. Polyamine concentrations in the liquid seaweed concentrate Kelpak® 31

Table 2.2. Average polyamine ratios in the stipes, fronds and seaweed concentrate Kelpak® 32

Table 2.3. Polyamine concentrations in the seaweeds from the divisions Phaeophyta (brown macroalgae), Chlorophyta (green macroalgae) and Rhodophyta (red macroalgae) 34

Chapter 4

Table 4. 1. The effect of 0.4% Kelpak® and 10^{-4} M polyamines (combination of Put, Spd and Spm) on growth parameters of okra (*Abelmoschus esculentus*) seedlings deprived of either nitrogen (-N), phosphorous (-P) or potassium (-K) 54

Table 4. 2. Nutrient concentrations in the seaweed concentrate Kelpak® **(data supplied by Dr Riaan Lourens, Kelpak® Technical Manager)** 57

Chapter 5

Table 5. 1. Amount of polyamines (μg.g^{-1}) in the roots, stems and fruits of okra plants treated with putrescine, spermidine and spermine (10^{-4} M) and 0.4% Kelpak® applied as a soil drench 63

Table 5. 2. Amount of polyamines (μg.g^{-1}) in the roots, stems and fruits of okra plants treated with putrescine, spermidine and spermine (10^{-4} M) and 0.4% Kelpak® applied as a foliar spray 64

LIST OF ABBREVIATIONS

ADC	Arginine decarboxylase
ANOVA	Analysis of variance
DW	Dry weight
FS	Foliar spray
FW	Fresh weight
$HClO_4$	Perchloric acid
HS	Hoagland's nutrient solution
IAA	Indole-3-acetic acid
IAM	Indole-3-acetamide
IBA	Indole-3-butyric acid
mAU	Milli absorption unit
NaOH	Sodium hydroxide
ODC	Ornithine decarboxylase
PA	Polyamine
Put	Putrescine
SAM	S-adenosylmethionine
SD	Soil drench
Spd	Spermidine
Spm	Spermine
SVI	Seedling vigour index
SWC	Seaweed concentrate

Chapter 1 - Literature Review

1.1. Seaweed species used in agriculture and horticulture

The term seaweed refers predominantly to macroscopic (but with a few microscopic representatives), multicellular marine, red, green and brown algae **(LOBBAN and HARRISON, 1994)**. The oceans and seaweeds are rich in trace elements and there are approximately 490 seaweed species (107 genera) that have economical value **(TSENG, 1981)**. These seaweeds and their products are used in agriculture and horticulture as animal feed, soil conditioners, manure, growth stimulants and plant protectants against pests and diseases **(VERKLEIJ, 1992)**. In addition, alginates extracted from the seaweed are used in the manufacture of soaps, paints, leather finishers, insecticides toothpastes, lipsticks, medicines, stabilizers in food products and as clarifying agents in the production of beer **(HILLSON, 1977)**. The most heavily harvested seaweeds are from the family Phaeophyceae. Although there are other seaweeds with similar chemistry, the size and availability of the Phaeophyceae seaweeds make them the obvious choice for harvesting and utilization **(STIRK and VAN STADEN, 1997)**. The Phaeophyceae seaweeds occur mainly in seawater (99%) and are generally restricted to colder, nutrient rich waters. This group of algae is economically very important as they are processed for human consumption and fertilizers. The most commonly harvested Northern Hemisphere seaweeds include the kelps *Ascophyllum nodosum*, *Laminaria hyperborean*, *L. digitata*, *Fucus vesiculosis* and *F. serratus*. Southern Hemisphere kelp species that are most commonly harvested include *Ecklonia maxima* and *Durvillea potatorum* **(VERKLEIJ, 1992)**.

There are historical references that indicate that seaweed has been used as human food as early as 600-800 BC in China **(WAALAND, 1981)**. Coastal Asian populations have used seaweed as food and medicine by 300 AD. Dulse, a snack food prepared from the red seaweed *Palmaria palmata*, was eaten by the Norse in Iceland as early as the year 961 BC. Seaweed has been used from 1690 in the production of soda

ash (washing soda) and later in the production of iodine and potash. It was only in the early 1900's that the Western population started utilizing seaweed, especially in industry for the production of alginates and carrageenan **(CRAIGIE, 2011)**. From 1981-1994 seaweed production increased from 3.2 million tons to 7 million tons (FW). The most heavily utilized seaweeds are the brown seaweeds with 5.2 million tons (75%) harvested annualy, while 1.73 million tons red seaweeds and 0.035 million tons (0.5%) green seaweeds are harvested annually. The value of Japanese and Korean seaweed production is currently set at $US 1 billion and $US 0.5 billion, respectively **(GUIRY, 2011)**. Today, seaweed research is focused on biofuels, biostimulants in agriculture, bioremediation of soils, water and sewage and use as medicine **(CRAIGIE, 2011)**.

Seaweed is utilized in two main forms in agriculture; either as dry seaweed meals or as liquid seaweed concentrates (SWC). Seaweed meals slowly release minerals and nutrients when applied as soil additives. Besides the nutrient release, seaweed meals also improve the moisture retaining ability of soil and its crumb structure, thereby increasing the soil's aeration **(QUASTEL and WEBLEY, 1947)**. Plants grown in kelp-amended soils are more tolerant to drought, have higher germination rates, increased seedling growth and seedling establishment and are overall more productive than traditionally grown plants **(WEIERSBYE *et al.*, 2001)**. There are, however, many problems associated with applying seaweed meals to soil. **WEIERSBYE *et al.* (2001)** reported that kelp amendment increased the sodium (Na) and chlorine (Cl) levels in the soil. After repeated exposure, plants become salt-stressed. The salt stress together with the high processing costs associated with the production of seaweed meals outweighs the benefits of kelp amendment. Alternatively, the seaweed could be ashed but **BRAIN *et al.* (1973)** reported that ashing of seaweed destroys its growth promoting ability. This discovery changed the seaweed fertilizer industry radically. Soon thereafter it was reported that the active principals in seaweed was the more heat sensitive growth regulators rather than nutrients. This led to the development of liquid SWCs as novel agricultural plant growth enhancing extracts

that stimulated plant growth **(TEMPLE and BOMKE, 1988)** without the detrimental effects associated with seaweed meals.

The first SWC was produced by **MILTON (1952)**. The method patented by **MILTON (1952)**, produced a SWC by heating the seaweed at temperatures over 100°C while under pressure and included the use of alkaline chemicals to weaken the cell walls **(MILTON, 1952)**. Variations of this alkaline hydrolyzed method are still used by most companies producing SWCs today. *Ecklonia maxima* (Osbeck) Papenfuss is a brown algal species used to make Kelpak® products. Kelpak® Products (Pty) Ltd. is a kelp product manufacturing company situated in Simon's Town, Western Cape, South Africa. Since the method developed by **MILTON (1952)** might destroy the growth regulators in the seaweed, the company developed an unique cell burst method to prepare the SWC, thereby excluding the use of heat and chemicals. Collection and processing of *E. maxima* are as follows. *Ecklonia maxima* are sustainably harvested at Kommetjie, Western Cape Province (Figure 1a). Divers cut the seaweeds at the base (holdfast) and load it into boats (Figure 1b). Arriving at the factory in Simon's town the fronds and stems are separated (Figure 1c). Fronds and stipes are then cut into more manageable pieces and washed (Figure 1d, e). The seaweed is then minced into smaller pieces (Figure 1f). The minced seaweed material then passes from a high pressure chamber into a low pressure chamber, during which the material disintegrates yielding the concentrate.

Figure 1. 1. Harvesting and proc essing *Ecklonia ma xima*. **a**-Kommetjie, ha rvest site, **b**-loading seaweed on boat, **c**-separating fronds and stipes, **d**-cutting seaweed into smaller pieces, **e**-washing seaweed and **f**-mincing seaweed material.

1.2. Different physiological effects exerted by seaweed concentrates on the growth and development of plants

The growth promoting effect of SWC application is diverse, increasing overall plant growth and fruit yield in many crops **(ABETZ and YOUNG, 1983; FEATONBY-SMITH and VAN STADEN, 1983a, 1984; FINNIE and VAN STADEN, 1985; ALDWORTH and VAN STADEN, 1987; JEANNIN *et al.*, 1991; STEVENI *et al.*, 1992; CROUCH and VAN STADEN, 1993; ATZMON and VAN STADEN, 1994; CROUCH and VAN STADEN, 1994; VAN STADEN *et al.*, 1995; JONES and VAN STADEN, 1997; FERREIRA and LOURENS, 2002)**. Seaweed concentrates stimulate plant growth in different plants at different concentrations and are therefore diluted to a specific concentration as instructed by the manufacturer. Seaweed concentrates can be applied as a foliar spray, soil drench, root drench, seed drench, fruit dip and pulse treatment for cuttings **(CROUCH and VAN STADEN, 1994)**.

1.2.1. Effect of seaweed concentrate on rooting, seedling establishment and plant growth

The application of SWCs increases overall root growth and seedling establishment in a wide variety of plant species. When Kelpak® was applied to pine cuttings (*Pinus patula*), rooting was increased by 70% with increased root quality **(JONES and VAN STADEN, 1997)**. In a similar study **ATZMON and VAN STADEN (1994)** reported that the increase in shoot growth found for pine seedlings might have been as a result of the significant increases in root number and root length after Kelpak® administration. Similar results were found by **VAN STADEN *et al.* (1995)**, when three *Eucalyptus* species were treated with a 10% Kelpak® solution. The study showed that a single Kelpak® dose early on in the life of the *Eucalyptus* seedlings increased lateral root growth and overall plant size.

The application of Kelpak® to *in vitro* cultured tomato roots significantly improved root length and increased the number of lateral roots **(FINNIE and VAN STADEN, 1985)**. **ALDWORTH and VAN STADEN (1987)** reported that dipping cabbage roots in seaweed concentrate (1:500 dilution) for 5 min before transplanting, significantly

increased seedling growth and appearance. Three-to-four weeks after transplantation, mean stem diameter and plant length were superior to the controls. In a study using a mung bean rooting bioassay, it was reported that the application of SWC increased root growth linearly with a dilution spectrum ranging from 0.2 to 10% Kelpak® **(CROUCH and VAN STADEN, 1991)**.

The application of SWC early in the vegetative growth of *Triticum aestivum* (wheat) increased several growth parameters associated with wheat. Kelpak® applied as a soil drench increased rooting, shoot length, total vegetative mass, number of spikelets, number of kernels and average grain mass **(NELSON and VAN STADEN, 1986)**. The leaves of the treated wheat plants were found to be darker and senesced slower. Maize plants treated with a SWC prepared from *Ascophyllum nodosum*, increased the total vegetative fresh weight of maize plants by 15-25% **(JEANNIN *et al.*, 1991)**. The SWC Maxicrop, increased growth in hydroponically-grown barley by 25-45% **(STEVENI *et al.*, 1992)**, Maxicrop also increased the weight and mean heart diameter in lettuce and increased curd diameter in cauliflower **(ABETZ and YOUNG, 1983)**. It is evident that SWCs promote growth of a wide variety of plant species. Although not discussed in detail, Kelpak® also produced significant yield increases in canola plants (*Brassica napus*) **(FERREIRA and LOURENS, 2002)**, wintergreen (*Phaseolus vulgaris*) **(FEATONBY-SMITH and VAN STADEN, 1984)** and swiss chard (*Beta vulgaris*) **(FEATONBY-SMITH and VAN STADEN, 1983a)**.

Kelpak® increases production in many crops, all of which have not been recorded in the literature but have been tested by Kelpak® Products (Pty) Ltd. (Table 1.1) These results indicate that SWCs enhance rooting, seedling establishment and overall growth in a wide range of plants.

Table 1. 1. Yield increases for various crops treated with Kelpak® (experiments conducted by Kelpak® (Pty) Ltd.)

Crop	Yield increase (%)	Crop	Yield increase (%)
White seedless grapes			
Thompson (Sultana)	9	Prime	12
Superior	12		
Coloured seedless grapes			
Flame	17	Sun red	6
Crimson	17		
Seeded grapes			
Red Globe	16	Dauphine	8
Dan-Ben-Hannah	8		
Cucurbits			
Melon	5	Butternut	23
Watermelon	31	Pumpkin	21
Cucumber	12		
Vegetables			
Onion	31	Tomato	25
Potato	16		
Leaf/Head vegetables			
Head lettuce	16	Broccoli	15
Leafy lettuce	13	Cauliflower	13
Cabbage (large variety)	15	Cabbage (small variety)	9
Legumes			
Bean	26	Pea	17
Soya	17	Peanut	16
Nut crop			
Almond	27	Hazelnut	15
Macadamia	20	Pistachio	19
Rice			
Rice	13		

1.2.2. Effects of seaweed concentrate on flowering, fruit set and crop yield

The application of Kelpak® as a foliar spray enhanced overall dry mass of beans (*Phaseolus vulgaris*) by 24%. The increase in dry mass was attributed to an increase in rooting and fruit set. Further supplementation of treated plants with chemical fertilizer resulted in a 59% increase in total plant dry mass **(FEATONBY-SMITH and VAN STADEN, 1984)**. The application of Kelpak® increased the cytokinin levels in all plant parts compared to the control plants in which cytokinins were only detected in the fruits **(FEATONBY-SMITH and VAN STADEN, 1984). TEMPLE and BOMKE (1989)** reported that the SWCs prepared from *Macrocystis integrifolia* and *E. maxima* both increased bean yield by 24%. Another study on a bean crop showed that the SWC, prepared from the red seaweed *Kappaphycus alvarezii*, increased the yield of soybean (*Glycine max*) by 56% **(RATHORE *et al.*, 2009)**.

In an experiment conducted by **CROUCH and VAN STADEN (1992)**, Kelpak® was applied to tomato seedlings as a soil drench. Root growth and overall photosynthetic accumulation efficiency were enhanced. When Kelpak® was applied as a foliar spray, total fruit fresh weight was increased by 17% and the total number of harvestable fruits were improved by 10%. Flowering was also earlier in Kelpak®-treated plants with more flowers compared to control plants. It was suggested that the early flower set might be a consequence of the enhanced growth and development of the plants. Thus, the plants reached maturity in a shorter time period. Economically, this is very important. Plants with an earlier fruit set fetch better market prices **(CROUCH and VAN STADEN, 1992)**. Application of Kelpak® to three varieties of peppers (*Capsicum anuum*) increased fruit number and fruit size which would also lead to better returns **(ARTHUR *et al.*, 2003)**.

Kelpak® increased grain yield and culm diameter and reduced lodging in wheat. Greater yields were produced due to an increase in all cells but especially the cells of the vascular bundles **(NELSON and VAN STADEN, 1984b)**. A 50% increase in grain yield was also recorded for barley treated with Kelpak® **(FEATONBY-SMITH and VAN STADEN, 1986)**. The increased yield was attributed to an increase in the

number of fertile spikelets per ear. The application of Kelpak® on marigold (*Tagetes patula*) seedlings increased seed production by 50% compared to the control plants. **(VAN STADEN *et al.*, 1994)**.

A phytohormone extract, designed to contain cytokinin, auxin and gibberellin, prepared from *M. integrifolia*, increased the production of bean plants but was not as effective as the SWC. The lower activity of the phytohormone extract can be attributed to one or a combination of reasons: destruction of the growth regulators during extraction, the concentration of the growth regulators was not optimal for stimulating growth, the growth regulators require other substances within the extract to function optimally or the extraction procedure did not extract other growth regulating substances present within the SWC **(TEMPLE and BOMKE, 1989)**. From this study it is evident that there are other as yet unknown growth regulating compounds in SWC that stimulate growth.

1.2.3. Effects of seaweed concentrate on nutrient-stressed crops

Nutrient deficiency in agriculture is an ever present concern for farmers. Huge sums of money are invested annually to increase the nutrient status of farm lands. Synthetic fertilizers have detrimental effects on the environment, which encourages the use of naturally occurring fertilizers **(CROUCH and VAN STADEN, 1993)**. Nothing in the market can replace the application of fertilizers. There are, however, some biological compounds which might relieve the stress experienced by the nutrient-stressed plants and thus reduce the fertilizer requirements of the plants.

Most of the studies concerned with the growth stimulatory effect of SWC were conducted on plants supplied with normal nutrient levels. There were, however, some studies that did investigate the effect of SWC on plant growth under conditions of nutrient-stress. **NELSON and VAN STADEN (1984a)** reported that treating nutrient-stressed greenhouse cucumbers with Kelpak® produce higher yields. The increased yield was a result of the growth regulators within the SWC that increased the root: shoot ratio and total dry mass of the plants. It was proposed that root growth was stimulated over and above that of shoot growth thereby increasing the overall

photosynthate accumulation efficiency of the plants. Kelpak® treatment increased phosphorous (P) levels in the cucumbers, but there was a reduction in nitrogen (N). The loss of N was attributed to the increased root growth which drew more nitrogen to the roots for assimilation into nitrogen containing compounds such as cytokinins **(NELSON and VAN STADEN, 1984a)**. **BECKETT and VAN STADEN (1989)** reported that Kelpak® did not affect wheat receiving an adequate supply of potassium (K) but significantly increased grain yield in plants receiving inadequate amounts of K. It was reported that Kelpak® reduced the K requirement of wheat. In a similar study, **BECKETT and VAN STADEN (1990b)** reported that the application of Kelpak® as a root flush increased the yield of wheat receiving a low nutrient supply (1% Hoagland's nutrient solution). No beneficial effect was observed for wheat which received an adequate supply of nutrients. The increase in yield in this study was not attributed to the cytokinins residing in the SWC, as the benzyladenine control (cytokinin equivalent of Kelpak®) did not increase yield. It was reported that Kelpak® did make a contribution to the nitrogen requirements of wheat. It is possible that other growth regulators within Kelpak® might have been degraded, supplying the wheat plants with nitrogen breakdown products. **BECKETT *et al.* (1994)** reported marked increases in yield of tepary beans receiving various concentrations of nutrients and Kelpak®. The yield of Kelpak®-treated tepary beans receiving 5% and 1% Hoagland's nutrient solution (HS) increased by 82% and 69%, respectively. These are remarkable increases, considering that 50% HS contains an adequate supply of nutrient to support normal plant growth. **CROUCH *et al.* (1990)** reported that the application of Kelpak® did not increase the yield of nutrient-stressed lettuce. Kelpak® application, did however, increase the yield and the calcium (Ca), potassium (K) and magnesium (Mg) content of lettuce plants receiving double strength HS by 52%, 46% and 37%, respectively.

Seaweed products have many other beneficial effects on plant growth. Application of several SWCs are able to inhibit nematode infection and gall formation in plants **(FEATONBY-SMITH and VAN STADEN, 1983b; CROUCH and VAN STADEN, 1993; WU *et al.*, 1998)**. Immersing fruit for one to two hours in SWC increased their

fruiting. Seaweed concentrate treatment increased fruit ripening rate of mangoes and bananas, increased shelf-life of capsicums and reduced the rate of degreening in limes **(BLUNDEN *et al.*, 1978)**.

