Overcoming Seed Dormancy and Development of *In Vitro* Propagation Protocols in Indigenous *Cucumis* Species for Use as Alternative Crops in Various Industries

by

Mmatshelo Yvonne Maila

THESIS
Submitted in fulfilment of the
requirements for the degree of

Doctor of Philosophy
in
Plant Production

in the

FACULTY OF SCIENCE AND AGRICULTURE
(School of Agricultural and Environmental Sciences)

at the

UNIVERSITY OF LIMPOPO

Supervisor: **Professor P.W. Mashela**
Co-supervisor: **Dr B. Nzanza**

2015

TABLE OF CONTENTS

		Page
DECLARATION		ix
DEDICATION		x
ACKNOWLEDGEMENTS		xi
LIST OF TABLES		xv
LIST OF FIGURES		xx
LIST OF APPENDICES		xxiv
ABSTRACT		xxvii

CHAPTER 1
GENERAL INTRODUCTION

1.1	Research problem	1
1.2	Problem statement	2
1.3	Motivation of the study	3
1.4	Aim and objectives	4
1.5	Reliability, validity and objectivity	4
1.6	Bias	5
1.7	Ethical considerations	5
1.8	Significance of the study	6
1.9	Format of thesis	6

CHAPTER 2
LITERATURE REVIEW

2.1 Work done on the research problem 7

2.1.1 Seed dormancy 7

2.1.2 Advantages of seed dormancy 8

2.1.3 General causes of seed dormancy 8

2.1.4 Forms of seed dormancy 9

2.1.4.1 Chemical dormancy 9

2.1.4.2 Physical dormancy 12

2.1.4.3 Physiological dormancy 15

2.1.5 Morphology of testa in cucurbits 16

2.1.6 Propagule selection for *in vitro* propagation and use of plant growth regulators 18

2.1.7 Suitable potting media for *in vitro* weaned plantlets 21

2.1.8 Degree of nematode resistance in plants 23

2.2 Research gaps 26

CHAPTER 3
SEED DORMANCY AND *IN VITRO* SEEDLING PERFORMANCE OF TWO INDIGENOUS *CUCUMIS* SPECIES

3.1 Introduction 27

3.2 Materials and methods 28

3.2.1 Location and seed preparation 28

3.2.2 Culture conditions and experimental design 29

3.2.3 Data collection 30

3.2.4 Data analysis 30

3.3 Results 31

3.3.1 *Cucumis africanus* seeds 31

3.3.1.1 Germination percentage in *Cucumis africanus* 31

3.3.1.2 Seedling performance in *Cucumis africanus* 32

3.3.2 *Cucumis myriocarpus* seeds 33

3.3.2.1 Germination percentage in *Cucumis myriocarpus* 34

3.3.2.2 Seedling performance in *Cucumis myriocarpus* 34

3.4 Discussion 42

3.4.1 Seed germination 42

3.4.2 Existence of chemical dormancy 42

3.4.3 Existence of physical dormancy 44

3.4.4 Seedling performance 46

3.5 Conclusion 48

CHAPTER 4
MORPHOLOGY OF SEED TESTA IN INDIGENOUS *CUCUMIS* SPECIES

4.1 Introduction 50

4.2 Materials and methods 50

4.2.1 Location 50

4.2.2 Seed preparation 51

4.2.3 Identification of layers 51

4.3 Results 51

4.4 Discussion 57

4.4.1 Variation in testa thickness 57

4.4.2 Epidermal layer 57

4.4.3 Hypodermal layer 58

4.4.4 Sclerenchyma layer 59

4.4.5 Aerenchyma layer 59

4.4.6 Chlorenchyma layer 60

4.4.7 Existence of “water gaps” 60

4.4.8 Elimination of physical dormancy 61

4.5 Conclusion 62

CHAPTER 5
OPTIMUM PLANT GROWTH REGULATOR CONCENTRATIONS FOR *IN VITRO* PROPAGATION OF TWO INDIGENOUS *CUCUMIS* SPECIES

5.1 Introduction 63

5.2 Materials and methods 64

5.2.1 *In vitro* propagule selection 64

5.2.1.1 Location and source of explants for propagule selection 64

5.2.1.2 Preparation of medium 64

5.2.1.3 Preparation of explants and culturing 64