1.3. Growth regulating substances in seaweed concentrates

Growth regulators such as cytokinins **(BRAIN *et al.*, 1973; TAY *et al.*, 1985; BECKETT and VAN STADEN, 1990b)** and auxins **(CROUCH and VAN STADEN, 1992)** play a significant role in increasing growth of plants treated with seaweed extracts. Cytokinins are a group of naturally occurring growth promoters which have shown masking effects for some nutrient deficiencies **(BECKETT and VAN STADEN, 1990a). BECKETT and VAN STADEN (1990a)** reported that the application of Kelpak® increased the uptake of Zn in plants that received no macronutrients but did not increase the uptake of Zn in plants which received sufficient macronutrients.

1.3.1. Auxin in plants and seaweeds

Auxins were the first group of plant hormones discovered in plants and are found in almost all cells of a plant but have higher concentrations in meristematic regions and actively growing organs. Auxins, such as indole-3-acetic acid (IAA), are characterized by their ability to stimulate cell elongation in stems and coleoptile sections, root initiation, vascular differentiation, tropic responses and the development of axillary buds, flowers and fruits **(HOPKINS and HÜNER, 2004)**.

Endogenous auxins are also present in seaweeds. Only IAA and indole-3-acetamide (IAM) were detected in *Ulva fasciata* and *Dictyota humifusa*, seaweeds collected from the South African south coast **(STIRK *et al.*, 2009)**. Indole-3-acetic acid and IAM were also the main auxins detected within several red algae from Brazil **(YOKOYA *et al.*, 2010)**. It is evident that auxins, especially IAA and IAM, are common constituents within macroalgae.

The auxins, indole-3-acetic acid (IAA), indole-3-carboxylic acid, N,N-dimethyltryptamine, indole-3-aldehyde and iso-indole,1,3-dione, were identified by **CROUCH *et al.* (1992)** using GC-MS in the commercial seaweed concentrate, Kelpak®. Indole-3-acetic acid and several indole-conjugates were the main auxins in the SWCs, Kelpak® prepared from *E. maxima* and ISP alginates prepared from *Macrocystis pyrifera* **(STIRK *et al.*, 2004)**. **HONG *et al.* (1995)** found auxin-like activity in Cytex (prepared from a Fucaceae seaweed, species unknown), a commercial seaweed extract. They reported the bioactivity of the extract to be equivalent to 0.3 $mg.l^{-1}$ IAA.

1.3.2. Cytokinin in plants and seaweeds

Seaweeds contain a considerable amount of cytokinins, including zeatin, dihydrozeatin, isopentenyladenine and isopentenyladenosine. Cytokinins are N^6-substituted derivatives of the purine base adenine. They have characteristic abilities, in combination with auxin, to stimulate cell division, shoot and root differentiation, growth of lateral buds, leaf expansion, chloroplast development and to retard leaf senescence. One of the main sites of cytokinin synthesis is within the roots, especially in mitotically active root tips, from where they are transported through the xylem to other plant parts **(HOPKINS and HÜNER, 2004)**.

The cytokinins and their riboside and ribotide conjugates were the main cytokinins found in *Ulva fasciata* and *Dictyota humifusa*, with *trans*-zeatin, dihydrozeatin and aromatic cytokinins occurring in much lower concentrations **(STIRK *et al.*, 2009)**. **STIRK *et al.* (2003)** found that the dominant cytokinins in 31 seaweed species were *cis*-zeatin and isopentenyladenine. Aromatic cytokinins were present in very low concentrations and dihydrozeatin-type cytokinins occurred at low concentrations in only nine seaweed species. Cytokinins have also been identified in a number of SWCs and are thought to promote growth of plants to which SWCs have been administered **(SANDERSON and JAMESON, 1986)**. Using GC-MS and the soybean callus bioassay, **TAY *et al.* (1985)** discovered zeatin, zeatin ribosides and dihydrozeatin ribosides, with a total cytokinin concentration of 0.115 $mg.l^{-1}$, in a SWC

made from *Durvillia potatorum*. The cytokinins zeatin, dihydrozeatin, zeatin-O-glucoside, isopentenyl and isopentenyladenosine were identified in the SWC made from *Fucus serratus* **(STIRK and VAN STADEN, 1997; STIRK *et al.*, 2003)**.

The presence of cytokinins in the SWC S.M.3-Chase Organics was established by the addition of aqueous seaweed concentrate to the growth medium of the cytokinin requiring strain, *Atropa belladonna*. The seaweed concentrate initiated and increased callus cell growth comparable to growth medium containing kinetin **(BRAIN *et al.*, 1973)**.

1.4. Introduction to polyamines

Although the presence of cytokinins and auxins have been established in the SWC Kelpak®, the many beneficial effects of Kelpak® application to crops cannot be explained by these two plant hormones alone. It is probable that other growth promoting compounds are also present in Kelpak®. One likely group of compounds is polyamines.

1.4.1. Distribution of polyamines

Polyamines (PA) are a group of ubiquitous, polyvalent and bioactive compounds which contain more than one amine group. These compounds were first discovered by van Leeuwenhoek in the 1700's as three sided crystals in human semen and were named spermine because of their high concentration within human semen. Polyamines are found in almost all plants, animals and microorganisms **(HOPKINS and HÜNER, 2004)**. The three most common PAs are the diamine putrescine (Put, butan-1,4-diamine), the triamine spermidine (Spd, [N-(3-aminopropyl) butane-1,4-diamine]] and the tetraamine spermine (Spm, [NN'-bis-(3-amino-propyl) butane-1,4-diamine]) **(VALERO *et al.*, 2002)**. At normal physiological pH, PAs are polycationic, which enables them to bind to negatively charged nucleic acids (polyanionic), phospholipids of the plasma membrane and certain proteins **(HOPKINS and HÜNER, 2004)**. These compounds are able to bind to macromolecules, directly

affecting their biosynthesis and metabolic activity. They also bind to soluble substances to form conjugates such as phenolic acids **(GUZMÁN-URIÓSTEGUI *et al.*, 2002)**. Polyamines are synthesized from two amino acids—arginine and ornithine **(HOPKINS and HÜNER, 2004)**. Polyamines are found in the cell wall fraction, cytoplasm, vacuoles, mitochondria and chloroplasts. The PA content in plants varies from nanomolar to millimolar levels **(HAMANA and MATSUZAKI, 1982; KAKKAR and SAWHNEY, 2002)**. Polyamines are part of the cellular contents of all living cells and are intricately linked to our day-to-day lives. Polyamines probably occur in all living cells **(GALSTON, 1983)** and are therefore ingested by man every day. A study in Britain revealed that the average daily PA consumption ranges between 350-500 µmol per person per day. The major sources of PAs are fruit, cheese, meat and non-green vegetables **(BARDOCZ *et al.*, 1995)**.

Several studies have shown that there are appreciable amounts of PA within seaweed **(HAMANA and MATSUZAKI, 1982; BADINI *et al.*, 1994; LEE, 1998; MARIÁN *et al.*, 2000)**. These studies showed that the relative concentrations of Put, Spd and Spm ranged from 0.37-2000 $nmol.g^{-1}$, 0.2-235 $nmol.g^{-1}$ and 0.07-120 $nmol.g^{-1}$, respectively. It is evident that the PA content of seaweeds may vary among different seaweed species. Although no data was presented, **BADINI *et al.* (1994)** found that the PA content of *Ulva rigida* from the Northern Adriatic Sea fluctuated throughout a year of sampling with the highest concentration recorded for summer. It was proposed by **BADINI *et al.* (1994)** that the fluctuations in PA content could be a result of different growth phases and/or an increase in temperature.

1.5. Metabolism of polyamines within plants

1.5.1. Biosynthesis

As mentioned, PAs are positively charged molecules that bind readily to nucleic acids, acidic phospholipids, proteins and enzymes. Some enzymes are directly modulated by PAs. For higher plants and bacteria, the first step in PA biosynthesis is

the decarboxylation of either ornithine or arginine by the enzymes ornithine decarboxylase (ODC) and arginine decarboxylase (ADC), respectively (Figure 1.2) **(BOUCHEREAU *et al.*, 1999)**.

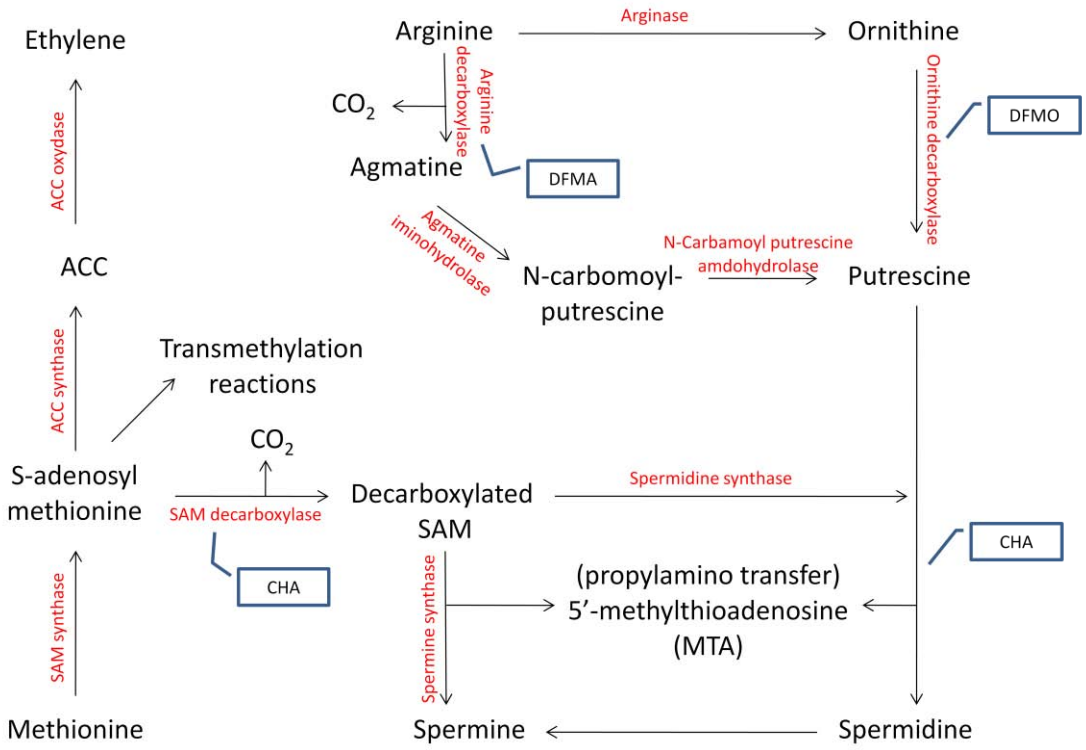

Figure 1. 2. Schematic representation of the biosynthesis of polyamines within plants. Adapted from **(KUSANO *et al.*, 2008)**.

For mammals and fungi, only the reaction catalyzed by ODC will lead to Put formation **(BOUCHEREAU *et al.*, 1999)**. A second enzyme, S-adenosylmethionine decarboxylase, induces the decarboxylated form of S-adenosylmethionine (aminopropyl) into the conversion of Put to Spd and Spm. The actual transfer of the aminopropyl group to Spd and Spm is catalyzed by the enzymes Spd synthase and Spm synthase (Figure 1.2), respectively **(TIBURCIO *et al.*, 1997)**. The subsequent addition of an aminopropyl moiety onto Put will form Spd and addition of an aminopropyl moiety on Spd will form Spm in the presence of the enzymes spermidine synthase and spermine synthase, respectively. The aminopropyl moiety is the

product of the decarboxylation reaction of S-adenosylmethionine (SAM) catalyzed by S-adenosylmethionine decarboxylase.

Polyamines and ethylene inhibit each other's biosynthesis as the two reactions share the common precursor SAM. The pool of free PA within the cell depends not only on synthesis but also on other processes such as degradation, conjugation and transport **(BOUCHEREAU *et al.*, 1999)**. The transport of PAs across the plasmalemma is energy-dependant and involves calcium. Calcium actively influences the transport through a cascade pathway which involves protein kinase and phosphatase activities **(BOUCHEREAU *et al.*, 1999).**

1.5.2. Polyamine degradation

Polyamine degradation is accomplished through a group of enzymes known as amine oxidases. Deaminating enzymes commonly associated with PA degradation are copper diamine oxidase and flavoprotein polyamine oxidase. The deamination breakdown product of putrescine is succinate and that of spermidine and spermine is β-alanine (Figure 1.2) **(BOUCHEREAU *et al.*, 1999)**.

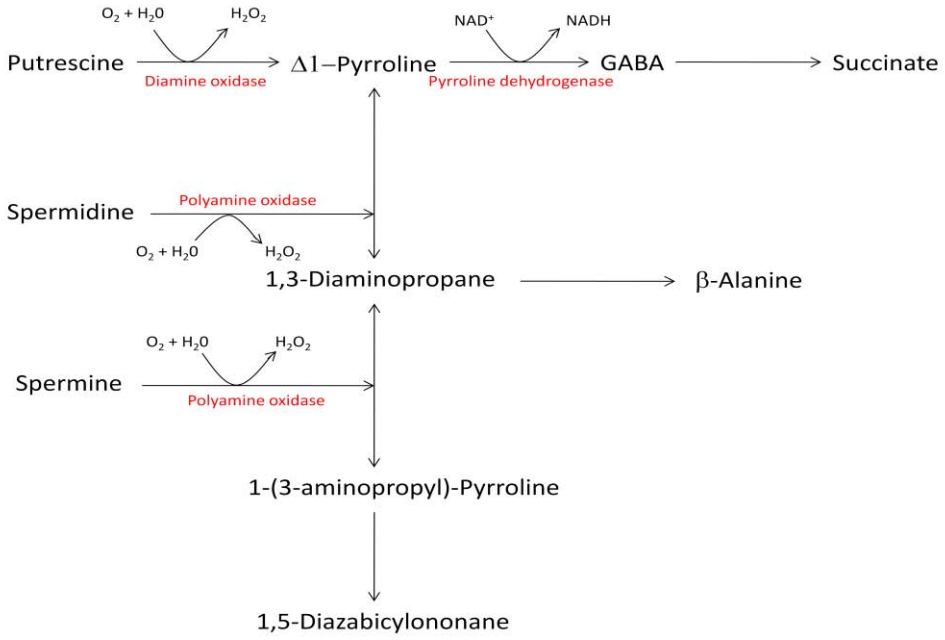

Figure 1. 3. Schematic representation of the degradation of polyamines by amine oxidases. Adapted from **(KUSANO *et al.*, 2008)**.

Δ1-Pyrroline is broken down to γ-aminobutyric acid which can be further oxidized and deaminized to produce succinic acid. This deamination pathway allows succinic acid to be incorporated into the Krebs cycle, thereby recycling carbon and nitrogen from Put **(BOUCHEREAU *et al.*, 1999)**.

Except for the free forms, PAs can occur bound to smaller molecules such as phenolic acids, conjugated forms and can be bound to larger macromolecules such as proteins (bound form). The most common conjugated polyamine is through an amide linkage to cinnamic acid. Conjugation is achieved through activated carboxyl groups provided by CoA. Polyamines bind to proteins, intra-cellularly or extra-cellularly, by post-translational covalent linkages catalyzed by transglutaminases **(BOUCHEREAU *et al.*, 1999)**. Polyamine conjugates may make up to 90% of a cell's PA content **(GALSTON and SAWHNEY, 1987)**.

1.6. Functions exerted by endogenous and exogenously applied polyamines

Studies have shown that PAs are responsible for a wide range of functions within a plant. Polyamines accumulate within plants in response to stress **(FLORES and GALSTON, 1982b; DAS *et al.*, 1995; GALSTON *et al.*, 1997; KAKKAR and SAWHNEY, 2002; SUDHA and RAVISHANKAR, 2002)**. They play an important role in developmental processes such as pollen maturation, germination and flower development **(SMITH, 1985; GERATS *et al.*, 1988; EVANS and MALMBERG, 1989)**. Physiological functions of PAs include the stabilization of cell membranes **(ROBERTS *et al.*, 1986; KAUR-SAWHNEY *et al.*, 1989; KAUR-SAWHNEY and APPLEWHITE, 1993)** and anti-senescence **(EVANS and MALMBERG, 1989)**. Because PAs are able to bind to nucleotides of RNA and DNA, they function in the regulation of gene expression, translation, modulation of cell signalling, modulation of ion channel activity **(KUSANO *et al.*, 2008)** and also interact with glutamate receptors **(KAKKAR and SAWHNEY, 2002)**. They are known to be associated with growth stimulating and growth regulating functions within plants **(GALSTON, 1983)**,

and to play a role in hormone-signalling pathways **(TIBURCIO *et al.*, 1997)**. Polyamines might also function as secondary hormone messengers **(SLOCUM and FLORES, 1991; SERGIEV *et al.*, 1995)**.

Polyamines are implicated in cellular processes such as chromatin organization, mRNA translation, ribosome biogenesis and cell proliferation **(IGARASHI and KASHIWAGI, 2000; THOMAS and THOMAS, 2001)**. The increased intracellular PA concentration during periods of rapid cell proliferation indicates that PAs are essential for cell growth and differentiation, but their roles in these cellular processes are still unknown **(GALSTON, 1983)**. Free PAs, either through endogenous presence or exogenous application, have an anti-senescence function in that they retard color change, increase fruit firmness, delay ethylene and respiratory gas emissions, induce mechanical resistance and reduce chilling symptoms in fruits **(VALERO *et al.*, 2002)**.

1.6.1. Effect of polyamines on plant growth and development

PISTOCCHI *et al.* (1987) reported that exogenously applied PAs were quickly taken up and transported into carrot cells, reaching maximum absorption after 1 min. The absorption of different PAs were highly localized. Putrescine was found mainly in the soluble cytoplasmic fraction, whereas spermidine was localized in the cell walls. The application of 10 µM calcium (Ca^{2+}) solution stimulated Put uptake. Spermidine uptake was stimulated linearly with an increase in Ca^{2+} concentration to 1 µM. The external application of PAs to plant systems is a valuable method to investigate the effect of PAs on plants **(PISTOCCHI *et al.*, 1987)**.

Polyamines have a wide variety of functions in the growth and development of plants **(BAIS and RAVISHANKAR, 2002)**. There is a direct correlation between PA concentration and metabolic activity. It was found that PA concentration increased with increased cell division. Cell wall elongation is accomplished when PA biosynthesis is inhibited, but is required for cell wall rigidity and cell-to-cell adhesion **(MONTAGUE *et al.*, 1978)**. Putrescine is the key regulator of ethylene and PA biosynthesis. Ethylene and PAs are also responsible for somatic embryogenesis **(FEIRER *et al.*, 1984; FIENBERG *et al.*, 1984; FEIRER *et al.*, 1985)**.

1.6.2. Effect of polyamines on root growth

It has been reported that PA are required by all active growing tissues. Using the mung bean bioassay, **FRIEDMAN *et al.* (1982)** reported that exogenously applied PAs do not promote root growth. The PA content within the plant tissue might, however, be enough for root growth and adding PAs above this level might be deleterious for growth **(FRIEDMAN *et al.*, 1985; WATSON *et al.*, 1998)**. A later study reported that auxins enhance PA synthesis which might be a requirement for auxin-stimulated root growth **(FRIEDMAN *et al.*, 1985)**. **BAIS and RAVISHANKER (2002)** reported that Put, Spd and Spm levels increased in hypocotyls before an increase in root number. When Put was added to Put-inhibited root cultures of chicory (*Cichorium intybus*), root growth was restored **(BAIS and RAVISHANKAR, 2002)**. There also exists a positive correlation between primary root growth and spermidine and spermine content. Treating plant with spermine and the spermidine inhibitor, cyclohexylammonium, decreased lateral root growth. Growth was restored by the exogenous application of putrescine to excised rice roots *in vitro*, which enhanced root elongation **(COUÉE *et al.*, 2004)**. The role of PAs in root growth seems to be occurring during the first two to three days after germination when active growth is taking place. As soon as lateral root initiation occurs, PA concentrations decrease **(SHEN and GALSTON, 1985)**. Polyamines promote root elongation by increasing root cell division. From these studies it is clear that PAs accumulate and promote growth where meristematic tissue occur and are not confined to certain plant organs.

1.6.3. Effect of polyamines on nutrient-deficiency

Little work has been done on the role PAs play during nutrient deficiency. **YOUNG and GALSTON (1984)** reported that Put levels along with ADC and ODC activity increased in oat plants (*Avena sativa*) under potassium stress. It was suggested by **YOUNG and GALSTON (1984)** that the increase in Put concentration might be a physiological adaptation to ionic stress for plants experiencing potassium deficiency.

Aims

The seaweed concentrate (Kelpak®) made from *Ecklonia maxima* (Osbeck) Papenfuss is known for its growth promoting effects on plants. These beneficial effects are currently attributed mainly to auxins and cytokinins that are known to be present in Kelpak®. However, their presence in SWC do not fully explain the significant growth promoting effect exerted by the SWC when administered to plants. The functions of PAs in plants correlate with the observed effects of SWC application. We have therefore turned our focus to PAs. Several studies have shown that marine algae contain appreciable amounts of PAs **(HAMANA and MATSUZAKI, 1982; BADINI *et al.*, 1994; LEE, 1998; MARIÁN *et al.*, 2000)**. Whether PAs are present within SWC's or are lost during the extraction process is unknown. This study was therefore primarily focused on answering the following questions: Are PAs present in Kelpak®?; Do PAs contribute to the growth promoting effect observed by Kelpak® application? The aims of this study were to:

- Characterize and quantify the PAs present in *E. maxima* and in the SWC prepared from *E. maxima*;
- To investigate if the PAs present in Kelpak® have an effect on the growth and development of plants; and
- To determine if the PAs in Kelpak® have the ability to overcome the detrimental effects of nutrient deficiency.

Chapter 2 - Seasonal variation of polyamines in *Ecklonia maxima* (Osbeck) Papenfuss and the seaweed concentrate Kelpak®

2.1. Introduction

The SWC Kelpak® made from *Ecklonia maxima* (Osbeck) Papenfuss is known for the growth promoting effect it has on plants. Although a variety of compounds have been discovered in the SWC, including hormones such as cytokinins and auxins, none of these fully explain the significant growth promoting effect exerted by the SWC when administered to plants. Other groups of compounds must be partly responsible for the growth stimulating effects observed from Kelpak® application. The focus of this study is polyamines (PAs), a group of bioactive compounds that might play a role in the growth regulating effect exerted by Kelpak®. The effects observed for PAs overlap with those found for the SWC prepared from *E. maxima* (Kelpak®) and thus PAs were studied to determine their possible role in plant growth regulation.

The aims of this part of the study were to determine the PA concentration and seasonal variation of the polyamines in *E. maxima*. The main objectives were to determine the concentrations of three common polyamines, putrescine, spermidine and spermine, in the fronds, stipes and SWC prepared from *E. maxima* on samples collected on a monthly basis over a 24 month cycle.

2.2. Materials and Methods

2.2.1. Acquisition and preparation of seaweed material and SWC for analysis

Ecklonia maxima material (stipes and fronds) and the liquid SWC marketed as Kelpak® were supplied by Kelp Products (Pty) Ltd. This company is situated in Simon's Town, Western Cape and has a license to harvest *E. maxima* from

Kommetjie, on the West coast of South Africa, for the commercial production of Kelpak®. Upon harvesting, the fronds and stipes of *E. maxima* were immediately washed, minced and vacuum-packaged at the factory and sent to the Research Centre for Plant Growth and Development, University of KwaZulu-Natal (Pietermaritzburg campus) within two days of harvesting. The SWC was prepared using a cold cell burst extraction procedure. During this process, stipes and fronds are minced into smaller pieces and passed through a high pressure chamber which creates potential energy in the seaweed material. The seaweed pieces are then passed through a low pressure chamber at a high velocity. The sudden change in pressure causes the energy contained within the seaweed pieces to be released, bursting the cell walls and thereby releasing the cellular contents. Thus an extract is obtained without the use of chemicals and heat. In total, 21 samples were received on a monthly basis from June 2009-June 2011. Immediately upon arrival in the Research Centre, the samples were dried as described below and stored at 10°C in plastic bottles.

The PA content of the *E. maxima* material (stipes and fronds) and SWC (Kelpak®) were analyzed using a Varian HPLC. The general method for the extraction of PAs from the SWC and from the dried seaweed material was the same except for some minor differences in terms of the mass of dried material used and subsequent changes made to the amount of chemicals used. The analytical method used for the identification and quantification of PAs was based on the method described by **FLORES and GALSTON (1982a)** and **VERKOELEN *et al.* (1988)** which includes the benzoylation of PAs in order to detect them on the HPLC system. It was necessary to modify the method for the *E. maxima* material due to the carrageenan (gelling compounds) present in the material which caused the extracts to gel so that they could not be filtered. The modified method is outlined in detail below.

2.2.2. Preparation and extraction of polyamines from the seaweed concentrate

The sample to be analyzed was standardized by measuring 20 ml Kelpak® sample into a centrifuge tube. The sample was frozen for 24 h at -10°C and then dried using

a freeze drier. After drying, the mass of the Kelpak® samples ranged between 450-600 mg. For each batch of SWC, three 100 mg replicates were analyzed.

For PA extraction, 10 ml 0.2 M perchloric acid ($HClO_4$) were added to 100 mg dried SWC powder. These samples were extracted in a fridge at 4°C for 1 h. After the extraction period, 10 ml 2 M sodium hydroxide (NaOH) were added to the extracts and left to react for 30 min at room temperature to allow the PAs to be deprotonated in solution. As the dry SWC dissolved completely, the centrifugation step was omitted from the method described by **FLORES and GALSTON (1982a)** and **VERKOELEN *et al.* (1988)**. To be able to detect the compounds with UV absorption, the PAs were benzoylated (Figure 2.1).

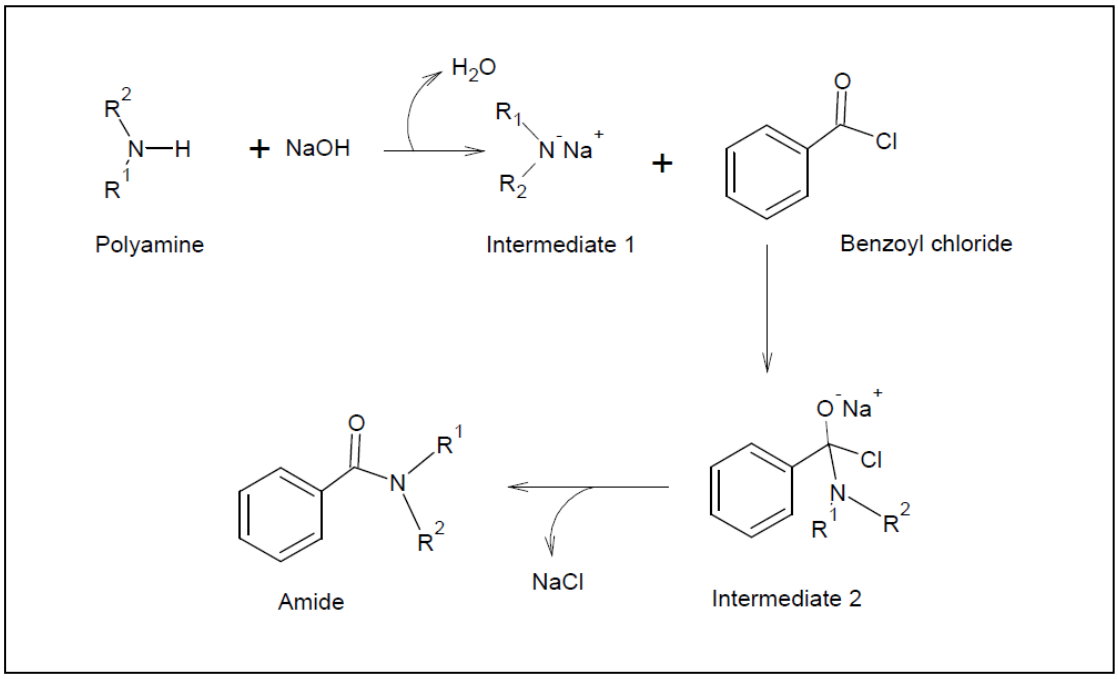

Figure 2. 1. Chemical reaction for the benzoylation of polyamines.

In order to detect PAs they are benzoylated to produce a benzamide analog which can be readily detected by UV monitoring on a HPLC system. The process involves adding NaOH and benzoyl chloride (Figure 2.2). Deprotonation of the PA with NaOH produces the sodamide intermediate 1, which nucleophylically attacks the benzoyl

chloride electrophile leading to intermediate 2 which expels NaCl to give the amide product **(LOUDON, 1995)**.

Benzoylation was achieved by adding 10 µl benzoyl chloride and shaking vigorously for 1 min. These samples were then left overnight at room temperature. The benzoylated PAs were then extracted by adding 5 ml chloroform. The samples were shaken vigorously to ensure that all the PAs dissolved into the denser chloroform layer. In order to separate the layers completely, the extract was centrifuged for 20 min at 614 *g* at 15°C (Beckman centrifuge Avanti J25I). The top aqueous layer was removed and discarded. As the chloroform layer could still contain impurities, 10 ml ultra-pure water were added and the extract shaken. These layers were separated again by centrifuging at 614 *g* at 15°C for 10 min. The denser (bottom) chloroform layer was then transferred to a small pill vial using a Pasteur pipette. This extract was dried completely under a flow of nitrogen and re-suspended in 300 µl 80% HPLC-grade methanol. Using a syringe filter system, the extract was filtered through a 0.22 µm Millipore membrane solvent filter, ready for HPLC analysis. This method is summarized in Figure 2.2.

2.2.3. Preparation and extraction of dry seaweed material

The frond and stipe seaweed material were frozen at -10°C immediately upon arrival from Kelp Products (Pty) Ltd. and then dried using a freeze drier. The dried material was then ground to fine powders in a mortar and pestle (using liquid nitrogen), to obtain a particle size of equal or less than 500 µm. Five hundred grams of dried stipe and frond powder were used per sample (3 replicates were tested per harvest). Samples turned into a gel as soon as it came into contact with water used to make the 0.2 M $HClO_4$ solution. This made it impossible to filter the extract through a 45 µm Millipore filter to remove the seaweed particles as stipulated in the methods of **FLORES and GALSTON (1982a)** and **VERKOELEN *et al.* (1988)**. When the normal ratio of liquid chemicals [5ml 0.2 M $HClO_4$ and 5ml 2 M NaOH] were used, the seaweed particles absorbed all the water and no extract could be recovered. To overcome this problem, double the amount of the liquid chemicals (10 ml 0.2 M

$HClO_4$ and 10 ml 2 M NaOH) were used during the extraction. To obtain an extract free of any particles, the samples were centrifuged at 15 344 *g* for 10 min at 15°C. The supernatant was transferred to another test tube to which 50 µl of benzoyl chloride were added. These tubes were shaken vigorously and left overnight. The rest of the procedure was as described above for the SWC extraction and is summarized in Figure 2.2.

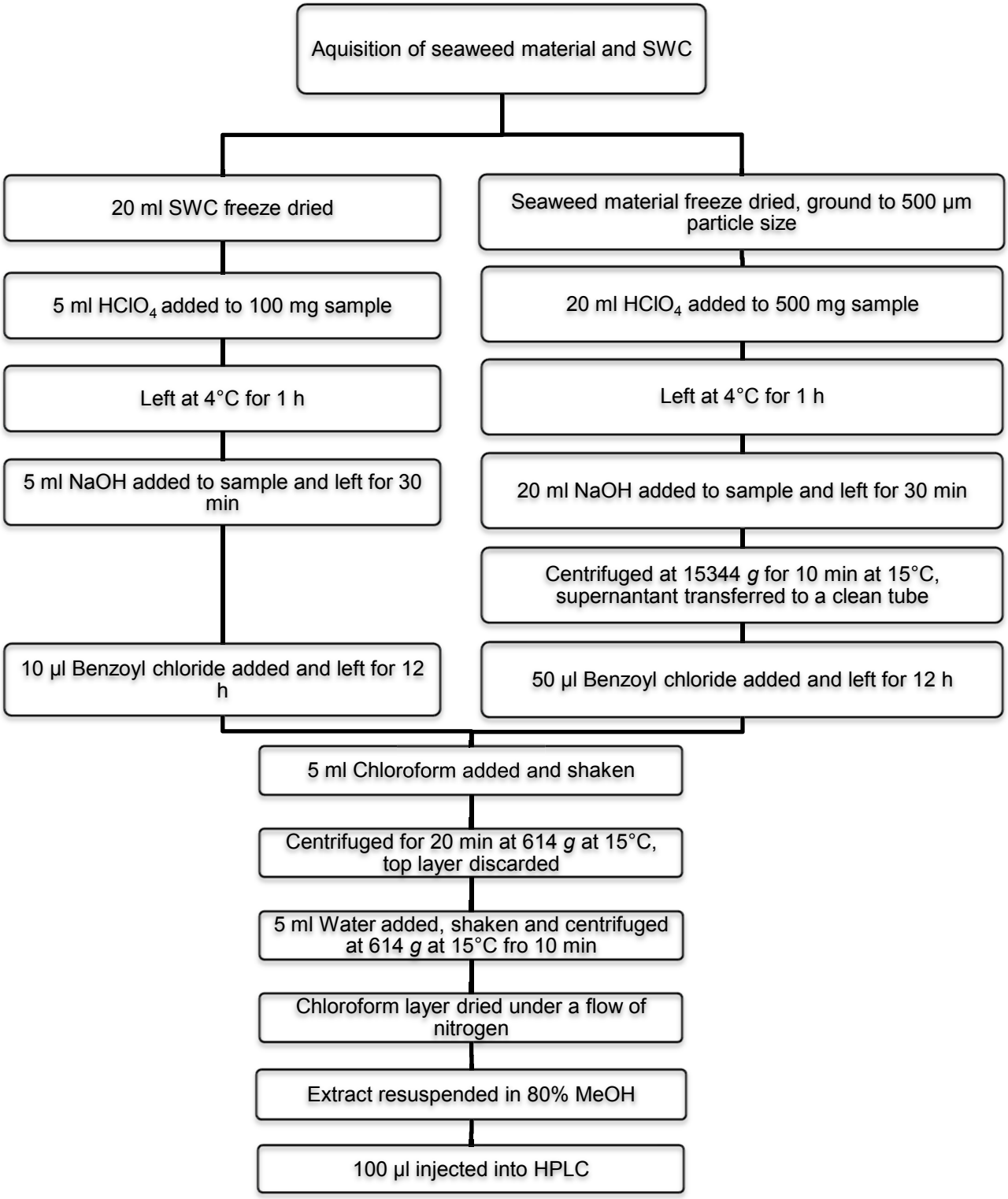

Figure 2. 2. Polyamine extraction procedure for the seaweed material and seaweed concentrate.

2.2.4. Preparation of the polyamine standard curves

The extraction procedure used for the standards was the same as described for the preparation and extraction of SWC, except that the washing with ultra-pure water was omitted. The standards used were as follows: putrescine dihydrochloride (161.07 $g.mol^{-1}$ Sigma®), spermine tetrahydrochloride (348.19 $g.mol^{-1}$ Sigma®) and spermidine (145.25 $g.mol^{-1}$ Sigma®). Standards were dissolved in ultra-pure water. Standard extraction curves for Put, Spd and Spm were constructed by injecting a range of concentrations (1×10^{-1} to 1×10^{-3} M) of each standard into the HPLC and then converting the milli absorption unit (mAU) values to the amount of compound present in each sample. By plotting the mAU values against their corresponding compound masses, a straight line graph was obtained (using Excel). From this, a straight line equation for each PA was calculated and this equation used to convert the mAU values for each sample **(VERKOELEN *et al.*, 1988)**.

2.2.5. HPLC analysis

One hundred microliters of the filtrate were injected (three replicates per sample) into a Varian HLPC. Separation of the PAs were achieved using a gradient elution program starting with 30% methanol: 70% TEA buffer (Appendix 1) changing to 100% methanol over 50 min. The flow rate was set at 1 $ml.min^{-1}$ through an Ultrasphere (250 mm × 4.6 mm) 5 µm ODS column. Absorption values were obtained using a ThermoFinnigan UV detector (UV 6000 LP) set at a wavelength of 254 nm. In order to establish that the Put, Spd and Spm peaks were the one's eluting at 19.6 min, 28.0 min and 30.1 min, respectively, the standards (with known concentration and corresponding mAU values) were added to certain samples during extraction. The increased size of the mAU peaks confirmed the presence of the polyamines in the extract.

2.3. Results

The retention times for the PA standards were 19.6 min, 28.0 min and 30.1 min for Put, Spd and Spm (Figure 2.3), respectively. Although some impurities were present in the stipe, frond and SWC extracts (Figure 2.4), these did not co-elute with the PA peaks. The stipes, fronds and SWC prepared from *E. maxima* collected over a two-year period were analyzed for PA content (Figure 2.5). Putrescine concentrations ranged from 15.98-54.46 $\mu g.g^{-1}$ in the stipe, 6.01-40.46 $\mu g.g^{-1}$ in the frond and 50.66-220.49 $\mu g.g^{-1}$ DW in Kelpak®. Spermine concentrations ranged from 1.02-35.44 $\mu g.g^{-1}$ in the stipe, 1.05-26.92 $\mu g.g^{-1}$ in the frond and 7.28-118.52 $\mu g.g^{-1}$ DW in Kelpak®. Although a standard curve was prepared for Spd, endogenous Spd was below the detection threshold in all the samples. Endogenous Put and Spm concentrations followed the same trend in the seaweed material throughout the two year period. There were two peaks annually with the highest concentrations for both Put and Spm recorded during winter (June to July) and the second peak during summer (October to February) in *E. maxima*. Putrescine and Spm concentrations were lowest during the months leading up to winter (March, April and May), followed by higher concentrations one month later (June) (Figure 2.5).

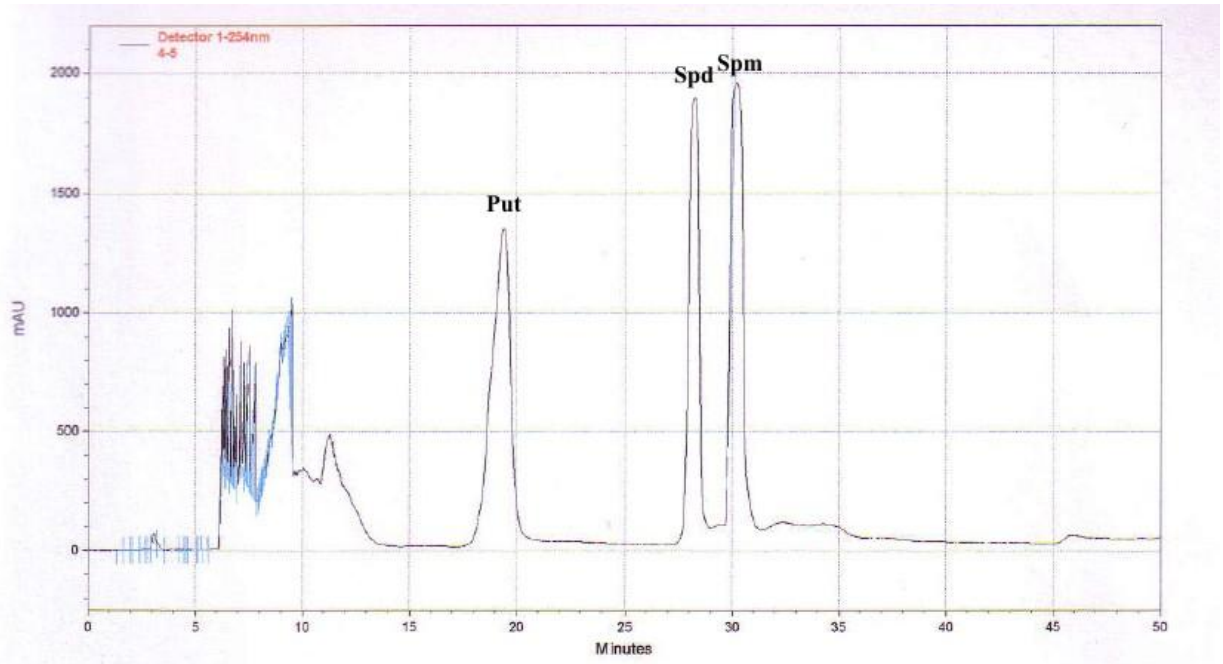

Figure 2. 3. Polyamine s tandards of pu trescine (29.81 µ g), spermidine (12.12 µg) a nd s permine (50.16 µg) eluting at 19.6, 28.0 and 30.1 min, respectively.

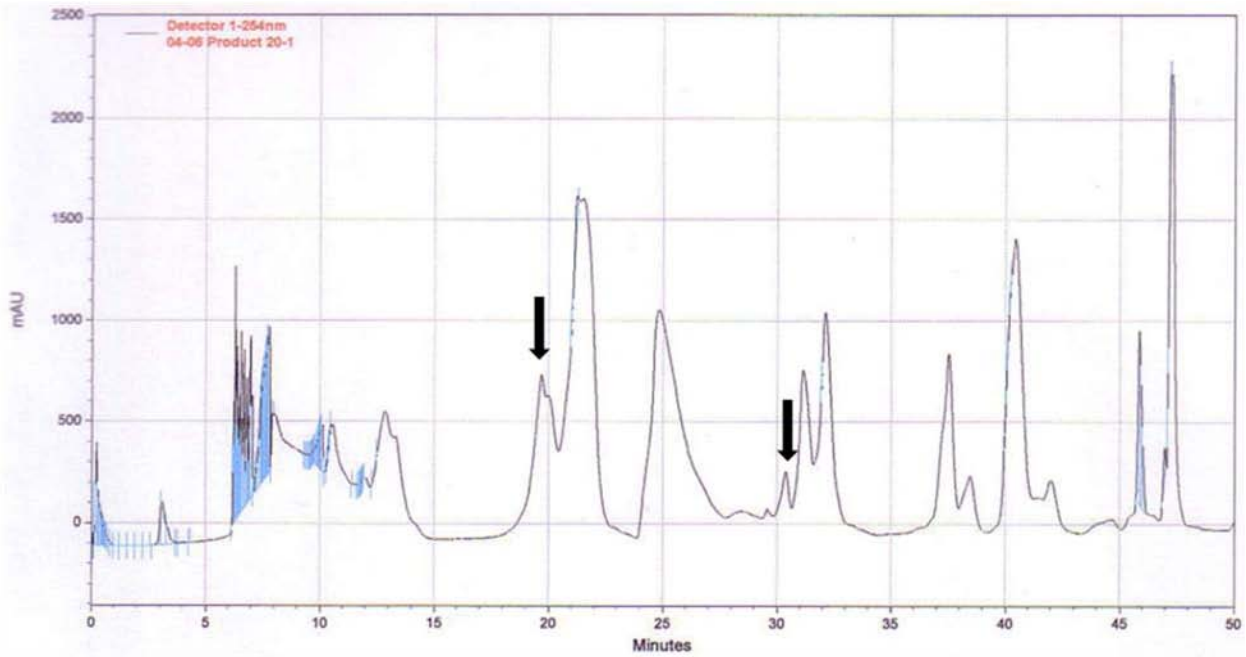

Figure 2. 4. HPLC sample of Kelpak® (harvested on 18 April 2011) showing putrescine (19.6 min) and spermine (30.1 min) (indicated with black arrows). No peak was detected for spermidine.

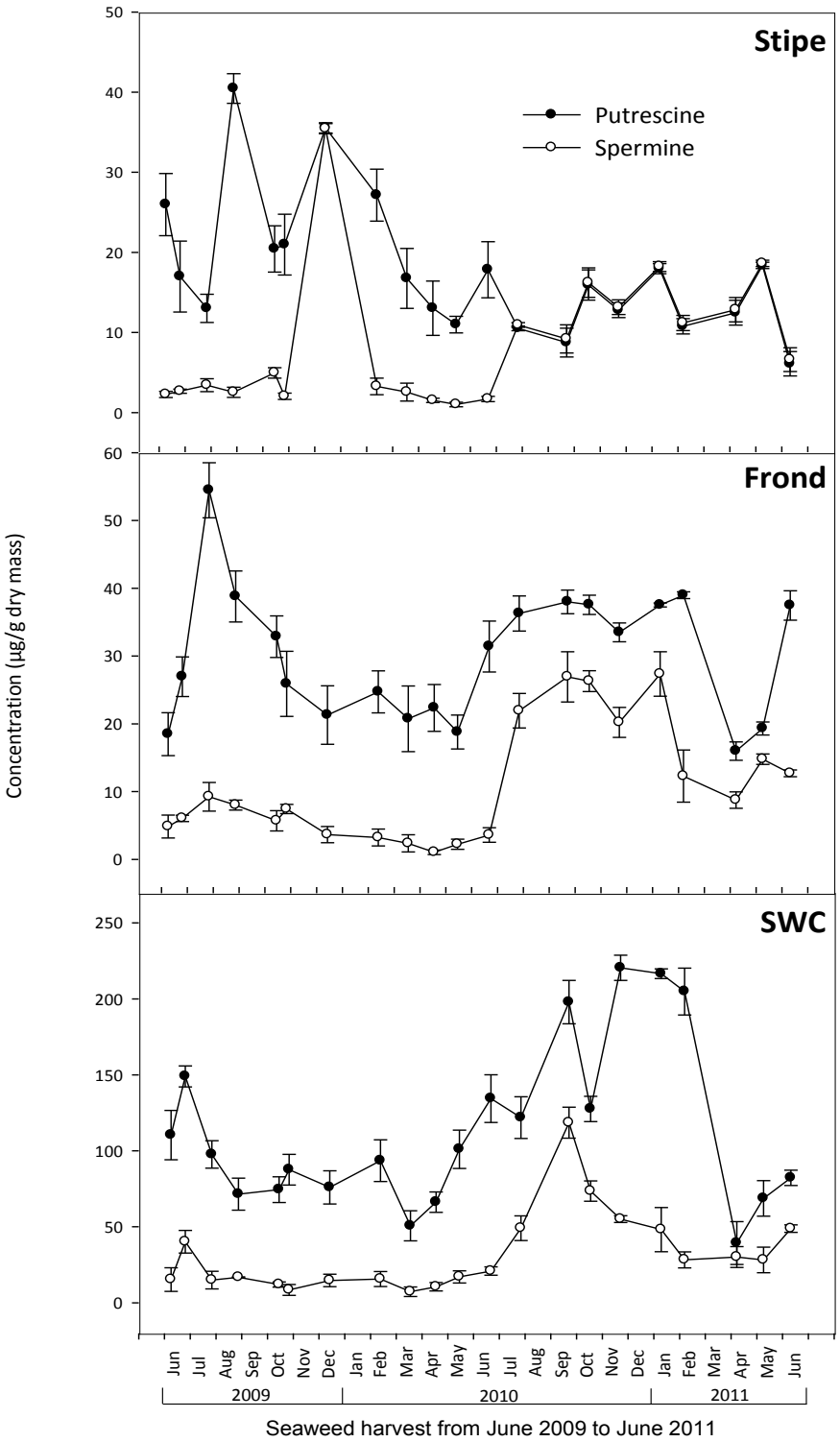

Figure 2. 5. Seasonal variation of the polyamines putrescine and spermine in the stipes, fronds and seaweed concentrate prepared from *E. maxima* over a two-year-cycle. Results are shown as mean ± SE (n=3).

The highest Put and Spm concentrations recorded for the stipe samples were 40.46 $\mu g.g^{-1}$ (August 2009) and 35.44 $\mu g.g^{-1}$ (December 2009), respectively. Putrescine and Spm concentrations within the fronds were slightly higher compared to the stipe with the highest concentrations being 54.46 $\mu g.g^{-1}$ (July 2009) and 27.35 $\mu g.g^{-1}$ (January 2011), respectively. The highest Put and Spm concentrations were 220.49 $\mu g.g^{-1}$ during December (2011) and 118.52 $\mu g.g^{-1}$ during September (2010), respectively.

Since Kelpak® is applied as a liquid, the amount of PAs present in the liquid SWC were summarized in Table 2.1. The Put:Spm ratio in the stipes and the fronds were 2:1 and 2.8:1 respectively (Table 2.2). The Put:Spm ratio in the SWC (3.6:1) were higher than the ratios established for the stipes and fronds. Although the difference was not that great, it does indicate that Spm breaks down quicker in the SWC than Put.

Table 2.1. Polyamine concentrations in the liquid seaweed concentrate Kelpak®

Sample	Put ($\mu g.ml^{-1}$)	Spm ($\mu g.ml^{-1}$)
Highest value	5.51 ± 0.21 (Dec 2011)	2.96 ± 0.25 (Sep 2011)
Lowest value	0.98 ± 0.35 (Apr 2011)	0.21 ± 0.089 (Oct 2009)
Average value	2.81 ± 0.27 (Jun 2009 to Jun 2011)	0.78 ± 0.13 (Jun 2009 to Jun 2011)

Table 2.2. Average polyamine ratios in the stipes, fronds and seaweed concentrate Kelpak®

Sample	Put : Spm
Stipes	2.1 : 1
Fronds	2.8 : 1
SWC	3.6 : 1

2.4. Discussion

The method described by **FLORES AND GALSTON (1982a)** and **VERKOELEN *et al.* (1988)** proved effective for extracting and quantifying PAs in the seaweed *E. maxima* and the SWC prepared from it. However, due to the carrageenan within the seaweed, the method had to be altered by increasing the amounts of solution used to obtain an extract. Only Put and Spm could be detected in *E. maxima* and Kelpak® with Spd falling below the detection threshold. Although the Put and Spm peaks were clear, there was some contaminants around the peaks in the chromatogram (Figure 2.4). This made the PA peaks shift slightly in some instances but not so much that the peaks co-eluted with other peaks.

Plant hormones are usually present at concentrations below 1 $\mu g.g^{-1}$ FW with ethylene and ABA being the exception, having much higher concentrations during particular environmental conditions. The amount of PAs within vascular plant tissue are higher, ranging from 1-800 $\mu g.g^{-1}$ (Put), 1-250 $\mu g.g^{-1}$ (Spd) and 0.5-150 $\mu g.g^{-1}$ (Spm) FW **(ALTMAN, 1989)**. Polyamine content may fluctuate between different tissues and organs in the same plant. The polyamine content of a plant may also be different during certain growth conditions and developmental stages **(ALTMAN, 1989)**. The PA concentrations obtained for *E. maxima* and Kelpak® from the present analysis falls well within the range of concentrations established for higher plants **(HARKESS *et al.*, 1992; BIONDI *et al.*, 1993)** and marine macroalgae (Table 2.3). Polyamine concentrations ranged from 218.8-747.1 $nmol.g^{-1}$ [(15.98-54.46 $\mu g.g^{-1}$)

Put] and 5.2-135.2 nmol.g^{-1} [(1.05-27.35 µg.g^{-1}) Spm] in the fronds and from 83.7-555.0 nmol.g^{-1} [(6.10-40.46 µg.g^{-1}) Put] and 5.0-175.2 nmol.g^{-1} [(1.02-35.44 µg.g^{-1}) Spm] in the stipes of *E. maxima*. This Put concentration was similar in range to concentrations reported by both **HAMANA AND MATSUZAKI (1982)** and **LEE (1998)** and slightly higher than concentrations reported by **MARIÁN *et al.* (2000)**. The Spm concentration in the stipes and fronds of *E. maxima* were similar to the concentrations found in the thallus of several green macroalgae species **(LEE, 1998)**. Spermine however, occurred in much higher levels in *E. maxima* than what has been reported for other macroalgae (Table 2.3). Both Put and Spm levels were much higher than reported by **BADINI *et al.* (1994)** for *Ulva rigida*. **HAMANA AND MATSUZAKI (1982)** evaluated a large group of eukaryotic algae for their PA content. The PA concentration within the Phaeophyta (brown seaweeds) ranged from 0.001-1.600 µmol.g^{-1} FW. In that study the highest PA levels were recorded for *Ecklonia cava*. It is evident from Table 2.3 that the relative PA concentration vary not only between the different seaweed divisions but also between closely related species and may also be influenced by time of harvest.

The PA concentrations measured in *Ulva rigida* (Chlorophyta) by **BADINI *et al.* (1994)**, were much lower than the concentrations found in other *Ulva* species by other research groups (Table 2.3). However, the ratio Put:Spm (1.7:1) was in the same order to the ratio established for the fronds (2.8:1) and stipes (2.1:1) of *E. maxima* (Table 2.2).

Table 2.3. Polyamine concentrations in the seaweeds from the divisions Phaeophyta (brown macroalgae), Chlorophyta (green macroalgae) and Rhodophyta (red macroalgae)

Material analyzed	Concentration (nmol.g^{-1})			Seaweed division	Species	Reference
	Putrescine	Spermidine	Spermine			
Fresh weight	1	1	1	Phaeophyta	*Undaria peterseniana*	(HAMANA and MATSUZAKI, 1982)*
	6	15	3		*Hizikia fusiforme*	
	1600	12	4		*Ecklonia cava*	
	119	3	2		*Myelophycus simplex*	
	457	4	4		*Padini arborescens*	
	372	6	0		*Sargassum thunbergii*	
	1407	7	6		*Sargassum fulvellum*	
	10	10	12	Rhodophyta	*Porhyra tenera*	
	1266	4	1		*Nemalion pulviratum*	
	1319	2	0		*Chondrococcus japonicus*	
	158	1	0		*Meristotheca papulosa*	
	147	1	1		*Gelidium amansii*	
	4	1	1		*Amphiroa dilatata*	
	1070	120	0	Chlorophyta	*Chlorella* sp.1	
	540	210	0		*Chlorella* sp.2	
	820	150	0		*Scenesdesmus* sp.	
	330	20	0		*Hydrodicton* sp.	
	370	30	10		*Enteromorpha prolifera*	
	760	2	4		*Ulva pertusa*	
	30	580	0		*Closterium* sp.	
	270	250	0		*Staurastrum* sp.	
	1000	10	0		*Stigeoclonium* sp.	
	1000	80	0		*Aegagropila sauteri*	
	100	170	0		*Spirogyra* sp.1	
	90	770	10		*Spirogyra* sp. 2	
	27	62	11		*Spirogyra* sp. 3	
	403	6	0		*Codium fragile*	

Dry weight	0.37-6.17	0.2-1.2	0.07-3.76	Chlorophyta	*Ulva rigida*	**(BADINI *et al.*, 1994)**
Dry weight	1100 700 450 1950 1800 2000 550	230 75 75 105 235 160 75	5 58 7.5 120 12 15 5	Chlorophyta	*Valoniopsis pachynema* *Chaetomorpha crassa* *Chlorodesmis caespitosa* *Boodlea composita* *Ulva reticulata* *Ulva lactuca* *Ulva fasciata*	**(LEE, 1998)**
Fresh weight	81 134 62	0.33 0.43 0.75	0.23 1.81 0.62	Phaeophyta Rhodophyta Rhodophyta	*Dictyota dichotoma* *Gelidium canariensis* *Grateloupia doryphora*	**(MARIÁN *et al.*, 2000)**

*Table adapted from **HAMANA and MATSUZAKI (1982)** . *The results were extracted from a larger study containing seaweeds from other divisions. Certain green macroalgae species were also omitted from the Table as they were not collected from the wild.*

This is the first report of PAs being detected in a SWC. The PA concentrations in the SWC Kelpak® ranged from 538.96-3024.55 nmol.g^{-1} DW (39.29-220.49 µg.g^{-1}) Put and 35.99-585.86 nmol.g^{-1} (7.28-118.52 µg.g^{-1}) DW Spm. Within every ml of Kelpak® there was an average of 2.81 µg.ml^{-1} Put and 0.78 µg.g^{-1} Spm (Table 2.1). The PA levels detected in Kelpak® were much higher than the PA levels in the fronds and the stipes of *E. maxima*. This can be attributed to the concentrating effect of the cold cell burst method which extracts the cellular content from all the cells in the seaweed material.

During the study period, two annual periods of high PA concentrations were observed (Figure 2.5). The first occurred during winter (June to August) and the second during late spring and early summer (October to December) with the latter having an extended period of high PA concentrations. Fluctuations in endogenous PA concentrations were also found in the green seaweed *Ulva rigida* with higher concentrations occurring during summer **(BADINI *et al.*, 1994)**. It was initially thought that the seasonal fluctuation in PA concentrations in *E. maxima* might be due to environmental factors. **TSENG (1981)** reported that temperature is one of the most important factors that determine growth in seaweeds. According to **TSENG (1981)**, every seaweed species has a maximal, optimal and minimal temperature for growth and development, therefore a change in water temperature could elicit a stress response in seaweeds. Polyamine concentrations were thus plotted against seawater temperatures (Figure 2.6). Water temperatures were the lowest (12°C) during late summer (January) and autumn (April). Contrary to preconceived ideas, the water temperature increased during winter and remained high until summer. During spring (September) when the water temperature was the highest (14.3°C), Put and Spm were also high (Figure 2.6). A sudden decrease in water temperature during summer (October) corresponded to the lower PA concentrations. The sudden decrease in temperature during October could have been caused by a cold Antarctic current **(STEGENA *et al.*, 1997)**. From Figure 2.6 it is evident, that PA levels do, to a large degree, correlate with water temperature.

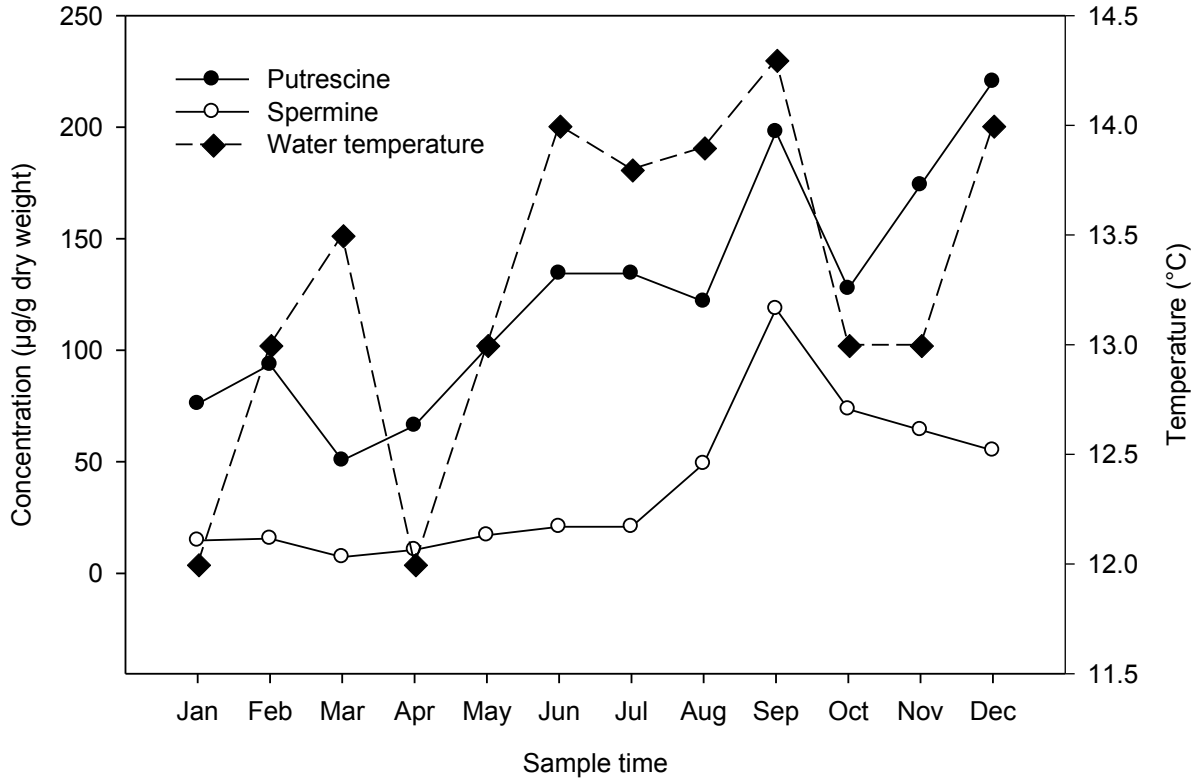

Figure 2. 6. Mean seawater temperatures at Kommetjie during 2007, the harvest site of *E. maxima* **(Courtesy of (LOURENS, 2011)/SOUTH AFRICAN WEATHER SERVICE)** and the seasonal variation of the polyamines putrescine and spermine in the seaweed concentrate prepared from *E. maxima* through an annual cycle.

Polyamines increase within plant tissue mainly due to stress **(LEE, 1998; BOUCHEREAU *et al.*, 1999; SUDHA and RAVISHANKAR, 2002; KUSANO *et al.*, 2008; MOSCHOU *et al.*, 2008)** and active growth **(GUZMÁN-URIÓSTEGUI *et al.*, 2002; VALERO *et al.*, 2002)**. In *E. maxima*, Put and Spm increased during the growing season when water temperatures were higher (Figure 2.6). This was expected due to the direct role PAs play in cell growth **(GALSTON and SAWHNEY, 1987)**. Seaweeds grow optimally at specific temperature ranges. This is largely due to the enzymes within the seaweed that function at an optimum temperature thereby increasing the metabolic rate of the seaweed **(LOBBAN *et al.*, 1985a)**. The low PA levels in *E. maxima* during March to April could therefore be due to the decrease in water temperature during this period which affects enzyme activity and thus ultimately growth.

PAPENFUSS (1942) found that the distribution of *E. maxima* was limited to the cooler waters between Port Nolloth and Papenkuilsfontein. The average water temperature (between 1992-2007) at Gansbaai (close to Kommetjie where the seaweed was harvested), was 13.29°C (Figure 2.6). *Ecklonia maxima* grows best in water where the temperature does not exceed 14.6°C **(PAPENFUSS, 1942)**. Water temperature decreases to 12°C during March and April and increased sharply by 2°C during the winter months (Figure 2.5). Considering that the yearly standard temperature variation was 0.76°C, it is possible that a sudden increase in water temperature, even as low as 2°C, will initiate an active growth period in *E. maxima*, thereby increasing the PA concentrations within the tissues.

Polyamine levels were high in summer which correlated well with the growing season of the seaweed. (Figure 2.5). Another growth limiting factor for seaweeds are the availability of nutrients which regulates the growth, reproduction and biochemistry of seaweeds **(DEBOER, 1981)**.The lower water temperature (13°C) during the summer is caused by off-shore South-Easterly winds. These winds direct water currents and in turn the flow of nutrient rich waters from the Antarctic into the kelp beds (upwelling) at the base of the Western Cape. Primary producers grow more rapidly in these nutrient rich conditions **(STEGENA *et al.*, 1997)**. Upwelled water that enter the kelp beds contain between 6-8 µM phosphate and 10-20 µM nitrate. The decomposition of plankton and debris which has sedimented out of the water column are the source of the nutrients in upwelled water **(STEGENA *et al.*, 1997)**. The increased nutrient flush together with the increased light conditions during this time explains why the seaweeds grow actively during the summer **(FEATONBY-SMITH, 1984)**.

Ecklonia maxima grows on the Western Cape coast which is in a winter rainfall area. The water motion on the coast is rough at this time of the year. The surface shear stress created by the back and forth movement of the water (surge) over the sea bed is strong enough to rip seaweed from the seabed. Although the water currents benefit the seaweed by bringing more nutrients and oxygen, excessive water motion can cause a stress response in the seaweed **(LOBBAN *et al.*, 1985b)**. The increased wave action that occurs during winter could therefore induce PA synthesis in *E. maxima*. The high PA levels in winter could therefore be

explained by the increased wave action and higher water temperatures causing stress conditions.

The trends established for Put and Spm in this study followed roughly the same pattern throughout the 24 month study period (Figure 2.5). The ratio of Put:Spm was almost constant throughout the study period (Table 2.2). The first eight samples were collected and freeze dried before the onset of analysis. The PA concentrations in these samples (Figure 2.5) were lower, suggesting that the PAs were degraded during storage. However, they still fitted the seasonal pattern established for the rest of the study period.

To date, studies concerning the growth regulators present in Kelpak® have focused on cytokinins and auxins. **MOONEY and VAN STADEN (1984b)** determined seasonal variation in the cytokinin-like activity in the reproductive laterals, vegetative laterals and the holdfast in *E. maxima*. The highest cytokinin-like activity was recorded for autumn (March), late winter (July to August) and summer (November). Lowest activity was found for middle winter (May, June, August and late summer (December). The PA pattern found in this study was similar to the cytokinin pattern described by **MOONEY and VAN STADEN (1984b)**. The main difference was that instead of a steep increase found in cytokinin-like activity during March, there was a decrease in PA concentration (Figure 2.5). Peaks in PA concentration overlapped with peaks found for cytokinin-like activity during July and November. Cytokinin-like activity and PA concentrations were low during winter (May-June). The simultaneous increase in both cytokinin-like activity and PAs indicate that these growth regulators might be linked in the growth stimulating processes. It is well known that growth regulators work synergistically to perform certain functions in plants. It has been suggested that PAs play a role as secondary hormone messengers **(SERGIEV *et al.*, 1995; KAKKAR and SAWHNEY, 2002)**. The presence of higher PA levels in actively growing tissues of *E. maxima* could induce cytokinin synthesis. Polyamine concentration might also increase as a direct response to the increased cytokinin levels, as has been shown by **SERGIEV *et al.* (1995)**. However, the pattern that the endogenous PAs follow during the life cycle of *E. maxima* as well as following

the same trend as the cytokinin-like activity suggests that these compounds may play a role in the hormone cascade during active growth.

The mechanism by which PAs elicit their function is still unknown. Just as terrestrial plants conform to their environment, the same holds true for seaweeds conforming to the rhythm of the ocean. The oceans and seaweeds are subjected to the lunar rhythm. **MOONEY and VAN STADEN (1984a)** reported that cytokinin-like activity in *S. heterophyllum* coincided with lunar rhythms, reaching high activity just before gamete release. Better insight might be obtained if the concentrations of PAs are monitored in conjunction to the lunar cycle. This could lead to a better understanding of the role of PAs in the physiology of seaweeds. The lunar cycle could then also be compared to the physiology of the seaweed during other periods in the life-cycle of the seaweed. However, this would be difficult to accomplish in the case of *E. maxima* as rough sea conditions often limit harvesting for weeks at a time.

Polyamines play a vital role in maintaining homeostasis within seaweeds. The fact that the PA levels fluctuated within *E. maxima* throughout the annual cycle suggest that they are synthesized and regulated according to the needs of the plant. The pattern found for PAs in *E. maxima* for the years 2009-2010 and 2010-2011 had overlapping general maxima and minima concentration peaks, which indicate that the requirements for PAs are different during the various seasons. The concentration of PAs within the fronds and stipes of *E. maxima* are similar to what has been established for other seaweeds.

This is the first report of PAs being present in a SWC. The beneficial effects of these PAs when the SWC is applied to crop plants is considered further in the following Chapters. Considering the specificity of seaweed to their niche environment and that the optimal water temperature for *E. maxima* is 14.6°C **(PAPENFUSS, 1942)**, a change in water temperature (due to global warming and melting of the ice caps) will have a serious effect on the growth of seaweed. Polyamine levels which are associated with periods of active growth, indicate that when water temperature decreases by 1°C, enzymes do not function optimally and growth is limited. Therefore, global warming might not just effect the growth of the seaweeds but could completely destroy seaweed communities.

Chapter 3 - Comparison of Kelpak® and polyamines on root growth

3.1. Introduction

Numerous studies have reported that Kelpak® increases rooting significantly in: tree seedlings **(ATZMON and VAN STADEN, 1994; VAN STADEN *et al.*, 1995; JONES and VAN STADEN, 1997)**, tomatoes **(FINNIE and VAN STADEN, 1985)**, cabbage **(ALDWORTH and VAN STADEN, 1987)**, mung beans **(CROUCH and VAN STADEN, 1991)**, wheat **(NELSON and VAN STADEN, 1986)**, barley **(STEVENI *et al.*, 1992)**, canola **(FERREIRA and LOURENS, 2002)**, wintergreen **(FEATONBY-SMITH and VAN STADEN, 1984)** and swiss chard **(FEATONBY-SMITH and VAN STADEN, 1983a)** indicating that there are active compounds within Kelpak® that increase rooting **(CROUCH and VAN STADEN, 1991)**. These root promoting compounds in Kelpak® were identified as auxins **(CROUCH and VAN STADEN, 1992)**. Since then, several auxins have been identified in seaweeds **(STIRK *et al.*, 2009; YOKOYA *et al.*, 2010)** and SWCs **(CROUCH *et al.*, 1992)** and are thought to be responsible for the root stimulating effect of the SWC.

It was initially thought that PAs do not have any effect on root growth **(FRIEDMAN *et al.*, 1982)**. A later study reported that auxins enhance PA synthesis and that they are required for auxin-stimulated root growth **(FRIEDMAN *et al.*, 1985)**. Auxin-stimulated root growth is accompanied by a doubling in Put levels and a 1.5 increase in Spd levels in mung beans **(FRIEDMAN *et al.*, 1982; BAIS and RAVISHANKAR, 2002)**. It was suggested that the endogenous PA content in plants might be sufficient for root growth and that the addition of PAs above the cellular PA content might be deleterious for growth **(FRIEDMAN *et al.*, 1985; WATSON *et al.*, 1998)**. Auxins enhance PA synthesis and are a prerequisite for auxin-stimulated root growth, with Put, Spm and Spd being required for the production of roots **(FRIEDMAN *et al.*, 1985)**. Rooting is inhibited by PA-inhibitors and can be restored by the reintroduction of PAs **(BAIS and RAVISHANKAR, 2002; COUÉE *et al.*, 2004)**. Putrescine applied in combination with auxin induced

significantly more roots in *Withania somnifera* cultured shoots that were already supplemented with only auxins **(SIVANANDHAN *et al.*, 2011)**.

It is clear that PAs are essential for auxin-stimulated root growth. How PAs elicit this function is still unknown. Kelpak® contain auxins and promotes root growth. Routine screening of Kelpak® samples in the mung bean bioassay shows that Kelpak®, produces more roots than individually applied auxins in the mung bean rooting bioassay. This could be attributed to the various auxins in Kelpak® that function in combination with other compounds to produce more rooting or to other compounds present in Kelpak®. The aim of this study was to compare the rooting effect of Kelpak® which contains both PAs (Chapter 2) and auxins **(STIRK and VAN STADEN, 1997)** to the response obtained when combinations of exogenous PAs and auxins are used.

3.2. Materials and Methods

The mung bean bioassay **(HESS, 1961)** was used to determine the effect of PAs on rooting. Mung bean, *Vigno mungo* L., seeds (250 g) were placed in plastic containers and surface sterilized by covering them with 3.5% sodium hypochloride for 20 min. The seeds were then thoroughly rinsed with tap water and imbibed for 6 h under a thin layer of water. Thereafter the imbibed seeds were scattered equally onto pre-moistened vermiculite in seedling trays. The seeds were covered with a thin layer of vermiculite. The seedling trays were placed in water trays in a Conviron growth cabinet at 25°C, 16 h light; 8 h dark and a light intensity of 115 $\mu mol.m^{-2}.s^{-1}$. Seedlings were left to germinate and grow in the Conviron for 10 days. Tap water was added to the water trays when necessary to keep the vermiculite moist.

After 10 days, uniform hypocotyl cuttings (12 cm in length) were prepared by removing the roots and cotyledons, leaving only the stem and two leaves intact. The pulse treatments consisted of 5 cuttings being placed into vials (90 × 24 mm) containing 20 ml (vials filled to 6 cm) of the respective test solutions for 6 h. The vials were placed 5 cm apart in trays in a Conviron and left at 25 ± 2°C at a light intensity of 115 $\mu mol.m^{-2}.s^{-1}$ for 6 h. The treatments were: distilled water, Kelpak®

(1, 2, 5, 10, 20, 50% dilution), indole-3-butyric acid (IBA), Put, Spm and Spd applied at 10^{-3}, 10^{-4}, 10^{-5} 10^{-6} and 10^{-7} M. In addition Put, Spm and Spd (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} M) treatments were also supplemented with 10^{-4} M IBA. The IBA solution container was covered with foil to limit light degradation of IBA. There were four replicates for every treatment (20 seedlings per treatment) with a total of 42 treatments.

After 6 h, the mung bean cuttings were removed from the treatment solutions, the stems gently rinsed with tap water and then transferred into vials containing tap water (20 ml, 6 cm). These were placed back into the Conviron for 10 days. The vials were filled with tap water as needed. After 10 days the number of roots formed were recorded. The experiment was conducted in triplicate (August 2010; September 2010 and February 2011). The results were analyzed using one-way ANOVA and means separated using Duncan's Multiple Range Test (Genstat® Fourteenth Edition). Standard errors of the means were calculated using Excel (Microsoft Office).

3.3. Results

The mung bean rooting bioassay is a well established and reliable bioassay to determine the root promoting ability of any compound that can be absorbed by the mung bean cuttings. The number of roots produced by the cuttings treated with PAs (Put, Spm and Spd) were not much higher than the water control regardless of the PA concentration (Figure 3.1 a, c and e). It was observed that the 10^{-3} M PA solutions were toxic, destroying the bottom 2-3 cm of the cuttings. This decreased root production, except for Put which produced more roots at 10^{-3} M than the other concentrations (Figure 3.1).

The rooting response of the mung beans to IBA was dose-dependent with the number of roots increasing with higher IBA concentrations with maximum rooting with 10^{-4} M IBA (Figure 3.1 g). The three PAs supplemented with 10^{-4} M IBA produced more roots than the non-supplemented PA solutions. The PA solutions were supplemented with 10^{-4} M IBA as this was found to be the optimum

concentration for root initiation. A synergistic effect was observed between Put and 10^{-4} M IBA, with these treatments producing significantly more roots than Put or IBA applied individually (Figure 3.1 f).

Treatment with Kelpak® produced a good dose-dependent rooting response in the mung bean cuttings with maximum rooting achieved with 20% Kelpak®. Higher Kelpak® concentrations were inhibitory (Figure 3.1 h). When the concentrations of each treatment that produced the best rooting response were statistically analysed, 20% Kelpak® and 10^{-3} M Put + 10^{-4} M IBA produced the highest average number of roots and was significantly higher than the other treatments (Figures 3.1 h and 3.2).

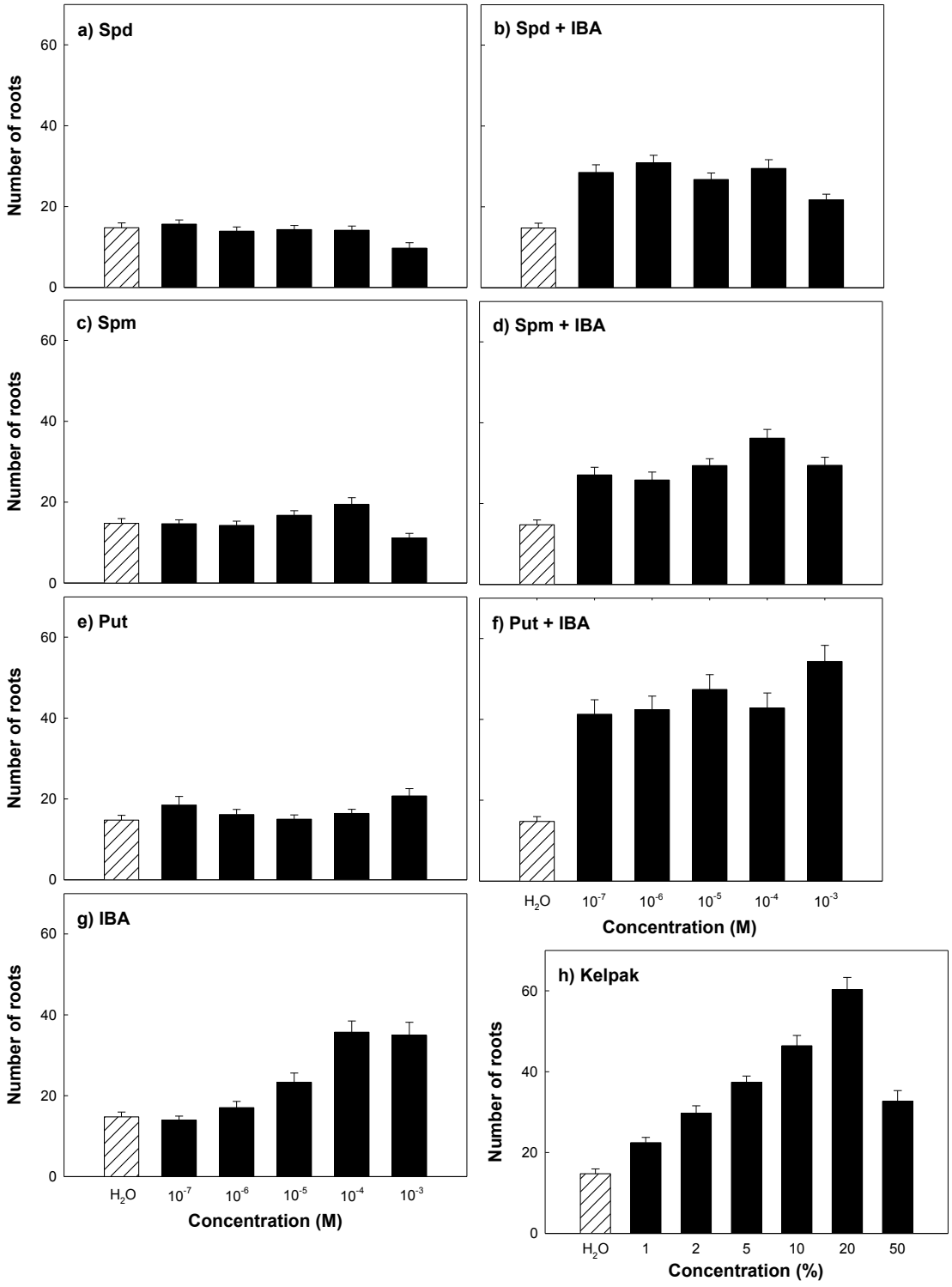

Figure 3. 1. The effect of polyamines, IBA, combinations of IBA and polyamines and Kelpak® on rooting in the mung bean rooting bioassay. Treatments were as follows: **a**-Spermidine, **b**-Spermidine + 10^{-4} M IBA, **c**-Spermine, **d**-Spermine + 10^{-4} M IBA, **e**-Putrescine, **f**-Putrescine +10^{-4} M IBA, **g**-IBA and **h**-Kelpak®.

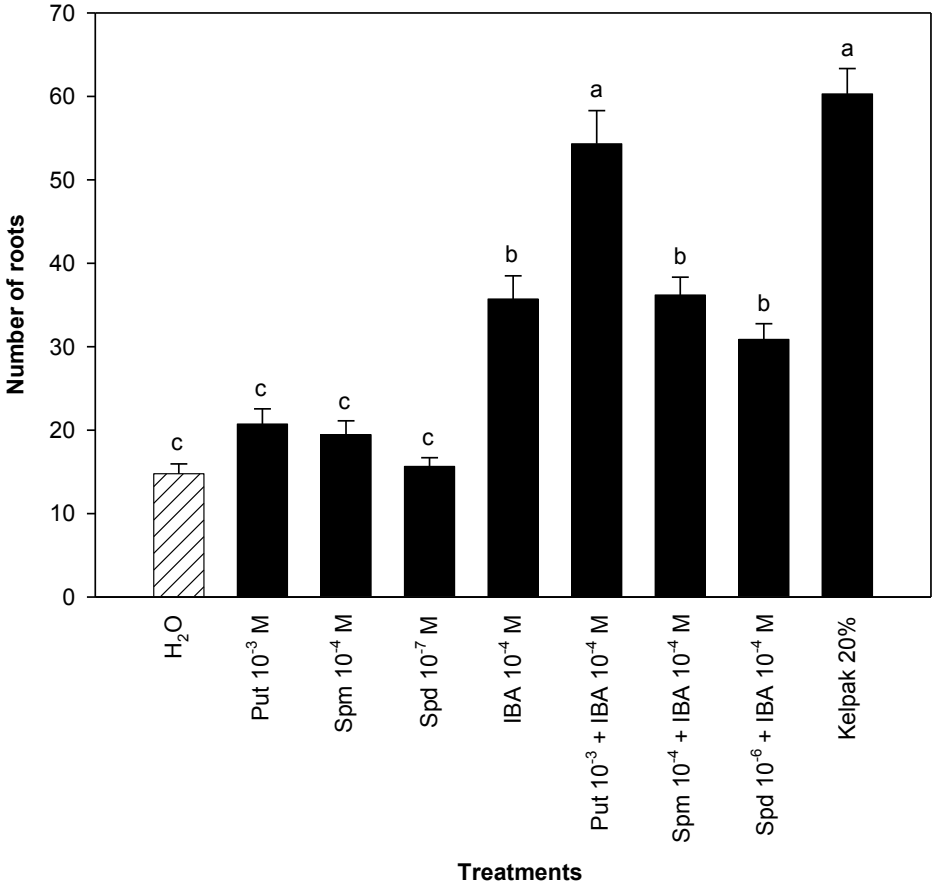

Figure 3. 2. Comparison between the best rooting values found for the polyamine treatments, IBA, combinations of polyamines and 10^{-4} M IBA and Kelpak®. Significant differences between values within each treatment are annotated by the letters a, b and c at $P<0.05$, calculated with Duncan's Multiple Range Test (n=60).

3.4. Discussion

Monthly screening of Kelpak® indicated that Kelpak® regularly produced more rooting in the mung bean bioassay than the IBA control. As Kelpak® contains several auxins, which are thought to be responsible for the root promoting effect of Kelpak®, the present experiments were conducted to investigate if PAs in combination with auxin produce significantly more roots. Kelpak® contains other compounds, such as PAs (Chapter 2) that might promote root growth.

Auxins are the main growth regulators that stimulate rooting **(HOPKINS and HÜNER, 2004)**. Although auxins require PAs to stimulate growth **(BAIS and RAVISHANKAR, 2002; COUÉE *et al.*, 2004)** high exogenous PA concentrations may be deleterious for growth **(FRIEDMAN *et al.*, 1985; WATSON *et al.*, 1998)**.

The inability of individually applied PAs to stimulate rooting was confirmed in this study (Figure 3.1 a, c and e). At high concentrations (10^{-3} M and 10^{-4} M) individually applied PA solutions were toxic, destroying the bottom 2-3 cm of the mung bean cuttings, although the cuttings did produce some roots above the damaged area. Indole-3-butyric acid produced significantly more roots than the water control. When Put and 10^{-4} M IBA were combined, a synergistic relationship was observed in the mung bean cuttings with 10^{-3} M Put + 10^{-4} M IBA producing an average of 54 ± 3 roots which were significantly more than when either 10^{-3} M Put (21 ± 2 roots) or 10^{-4} M IBA (36 ± 3 roots) were applied individually. Combining Spm and Spd with IBA did not produce significantly more roots than individually applied IBA (Figure 3.2). Why only Put showed this synergistic relationship with 10^{-4} M IBA is unknown.

HAUSMAN *et al.* (1995) reported that Put levels increased sharply with the addition of auxin to poplar tree shoots. It was suggested that Put functions as a secondary messenger, correlating with peaks in mitotic activity. The increase in PA content in the mung bean seedlings in the present study might have elicited a rooting response in the cuttings by either activating the genes for auxin synthesis or activating the auxins directly. Exogenously applied Put functioning as a possible hormonal secondary messenger could possible also have inhibited the process.

The increased rooting suggests that Put plays an active role in auxin-stimulated root growth. Auxins stimulate the formation of new root meristematic tissue along the mung bean stem. When the new meristematic tissue is formed, Put will function in increasing cell growth and differentiation. Since PAs play an important role in DNA replication during cell growth, it is possible that Put will influence root growth at the cellular level, thereby increasing the formation and growth of roots during auxin-stimulated root growth. More in depth studies will have to be conducted to determine the precise mode of action of PAs during auxin-stimulated root growth.

It is thought that the auxins in Kelpak® are partly responsible for its root-promoting ability. As, the Put-auxin combination produced almost the same number of roots as the Kelpak® treatment, it is likely that the PAs present in Kelpak® work

synergistically with auxins to produce roots. This would be difficult to test for, because removing PAs from Kelpak® eg. by heating, will destroy other growth regulators present in Kelpak®. The effect of the PAs in Kelpak® can be tested by inhibiting PA synthesis in the plants. If root growth recommences after Kelpak® application, it will indicate that Kelpak® has sufficient amounts of PAs to stimulate root growth.

Chapter 4 - Effect of Kelpak® and polyamines on okra seedlings grown in nutrient deficient conditions

4.1. Introduction

Kelpak® increases growth and yield in nutrient-stressed plants **(NELSON and VAN STADEN, 1984a; BECKETT and VAN STADEN, 1989**, **1990b; BECKETT *et al.*, 1994)**. Kelpak® significantly increased growth of greenhouse-grown cucumbers and tepary beans under a range of nutrient conditions **(NELSON and VAN STADEN, 1984a; BECKETT *et al.*, 1994)** and wheat receiving a low supply of nutrients **(BECKETT and VAN STADEN, 1989**, **1990b)**. Growth was only increased in lettuce plants when receiving double the amount of required nutrients **(CROUCH *et al.*, 1990)**. It must be noted that SWCs significantly increase growth and yield in wheat **(NELSON and VAN STADEN, 1984b**, **1986)** and lettuce **(ABETZ and YOUNG, 1983)** under normal field conditions. The simplest explanation of these reports is that different plants have different nutrient requirements and are therefore affected differently by SWC application. The nutrient requirements of a plant can be established by recording the nutrient status of the plant during growth and by understanding the function of every nutrient necessary for plant growth **(ULRICH, 1952)**.

It was suggested that the growth regulators in Kelpak® are responsible for the increased growth in plants experiencing nutrient-stress. The dose at which Kelpak® was administered was too low for the nutrients in the SWC to have any significant effect in relieving nutrient deficiency in the plants **(BECKETT and VAN STADEN, 1990a)** and thus cytokinins were considered responsible for the increased growth of nutrient-stressed plants **(BECKETT and VAN STADEN, 1990a)**. However, other growth regulators that may be present in SWCs may also alleviate the nutrient-stress experienced by the plants **(BECKETT and VAN STADEN, 1989)**. The aims of this part of the study were therefore to establish if Kelpak® which was previously shown to contain PAs (Chapter 2) and exogenously

applied PAs are able to alleviate the stress caused by nitrogen-, phosphorous- and potassium-deficiency on okra, *Abelmoschus esculentus*, seedlings.

4.2. Materials and Methods

The experiment was conducted in February 2011 in a greenhouse located at the University of KwaZulu-Natal Botanical Gardens, Pietermaritzburg Campus. *Abelmoschus esculentus* (okra-Clemson spineless lot no: YR 025 AY) seeds were purchased from McDonald's Seed Company, Pietermaritzburg, South Africa and stored in plastic containers containing silica gel at 4°C until planted. One seed was planted per pot (10 cm pot diameter, 250 ml) containing sterile quartz sand. Pots were randomly arranged on metal benches 1 m from the ground. Seedlings were subjected to natural light conditions at 20-30°C.

The experiment was designed to determine the effect of Kelpak® and PAs on okra seedlings treated with half-strength (50%) Hoagland's nutrient solution (HS) **(HOAGLAND and SNYDER, 1933)** and individually applied HS without nitrogen (N), phosphorous (P) or potassium (K). The concentrations of N, P and K in 50% HS were 253, 20.4 and 96.4 $g.l^{-1}$, respectively. The sources of N, P and K were removed and replaced with alternative chemicals. Following the Hoagland's recipe, stock solutions were prepared using macro- and micro-nutrient chemicals. The nitrogen sources, calcium nitrate [$Ca(NO_3)_2$] and potassium nitrate (KNO_3), were replaced with calcium chloride ($CaCl_2$) and potassium chloride (KCl) at 110.27 and 55.9 $g.l^{-1}$, respectively. The phosphorous source, potassium dihydrogen orthophosphate (KH_2PO_4), was replaced with KCl at 55.9 $g.l^{-1}$. The potassium sources, KNO_3 and KH_2PO_4, were replaced with sodium nitrate ($NaNO_3$) and sodium dihydrogen phosphate (NaH_2PO_4) at 63.74 and 20.69 $g.l^{-1}$, respectively. To make 50% HS, 5 ml were taken from each Hoagland's stock solution and made up to 1.5 l. The N- and K-deprived solutions were prepared by taking 5 ml from the replacement solutions. For the P-deprived solution, 5 ml KH_2PO_4 was replaced with 1 ml KCl. Treatments were represented as -N, -P and -K.

The quartz sand was kept moist with automated sprinklers, watering for 10 min every day for 8 weeks. The four basic treatments were 50% HS, 50% HS deficient in N (HS-N), 50% HS deficient in P (HS-P) and 50% HS deficient in K (HS-K) which served as the controls. In addition, seedlings were either treated with the basic treatment supplemented with Kelpak® made up to an effective concentration of 0.4% or with the basic treatment supplemented with the PA solution made up to an effective concentration of 10^{-4} M. The standards used to make the 10^{-4} M PA solution were as follows: putrescine dihydrochloride (161.07 $g.mol^{-1}$ Sigma®), spermine tetrahydrochloride (348.19 $g.mol^{-1}$ Sigma®) and spermidine (145.25 $g.mol^{-1}$ Sigma®). There were 11 seedlings per treatment. This gave a total number of 12 treatments (test solutions). Seedlings were treated with 50 ml of each solution, twice a week.

Seedlings were harvested after eight weeks and various growth parameters recorded. Shoot and root length were measured and number of roots counted. The number of leaves were counted and the leaf area measured using a leaf area meter (LI-3100, LI-Cor Inc.). The fresh weights of the stems and roots were recorded while also recording stem diameter. The dry weight of the stems and roots were measured by drying the material in an oven at 35°C. The dry mass of the stems and roots were recorded three weeks later when all the material was completely dry.

Seedling vigour was determined using the seedling vigour index (SVI) indicator **(KULKARNI *et al.*, 2007)**. Seedling vigour index was recorded from data from week 8, SVI = [stem thickness (mm)/seedling length (mm) + root fresh weight (g)/shoot fresh weight (g)] × [shoot fresh weight (g) + root fresh weight (g)]. The results were analyzed using one-way ANOVA and means separated using Duncan's Multiple Range Test (Genstat® Fourteenth Edition). Standard errors of the means were calculated using Excel (Microsoft Office).

4.3. Results

From a visual inspection of the seedlings (Figure 4.1), definite differences were observed between the control seedlings and seedlings treated with Kelpak® and PAs. Growth of seedlings deficient in N were severely stunted. The leaves were small and light green to yellow in color (Figure 4.1b). The growth of the P-deficient seedlings (control) were stunted, leaves were smaller, started yellowing from the edges and showed signs of necrosis (Figure 4.1c). The control seedlings deficient in K appeared stunted and leaves had a blotchy appearance (Figure 4.1d).

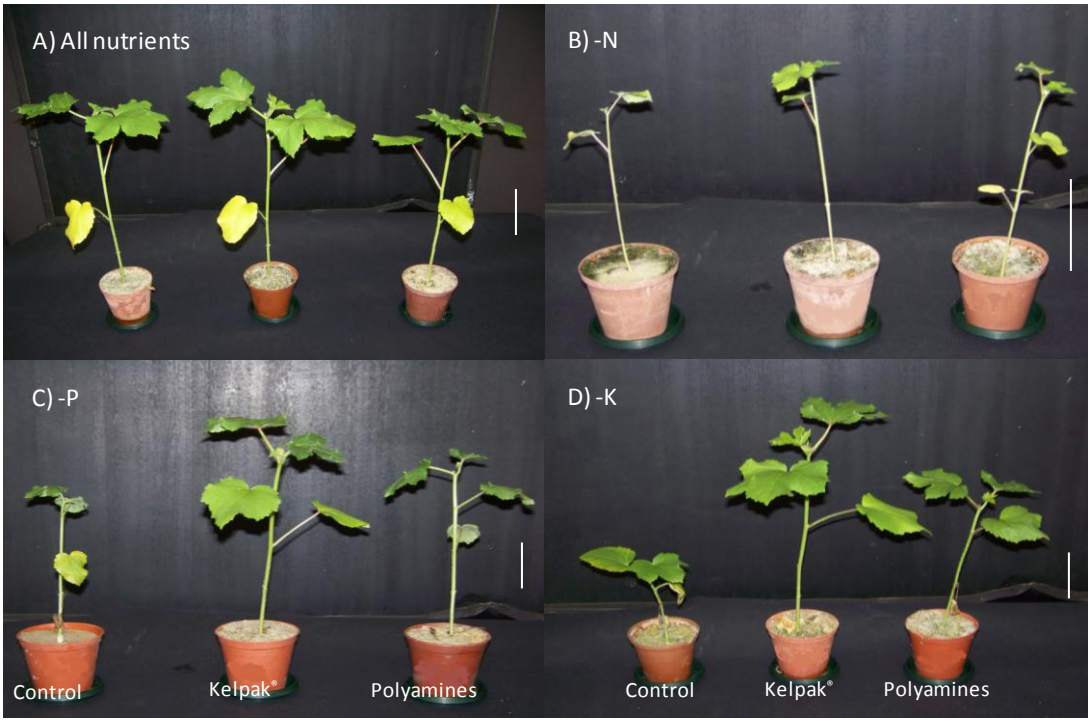

Figure 4. 1. The effect of 0.4% Kelpak® and 10^{-4} M PAs on okra, *Abelmoschus esculentus*, seedlings in relieving nutrient deficiencies (-N, -P and -K) after 8 weeks growth. Scale bars = 100 mm.

Only root dry weight was significantly higher than in the controls when treated with PAs. Highest values, for all the growth parameters tested, were recorded for all the Kelpak® treatments even if it was not significantly different from the control (Table 4.1). Neither the Kelpak® treatment nor the PA treatment had any significant effect on N-deficient seedlings compared to the control treatments. Kelpak® and PA application had no significant effect on okra seedlings grown in adequate nutrient conditions (50% HS) apart from the PA treatment increasing

root dry weight (Table 4.1). Kelpak® significantly increased all the growth parameters (root length for P deficiency being the only exception) for the P- and K-deficiency treatments (Table 4.1).

Kelpak® and PAs had no significant effect on the seedling vigour index of seedlings receiving an adequate supply of nutrients (50% HS Figure 4.2a). However, Kelpak® treatment significantly increased seedling vigour of seedlings subjected to the different nutrient deficiencies. Seedling vigour was also significantly higher in N-deficient seedlings treated with PAs but not in the -P and -K treatments.

Table 4. 1. The effect of 0.4% Kelpak® and 10^{-4} M polyamines (combination of Put, Spd and Spm) on growth parameters of okra (*Abelmoschus esculentus*) seedlings deprived of either nitrogen (-N), phosphorous (-P) or potassium (-K)

Treatment	Shoot length (mm)	Root length (mm)	Leaf (no.)	Root (no.)	Stem thickness (mm)	Shoot fresh weight (g)	Root fresh weight (g)	Shoot dry weight (mg)	Root dry weight (mg)	Leaf area (cm^2)
All nutrients										
Control	282 ± 19 a	192 ± 11 a	4.5 ± 0.1 a	21 ± 1.7 a	3.6 ± 0.2 a	9.1 ± 1.1 a	4.4 ± 0.4 a	1170 ± 176 a	308 ± 35 b	46 ± 5 a
Kelpak (0.4%)	313 ± 19 a	182 ± 15 a	4.3 ± 0.1 a	27 ± 2.5 a	3.6 ± 0.2 a	9.4 ± 0.9 a	4.7 ± 0.5 a	1370 ± 160 a	382 ± 40 b	44 ± 4 a
Polyamines (10^{-4} M)	289 ± 31 a	193 ± 20 a	4.6 ± 0.4 a	25 ± 3.3 a	3.8 ± 0.4 a	10.4 ± 1.4 a	5.4 ± 0.7 a	1491 ± 222 a	411 ± 61 a	42 ± 4 a
No nitrogen (-N)										
Control	173 ± 20 a	146 ± 21 a	2.7 ± 0.3 a	16 ± 1.8 a	1.8 ± 0.2 a	1.1 ± 0.1 a	0.84 ± 0.11 a	171 ± 22 a	60 ± 8 a	11 ± 4 a
Kelpak (0.4%)	210 ± 21 a	175 ± 15 a	3.1 ± 0.1 a	17 ± 2.0 a	2.0 ± 0.1 a	1.9 ± 0.2 a	1.32 ± 0.18 a	258 ± 32 a	97 ± 12 a	16 ± 4 a
Polyamines (10^{-4} M)	202 ± 24 a	147 ± 18 a	3.1 ± 0.3 a	16 ± 2.3 a	1.9 ± 0.2 a	1.9 ± 0.3 a	1.2 ± 0.19 a	290 ± 44 a	87 ± 13 a	10 ± 1 a
No phosphorus (-P)										
Control	213 ± 12 b	188 ± 10 a	2.7 ± 0.2 b	22 ± 1.6 b	3.3 ± 0.2 b	3.4 ± 0.4 b	2.0 ± 0.2 b	452 ± 53 b	164 ± 19 b	17 ± 2 b
Kelpak (0.4%)	300 ± 4 a	187 ± 9 a	4.5 ± 0.1 a	31 ± 1.1 a	4.1 ± 0.1 a	9.2 ± 0.4 a	5.5 ± 0.3 a	1511 ± 86 a a	470 ± 26 a	36 ± 1 a
Polyamines (10^{-4} M)	216 ± 28 b	192 ± 24 a	2.6 ± 0.3 b	23 ± 3.3 b	2.7 ± 0.3 b	4.0 ± 0.6 b	2.7 ± 0.4 b	538 ± 95 b	216 ± 37 b	16 ± 2 b
No potassium (-K)										
Control	161 ± 21 b	179 ± 21 a	3.6 ± 0.3 a	18 ± 2.8 b	2.8 ± 0.3 b	3.7 ± 0.6 a	2.2 ± 0.4 b	570 ± 111 b	168 ± 35 b	24 ± 3 b
Kelpak (0.4%)	283 ± 18 a	195 ± 7 a	4.0 ± 0 a	32 ± 3.5 a	3.7 ± 0.3 a	9.2 ± 1.3 b	6.0 ± 0.5 a	1641 ± 238 a	505 ± 59 a	46 ± 4 a
Polyamines (10^{-4} M)	168 ± 25 b	191 ± 22 a	3.8 ± 0.4 a	24 ± 3.1 b	2.7 ± 0.3 b	4.5 ± 0.7 a	3.0 ± 0.5 b	672 ± 121 b	235 ± 42 b	26 ± 4 b

Data are the means of 11 replicates ± SE. Significant differences between values within each treatment is annotated by the letters a and b at P<0.05, calculated with the Duncan's Multiple Range Test

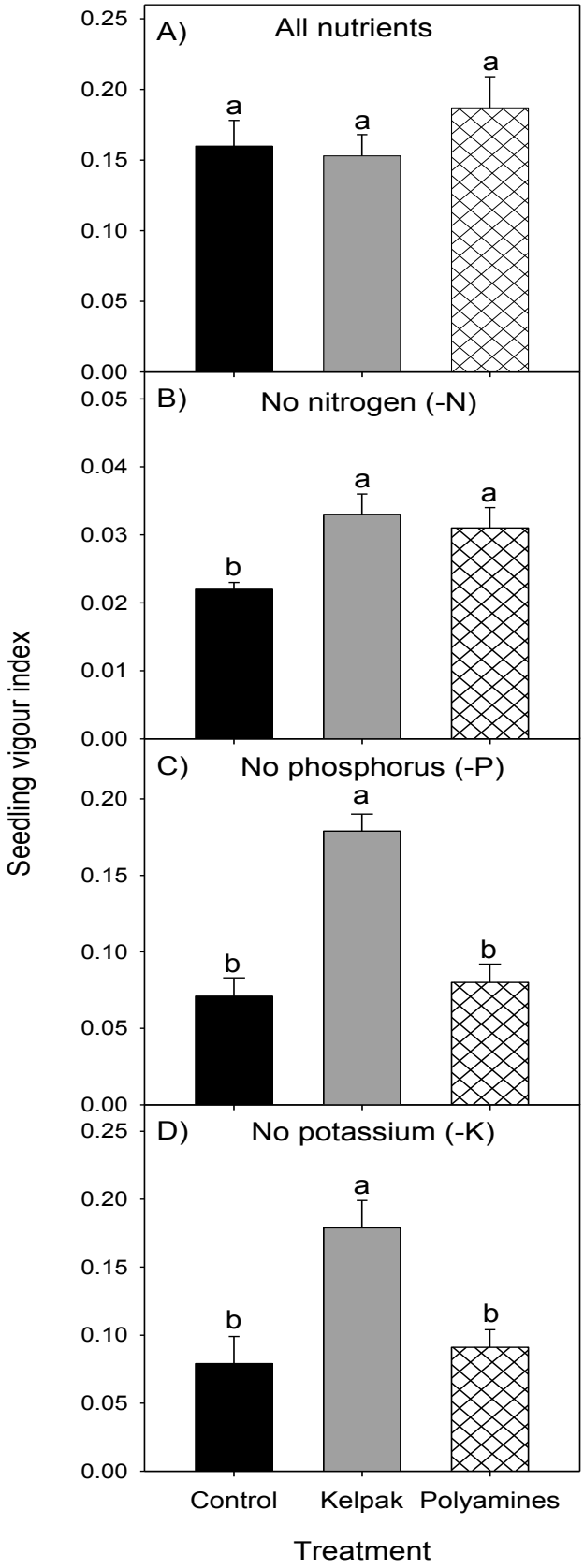

Figure 4. 2. Effect of 0.4% Kelpak® and 10^{-4} M polyamines on the Seedling Vigour Index (SVI) of okra, *Abelmoschus esculentus*, seedlings deprived of nitrogen (-N), phosphorous (-P) and potassium (-K). Significant differences between values within each treatment is indicated by the letters a and b at $P<0.05$, calculated with the Duncan's Multiple Range Test (n=11).

4.4. Discussion

Studies concerned with nutrient deficiency are usually conducted with weak nutrient solutions, still containing the nutrients being tested for. In this study, however, the nutrients of the nutrient deficiencies being tested for were completely removed from the nutrient solution (as described in the Methods and Materials section of this Chapter). The rationale behind this was to investigate if Kelpak® and/or PAs were able to lower the nutrient requirements of okra and to determine if the solutions were able mask the detrimental effect associated with the various nutrient deficiencies. From a visual inspection, Kelpak®-treated plants appeared healthier and did not show nutrient-deficiency-symptoms (Figure 4.1). Although PA-treated seedlings appeared to be healthier and larger, almost no significant differences were recorded in the measured growth parameters for PA treatment compared to the control treatments (Table 4.1).

Kelpak® increased yield and nitrogen levels in tepary beans under low nutrient levels **(BECKETT *et al.*, 1994)** but the increase in N levels could not be explained. It is possible the increase in N levels may have been a result of N-containing growth regulators (auxins and cytokinins) within the SWC that were absorbed by the plants. Polyamines, which contain N, have been quantified in Kelpak® (Chapter 2) and could be supplying the seedlings with nitrogen. Organic molecules such as organic acids, methionine and even PAs in SWCs could also increase nutrient absorption in plants by chelating to the nutrients, thereby increasing their absorbance **(LYNN, 1972) as cited by (CROUCH *et al.*, 1990)** or indirectly by increasing the absorptive area of the roots by stimulating root growth.

The nutrients present in undiluted Kelpak® are very low (Table.4.2) and even lower at a 0.4% dilution, as used in the current experiment. At this rate of application, Kelpak® treatment only contributed 80 µg, 60 µg and 140 µg N, P, and K per application (Table 4.2) to the total nutrient status of the seedlings, respectively. Thus the amount of nutrients in Kelpak® are insufficient to relieve the nutrient stresses. The increase in growth and seedling vigour are therefore attributed to the growth regulators present in Kelpak®. Another reason for the high SVI for the Kelpak® treatment (Figure 4.2) is that the increased root growth

increased the absorptive area of the roots of the seedlings thereby increasing absorption of the limited nutrients available.

Determining the nutrient requirements of plants is not an exact science. Generally, the nutrient requirements of plants are estimated by quantifying the amount of nutrients within the plant. The amount of N, P and K required by okra plants are 4.95%, 0.49% and 1.41% of the total mass of the plant **(AKANDE *et al.*, 2010)**. Values were quantified from okra plants receiving N, P and K fertilizer at the rate of 20:10:10, respectively. Taking the average seedling weight as 13.5 g (50% HS treatment) and using the values reported by **AKANDE *et al.* (2010)**, the amount of N, P and K required by the 8-week-old okra seedlings were 670 mg, 70 mg and 190 mg, respectively. The amount of N, P and K supplied by the 0.4% Kelpak® treatment during the full duration of the experiment were 1.28 mg, 0.96 mg and 2.24 mg, respectively (neglecting nutrient loss during application). Therefore, the amount of nutrients potentially supplied by Kelpak® was too low to meet the nutrient requirements of the okra seedlings. Kelpak® treatment reduced the amount of nutrients required by the okra seedlings and effectively masked the deficiency symptoms and growth limiting effect of P- and K-deficiency.

Table 4. 2. Nutrient concentrations in the seaweed concentrate Kelpak® **(data supplied by Dr Riaan Lourens, Kelpak® Technical Manager)**

Nutrient	Amount (%; m : v)	Mass (mg) per 1000 ml Kelpak®	Mass (mg) per 1000 ml 0.4% Kelpak®	Mass (µg) per 50 ml Kelpak®	Total mass (mg) received during the trial
N	0.04	400	1.6	80	1.28
P	0.03	300	1.2	60	0.96
K	0.7	7000	28.0	140	2.24

Although the role of PAs during nutrient stress is unknown, Put synthesis is induced by K-deficiency **(YOUNG and GALSTON, 1984)**. The exogenous application of PAs increased the SVI of N-deficient okra seedlings (Figure 4.2). These compounds are known for their ability to stimulate cell activity and could

have increased the growth in the roots and/or stems. In addition, the PAs that were absorbed by the seedlings, may have been degraded and the free N assimilated into proteins and other nitrogen containing compounds. The SVI of the Kelpak® treatment was similar to that of the PA treatment (Figure 4.2). The implication of this is that the PA present in the SWC as well as some other amino acid- and N-containing compounds could have been broken down and used by the seedlings, increasing the SVI of the N-deficient seedlings (Figure 4.2).

Several studies have shown that nutrient supply affects cytokinin export from the roots to other plant parts. Cytokinin export decreased in potato plants subjected to N-deficiency **(SATTELMACHER and MARSCHNER, 1978)**, tomato plants subjected to P-deficiency **(MENARY and VAN STADEN, 1976)** and in sunflower subjected to P- and K-deficiency **(EL-D *et al.*, 1979)**. The okra seedlings, deficient in all three of the nutrients, would therefore have a limited distribution of cytokinins. Kelpak® serves as an alternative supply of cytokinins to the okra seedlings, thereby lowering their cytokinin requirement.

Kelpak® increased the SVI and most growth parameters of okra seedlings deficient in N, P and K (Table 4.1). In reality, soil may become low in certain nutrients but very seldom will it be completely depleted of the nutrients. This being a worst case scenario, Kelpak® was effective in relieving P and K deficiency and also increased the SVI of N-, P- and K-deficient seedlings (Figure 4.2). Kelpak® application will alleviate P- and K-deficiency in the field and render the plants healthier. The PA solution was only able to significantly increase root dry weight (Table 4.1) and the SVI of N-deficient seedlings (Figure 4.2). The PA solution did, however, produce the highest values for most of the growth parameters for okra seedlings receiving an adequate supply of nutrients. It was reported that PAs do not increase rooting directly **(FRIEDMAN *et al.*, 1982)**, but rather work together with auxins to stimulate root growth **(FRIEDMAN *et al.*, 1985)**. The normal levels of auxins in the roots could therefore have benefitted from the extra supply of PAs, thus increasing growth (Table 4.1). Increased root growth increased the amount of resources available for growth thus increasing shoot growth (1491 mg ± 222 mg DW) above the control (1170 mg ± 160 DW) and Kelpak® treatment (1370 mg ± 176 mg DW) (Table 4.2).

Except for their function during cellular growth in actively dividing tissues of seedlings receiving an adequate supply of nutrients and supplying N to N-deficient seedlings, PAs had no real effect in relieving N-, P- and K-deficiencies. Polyamines seem to function with auxins (Chapter 3) especially when sufficient nutrients are available. The PAs present in Kelpak® should function in the same way. Auxins play a vital role in Kelpak®-stimulated growth. The effect of auxins could be enhanced by the PAs found in Kelpak®. Although Kelpak® contain PAs, it remains a weak source of N and should not be applied to seedlings as a source of N. Seedlings grown in P- and K-deficient soils will benefit from Kelpak® treatment. The seedlings will absorb more nutrients present in low concentrations. It is recommended that Kelpak® be included as an organic biostimulant to any fertilizing program, especially for areas prone to nutrient deficiency. Kelpak® has the ability to increase yield and decrease fertilizing costs.

Chapter 5 - Uptake and transport of polyamines by okra plants

5.1. Introduction

Since PAs are produced by all cells, long distance transport was first thought to be unimportant **(YOUNG and GALSTON, 1983)** but it has since been shown that PAs are transported. Intra- and intercellular transport of PAs occur through the tonoplast and plasmalemma **(PISTOCCHI *et al.*, 1988)**. The rate of PA synthesis differs between cells of different plant tissues **(FRIEDMAN *et al.*, 1986)**. Irrespective of their synthesis in other plant organs, large amounts of PAs synthesized in the roots are transported to other plant parts to maintain PA levels **(FRIEDMAN *et al.*, 1986; RABITI *et al.*, 1989)**.

Endogenous PA levels are regulated within the cells by biosynthesis, **(BOUCHEREAU *et al.*, 1999)**, conjugation, transport and degradation **(BAGNI and PISTOCCHI, 1991; ANTOGNONI *et al.*, 1998)**. Although long distance transport of PAs occurs through both the xylem and phloem **(RABITI *et al.*, 1989; ANTOGNONI *et al.*, 1998)**, transport occur mainly through the xylem as the plant matures **(RABITI *et al.*, 1989)**. Only free PAs are transported in the xylem and phloem. Conjugated PAs are restricted to the vacuoles and might not be able to enter the cytosolic fluid of the sieve tubes **(FRIEDMAN *et al.*, 1986; ANTOGNONI *et al.*, 1998)**.

Kelpak® contains on average 2.81 $\mu g.ml^{-1}$ Put and 0.78 $\mu g.ml^{-1}$ Spm (Table 2.1). When Kelpak® is applied as a soil drench or as a foliar spray, it is likely that some of the PAs are absorbed by the plant and transported to other plant parts as required. The aim of this experiment was to investigate if polyamines present in Kelpak® are absorbed by the plants and if these PAs are translocated to the fruits.

5.2. Materials and Methods

5.2.1. Growth conditions of the plant material

The experiment was conducted from 15 September 2010 to 15 January 2011 in a shade house located at the University of KwaZulu-Natal Botanical Gardens, Pietermaritzburg Campus. Okra, *Abelmoschus esculentus* (Clemson spineless lot no: YR 025 AY), seeds were purchased from McDonald's Seed Company, Pietermaritzburg, South Africa and stored in plastic containers containing silica gel at 4°C until planted. One seed was planted per pot (25 cm diameter, 5000 ml) containing potting mixture and the pots were arranged randomly in the shade house. Pots were watered using a drip irrigation system, being watered for 10 min twice a week. Plants were subjected to natural light (40% shade cloth) with temperatures ranging between 15-40°C.

The experiment was designed in two phases that differed only in the mode of application of the test solutions soil drench (SD) or foliar spray (FS). The treatments were Put, Spm, Spd (10^{-4} M), Kelpak® (0.4%) and water. Every two weeks, 100 ml of the respective test solutions were applied to 9 okra plants as a SD. The same treatments were also applied to 9 plants each as a FS. For foliar application, the test solutions were applied with spray bottles containing the test solution with two drops Tween 20 (wetting agent). The stems and leaves of the plants were sprayed until run-off was achieved. In total, 90 plants were treated. Plants were harvested after 4 months, once the fruits had reached maturity. The roots, stems and fruits were removed, rinsed with water and dried for four weeks at 35°C in a drying oven.

5.2.2. HPLC analysis of okra plant material for polyamine content

After the plant material was dried, the roots, stems and fruit were ground to fine powders using an Ultra Centrifugal Mill (Type ZM 200, Retsch®). To determine the amount of PAs in the plant material, 200 mg of dry powder from every plant part was analyzed in triplicate for each treatment and mode of application. Extraction was achieved by adding 5 ml 0.2 M $HClO_4$ and leaving the extracts at 4°C for 1 h. Thereafter, 5 ml 2 M NaOH were added to the extracts. The samples were then centrifuged for 10 min at 15 344 *g* to recover the supernatant. In order to detect

the PAs, they were benzoylated with 10 µL benzoyl chloride, shaken vigorously and incubated overnight. The remainder of the extraction procedure was as described in the Materials and Methods section, Chapter 2. The results were analyzed using one-way ANOVA and means separated using Duncan's Multiple Range Test (Genstat® Fourteenth Edition).

5.3. Results

Generally the highest PA levels were detected in the roots of the okra plants (Tables 5.1 and 5.2). Spermidine occurred at higher levels in the roots, stems and fruits, compared to Put and Spm levels, irrespective of the mode of application (SD or FS). No significantly different Put concentrations were detected in the roots, stems and fruit when the test solutions were applied as a SD. Although not significantly different, Put did occur at higher concentrations in the roots and stems when treated with Put (SD). Similarly Spd concentrations were higher in the stems and fruit when treated with Spd as a SD. Spermine was on average, the lowest when treated with Spm as a SD. All three PA treatments increased Spd levels significantly above the control.

The PA solutions applied as a SD did not increase Spm content significantly above the control plants in any plant part. Foliar applied PA solutions increased Put content in the roots significantly above the control. Foliar applied Spd significantly increased Put content in the stems compared to the controls. Putrescine and Spd also significantly increased Put content in the fruits compared to the control when applied as a FS. When applied as a FS, only Spm treatment could increase Spd content significantly above the controls in the roots, stems and fruit. Similarly, only foliar applied Spd was able to increase Spm content above the controls in the roots, stems and fruit.

Applied as a SD, Kelpak® was unable to increase the PA content significantly above controls or PA treatments in any plant part. When applied as a FS, Kelpak® was able to increase Spd content above the control in the fruits. Similarly, foliar applied Kelpak® increased Spm content significantly above Put and Spm treatments.

Table 5. 1. Amount of polyamines (μg.g^{-1}) in the roots, stems and fruits of okra plants treated with putrescine, spermidine and spermine (10^{-4} M) and 0.4% Kelpak® applied as a soil drench

	Putrescine (μg.g^{-1})				Spermidine (μg.g^{-1})				Spermine (μg.g^{-1})			
	Treatment	**Avg**	**Std err**	**Sig**	**Treatment**	**Avg**	**Std err**	**Sig**	**Treatment**	**Avg**	**Std err**	**Sig**
Roots	Control	6.18	1.60	a	Control	23.43	1.59	b	Control	14.64	0.57	a
	Putrescine	7.87	0.55	a	Putrescine	44.70	4.32	a	Putrescine	14.48	1.91	a
	Spermine	6.01	0.42	a	Spermine	42.70	1.00	a	Spermine	19.47	2.32	a
	Spermidine	6.10	0.11	a	Spermidine	37.41	7.67	a	Spermidine	20.25	1.39	a
	Kelpak	7.05	0.66	a	Kelpak	21.98	2.58	b	Kelpak	19.93	4.87	a
Stems	Control	4.92	1.68	a	Control	29.04	4.41	a	Control	12.63	3.95	a
	Putrescine	7.95	0.61	a	Putrescine	29.09	0.61	a	Putrescine	9.40	0.80	a
	Spermine	6.86	1.65	a	Spermine	16.69	2.11	b	Spermine	6.56	0.74	a
	Spermidine	5.66	0.41	a	Spermidine	36.09	1.03	a	Spermidine	8.10	1.04	a
	Kelpak	7.74	1.63	a	Kelpak	20.55	2.43	b	Kelpak	9.06	1.02	a
Fruits	Control	5.07	0.33	a	Control	23.10	1.55	ab	Control	18.77	2.61	ab
	Putrescine	4.56	0.52	a	Putrescine	19.61	1.97	bc	Putrescine	24.32	1.10	a
	Spermine	4.59	0.13	a	Spermine	20.22	0.28	bc	Spermine	16.69	2.87	b
	Spermidine	4.99	0.07	a	Spermidine	24.86	1.18	a	Spermidine	18.22	2.76	ab
	Kelpak	4.01	0.42	a	Kelpak	17.03	1.12	c	Kelpak	18.57	0.73	ab

Significant differences between values within each treatment is annotated by the letters a, b and c at P<0.05, calculated with Duncan's Multiple Range Test (n=27).

Table 5. 2. Amount of polyamines (µg.g^{-1}) in the roots, stems and fruits of okra plants treated with putrescine, spermidine and spermine (10^{-4} M) and 0.4% Kelpak® applied as a foliar spray

	Putrescine (µg.g^{-1})				Spermidine (µg.g^{-1})				Spermine (µg.g^{-1})			
	Treatment	**Avg**	**Std err**	**Sig**	**Treatment**	**Avg**	**Std err**	**Sig**	**Treatment**	**Avg**	**Std err**	**Sig**
Roots	Control	5.73	0.72	c	Control	40.48	6.57	b	Control	16.70	1.45	b
	Putrescine	12.50	0.92	a	Putrescine	19.50	0.71	c	Putrescine	15.05	1.15	b
	Spermine	9.71	0.83	b	Spermine	61.16	8.31	a	Spermine	17.97	0.46	b
	Spermidine	8.19	0.32	b	Spermidine	27.66	2.78	bc	Spermidine	28.83	2.94	a
	Kelpak	7.94	0.59	bc	Kelpak	41.86	3.88	b	Kelpak	14.55	1.14	b
Stems	Control	6.11	1.21	b	Control	29.45	6.34	b	Control	12.80	2.57	a
	Putrescine	6.19	0.58	b	Putrescine	17.69	4.00	b	Putrescine	7.59	0.16	a
	Spermine	5.51	0.20	b	Spermine	58.08	8.63	a	Spermine	18.22	5.28	a
	Spermidine	12.73	1.80	a	Spermidine	25.06	3.25	b	Spermidine	13.33	5.71	a
	Kelpak	6.04	0.07	b	Kelpak	21.52	1.74	b	Kelpak	9.63	1.56	a
Fruits	Control	5.28	0.07	b	Control	14.35	2.85	b	Control	10.66	2.44	ab
	Putrescine	8.18	0.22	a	Putrescine	17.53	1.53	ab	Putrescine	8.32	0.87	b
	Spermine	4.57	0.17	b	Spermine	16.80	1.29	ab	Spermine	10.17	1.27	b
	Spermidine	8.65	1.72	a	Spermidine	20.91	0.54	a	Spermidine	15.65	1.26	a
	Kelpak	6.26	0.23	ab	Kelpak	16.91	1.26	ab	Kelpak	12.56	1.73	ab

Significant differences between values within each treatment is annotated by the letters a, b and c at P<0.05, calculated with Duncan's Multiple Range Test (n=27).

5.4. Discussion

The majority of PAs are produced in the roots (irrespective of their synthesis in other organs) and are transported to other plant organs as required **(FRIEDMAN *et al.*, 1986)**. This was also found to be true for this experiment. Overall, applying PAs as a SD was not as effective in increasing the PA concentrations in the different plant organs as was FS (Table 5.1 and 5.2). Only Spd increased significantly in the plant tissues when treated with all three PA solutions as a SD (Table 5.1). Spermidine concentrations were much higher than the other PAs irrespective of the mode of application. The application of all three PAs as a SD increased Spd concentrations in the roots. The ratio of Put:Spd:Spm (FS) were 1:5:2, 1:5:3 and 1:2:1.5 in the roots, shoots and fruits, respectively. The excess PAs absorbed by the plant were probably converted into Spd. Spermidine is the main PA produced in the roots. Foliar application of PAs are readily converted to Spd in the roots. As Spd only needs to gain or lose one amine group, it can easily be modified into either Put or Spm after reaching its intended destination. Therefore, Spd might be the preferred PA for long distance transport.

No clear trends in PA concentrations were found when applying PAs to plants, with the exception of several partial trends found when PAs were applied as a FS. Foliar application of Put increased the Put content of the roots and fruits significantly compared to the control, but not in the stems. Spermine treatment increased Spd concentrations in the roots and stems, but not in the fruit. Spermine concentrations were also significantly higher in the roots and fruits when treated with Spd, but not in the stems. Spermidine treatment (FS) produced significantly higher Put, Spd and Spm in the fruits. As Spd only needs to gain or lose one amine group, it can easily be modified into either Put or Spm. The significant increases in all three PAs in the fruits could be a result of the foliar application of Spd. It is evident that Spm and Spd are easily inter-converted between the two forms. Applying Spm as a FS significantly increase Spd content in all the plant parts. Similarly, applying Spd as a FS produced significantly higher Spm concentrations in the roots and higher levels in the stems and fruit compared to the other treatments (Table 5.2). Since PAs can be converted

into either PA form (Put, Spd of Spm) **(COSTA and BAGNI, 1983; RABITI *et al.*, 1989)**, it is difficult to establish how the individually applied PAs affected each other's concentrations in the plant material. Since the plants were only harvested after the fruits reached maturity, the PAs in the plants will not be a true reflection of the PA content in actively growing plants. By the time the fruits reached maturity, the leaves had senesced. Since PAs would also have been transported to the leaves, important information was lost. If the experiment was to be repeated, harvesting should also be done before the leaves dropped just after the fruits are formed.

Kelpak® applied as a SD did not increase the PA content in the different plant organs. The PA concentrations in 0.4% Kelpak® were very low and therefore did not contribute many PAs. By applying the test solutions more frequently and by increasing the concentration of Kelpak®, the amount of available PAs would have been increased. However, since Kelpak® was applied at a rate similar to which farmers would apply it, increasing the concentration would not reflect the amounts absorbed by plants in the field. It is clear that the amount of PA contributed by Kelpak® is too low to affect the PA status of mature plants. In future experiments, PA content must be determined every 2 weeks from germination to establish if the Kelpak® solution contributed any PAs to the plants at the seedling and early growth stages.

From this study, it is evident that the foliar application of PAs to plants will change the endogenous PA levels in the plants. However, the application of a certain PA to a specific plant part does not necessarily mean that the endogenous levels will reflect the application, as the PAs might be converted into the different PAs or be degraded or conjugated to other molecules. The amount of Spd in the roots increased with PA application (SD of FS) and were transported to the other plant parts, increasing the Spd content significantly.

Chapter 6 - General Conclusions

Several studies have reported that PAs range from nanomolar to micromolar levels in plants and seaweeds. The concentrations of PAs found in *E. maxima* were in the same range established for other seaweeds. This is the first report of PAs being detected in a SWC. In Kelpak® there was an average of 2.81 $\mu g.ml^{-1}$ Put and 0.78 $\mu g.ml^{-1}$ Spm. Since the effects of Kelpak® on plant growth are similar to the functions of PAs, the possible role of PAs in Kelpak® on plant growth was investigated.

During the study period, two periods of high PA concentrations in *E. maxima* and Kelpak® were observed. The first peak occurred during winter and the second peak during late spring and early summer, with the latter having an extended period of high PA concentrations. The observed variation in PA content in *E. maxima* was due to a change in water temperature and stress brought about by rough wave action during the rainy season (winter) in the Western Cape Province. Polyamine content increased in the seaweed material when the water temperature increased to 14°C and decreased when the water temperature was below 13°C. The decrease in PA levels in *E. maxima* during March when water temperature was low, were attributed to the decrease in growth activity in the seaweed material due to the low water temperatures.

The seasonal variation of PAs established in this study for *E. maxima* were similar to the cytokinin pattern described by **MOONEY and VAN STADEN (1984b)**. Peaks in PA concentration overlapped with peaks found for cytokinin-like activity during July and November. The simultaneous increase in both cytokinin-like activity and PAs indicate that these growth regulators might be intrinsically linked to the growth stimulating processes. The presence of high PA levels in actively growing tissues of *E. maxima* could induce cytokinin synthesis. Polyamine concentration might also increase as a direct response to the increased cytokinin levels, as shown by **SERGIEV *et al.* (1995)**.

The role of PAs in the seaweed might be better understood if the PA concentrations were monitored in conjunction with the lunar cycle. This will lead to a better understanding of the physiology of seaweeds, especially the reproductive cycle which is often linked to the lunar cycle. The lunar cycle could then also be compared to the physiology of the seaweed during other months in the life-cycle of the seaweed.

Polyamines play a vital role in maintaining homeostasis within seaweeds. The fact that the PA levels fluctuated within *E. maxima* throughout an annual cycle suggests that they are synthesized and regulated according to the needs of the plant. Polyamine levels are associated with periods of active growth and stress conditions. Changes in any of the environmental conditions in these habitats may lead to the destruction of seaweed populations. The global industry that makes use of natural seaweed populations is worth billions of dollars. It is, therefore, important that these seaweed communities are sustainably harvested and monitored for future use.

Monthly screening of Kelpak® indicated that Kelpak® consistently resulted in more rooting in the mung bean bioassay compared to the auxin (IBA) control. When 10^{-3} M Put and 10^{-4} M IBA were combined in a treatment, a synergistic relationship developed, which increased rooting above separately applied Put and IBA. Together with the exogenously applied auxins, the additional supply of PAs enabled the mung bean cuttings to produce more roots. The increased rooting suggests that Put plays an active role in auxin-stimulated root growth. Auxins stimulate the formation of new root meristematic tissue along the mung bean stem. When the new meristematic tissue is formed, Put could promote active cell division.

It was thought that the various auxins in Kelpak® are responsible for its root-promoting ability. However, the Put/-auxin combination produced almost the same number of roots as Kelpak®. It is, therefore, possible that the PAs present in Kelpak® work in a synergistic manner with auxins to increase rooting. Further experimentation are required to establish the role of Put in auxin-stimulated root-growth. Furthermore it is also necessary to determine whether this synergistic effect will occur between

Put and different auxins, and whether this synergistic relationship is responsible for the improved rooting observed with Kelpak® application.

Kelpak® increases yield in plants receiving a low supply of nutrients. In this study, however, N, P and K were completely removed from 50% Hoagland's nutrient solution. Kelpak®-treated plants appeared healthier and did not show nutrient-deficiency-symptoms. Kelpak® treatment significantly increased seedling vigour of okra seedlings deprived of either P or K. Since Kelpak® contains insufficient amounts of nutrients to alleviate the nutrient deficiencies, the increased growth and seedling vigour was ascribed to the growth regulators (mainly auxins and cytokinins) in Kelpak®. Although PA-treated seedlings appeared to be healthier and larger, PAs had no significant effect in increasing seedling vigour in P- and K-deficient okra seedlings. PA treatment did, however, increase the vigour of N-deprived okra seedlings. This was attributed to the N-containing PAs that were degraded and metabolized by the seedlings. Besides possibly supplying the seedlings with N, the PA treatment had no physiological effect on the growth of the seedlings. Polyamine treatment also increased several growth parameters in okra seedlings receiving an adequate supply of nutrients. Since PAs play a role in auxin-mediated root growth, the presence of additional PAs could have increased the growth of the roots, thereby increasing overall growth. Polyamine treatment did not reduce the effect of any of the nutrient deficiencies. Under favourable growing conditions (adequate supply of nutrients) the PAs play a role in increasing growth, most probably increasing root growth, functioning in combination with other growth regulators.

In reality, soil may have (or may develop) low levels of certain nutrients but very seldom will a soil be completely depleted of the nutrients. Application of Kelpak® may therefore reduce the effect of P- and K-deficiency in the field and render plants healthier. The auxins and PAs present in Kelpak® may work synergistically in increasing yield in nutrient-stressed plants. Seedlings grown in P- and K-deficient soils will benefit from Kelpak® by increasing absorption of nutrients present in low concentrations. It is recommended that Kelpak® be included as an organic biostimulant to any fertilizing program, especially for areas prone to nutrient

deficiencies. Kelpak® has the unique ability to increase yield and decrease fertilizing costs.

Kelpak® applied as a soil drench to okra seedlings, did not increase the PA content in the different plant organs. The concentration of PAs in a 0.4% Kelpak® solution were very low and, therefore, did not contribute many PAs. It was established that applying PAs as a soil drench was not as effective as a foliar application in increasing the PA levels in the different plant organs of the okra seedlings. Applying PAs as a soil drench significantly increased Spd concentrations in the plant organs. Spermidine concentrations were much higher than the other PAs. The excess PAs absorbed by the plant were probably converted into Spd. The PAs were produced in the roots (especially Spd) and a large proportion of the PAs were transported to the shoots and ultimately to the fruits.

Since Spd only needs to lose or gain one amine group to be converted to Put or Spm, respectively, it could easily have been modified into either Put or Spm. By applying the PAs as a foliar spray, it could be absorbed directly into the tissue of the leaves, and into the roots via run-off. The amount of Spd in the roots increased with application of PAs (soil drench or foliar spray) and was transported to the other plant parts, increasing the Spd content significantly. Since Spd levels increased with the various PA applications, it seems likely that Spd is more readily transported than Put or Spm.

Ecklonia maxima, is a valuable marine resource from which the seaweed concentrate Kelpak® is produced. Although previously known to contain the growth regulators cytokinins and auxins, which stimulate growth, this study has shown that PAs are also present in the stipes and fronds of the seaweed as well as in the SWC. Together with the auxins and cytokinins in Kelpak®, the PAs seem to play a role in promoting root growth, and growth under normal growing conditions. It was also shown that Kelpak® has the ability to reduce P- and K-deficiency, producing healthier plants which may absorb more nutrients present at low concentrations. With ever increasing human populations and limiting food supplies, there is a pressing need to increase

food production and decrease input costs. The various growth regulators present in Kelpak® serves as a promising agricultural tool, that may aid in securing food production.

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Appendix

1) Preparation of 0.2 M perchloric acid

The 0.2 M perchloric acid ($HClO_4$) used in the extraction of PAs was prepared by adding 17.09 ml $HClO_4$ to 970 ml ultra-pure water, stirring continually and topping up to 1 l with ultra-pure water. To accommodate for impurities, the amount of $HClO_4$ required to make a 0.2 M solution was calculated as follows:

$$x = \frac{M \times MW}{\%\ \text{purity}} \div \text{specific density}$$

$$x = \frac{0.2 \times 100.84}{0.7} \div 1.68$$

$$x = 17.09\ \text{ml}.\text{l}^{-1}$$

2) Preparation of 2 M sodium hydroxide

The sodium hydroxide solution was prepared by dissolving 8 g NaOH (Saarchem®) in 100 ml ultra-pure water.

3) Preparation of TEA buffer

Triethylamine (TEA) buffer was prepared by adding 11.438 ml glacial acetic acid (analytical) to 970 ml ultra-pure water while stirring with a magnetic stirrer. The pH of the solution was monitored continuously from this point onwards (± pH 2.5). Triethylamine (Merck-Schuchardt®) was added 1 drop at a time with a Pasteur pipette until a pH of 3.5 was reached. The buffered solution was topped up to 1000 ml with ultra-pure water. The TEA buffer was stored at 10°C.