5.2.1.4 Experimental design, data collection and statistical analysis 65
5.2.2 Optimum plant growth regulator concentrations for shoot regeneration and root initiation 66
5.2.2.1 Location and source of explant material for optimisation of plant growth regulators 66
5.2.2.2 Shoot multiplication stage and culture conditions 66
5.2.2.3 *In vitro* rooting of regenerated shoots 67
5.2.2.4 Data analysis 67
5.3 Results 68
5.3.1 *In vitro* propagule selection 68
5.3.2 Selection of optimum plant growth regulator concentrations 80
5.3.2.1 Shoot multiplication stage 80
5.3.2.2 Root initiation stage 83
5.4 Discussion 91
5.4.1 Selection of *in vitro* propagule 91
5.4.1.1 Regeneration of plant tissues from various organs 91
5.4.1.2 Shoot regeneration from various organs 94
5.4.1.3 Suitable propagule 97
5.4.2 Optimum plant growth regulator concentrations 97
5.5 Conclusion 100

CHAPTER 6
ACCLIMATISATION OF *IN VITRO* PROPAGATED *CUCUMIS* PLANTLETS UNDER GREENHOUSE CONDITIONS

6.1 Introduction 101

6.2 Materials and methods 103

6.2.1 Acclimatisation and greenhouse conditions 103

6.2.2 Acclimatisation of *in vitro* plantlets 104

6.2.3 Experimental design and cultural practises 105

6.2.4 Data collection 106

6.2.5 Statistical analysis 107

6.3 Results 108

6.3.1 Acclimatisation 108

6.3.2 Suitable potting media 108

6.4 Discussion 114

6.4.1 Acclimatisation of *in vitro* plantlets 114

6.4.2 Potting media 115

6.5 Conclusion 119

CHAPTER 7
DEGREE OF NEMATODE RESISTANCE ON *IN VITRO*-PRODUCED PLANTLETS OF NEMATODE-RESISTANT INDIGENOUS *CUCUMIS* SPECIES

7.1 Introduction 120

7.2 Materials and methods 121

7.2.1 Location and preparation of materials 121

7.2.2 Preparation of plantlets 122

7.2.3 Treatments and experimental design 122

7.2.4 Data collection 123

7.2.5 Nematode extraction and counting 124

7.2.6 Data analysis 125

7.3 Results 125

7.3.1 Host-status 125

7.3.2 Host-sensitivity 126

7.4 Discussion 129

7.4.1 Host-status 129

7.4.2 Host-sensitivity 130

7.4.3 Nematode resistance 131

7.5 Conclusion 131

CHAPTER 8
SUMMARY, SIGNIFICANCE OF FINDINGS, FUTURE RESEARCH AND CONCLUSIONS

8.1 Summary 133

8.2 Significance of findings 133

8.3 Future research 134

8.4 Conclusions 135

REFERENCES 136

APPENDICES 172

DECLARATION

I declare that the thesis hereby submitted to the University of Limpopo, for the degree of Doctor of Philosophy in Agriculture (Plant Production) has not previously been submitted by me for a degree at this or any other university; that it is my work in design and in execution, and that all material contained herein had been duly acknowledged.

Maila M.Y. (PhD) **Date**

DEDICATION

To my three beloved daughters: Morategi, Mosibudi and Mogau

ACKNOWLEDGEMENTS

Glory unto the Almighty God, my Father and my Maker, Jehovah Elshadai Adonai, the Master of everything. With You in me, it is always Amen!

My heartfelt gratitude to the Land Bank Chair of Agriculture ─ University of Limpopo and the Flemish Interuniversity Council (VLIR) for generously funding various aspects of my doctoral studies in the Indigenous Cucurbitaceae Technologies (ICT) within the Green Technologies Research Programme. Also, VLIR provided a three year scholarship, which is greatly appreciated.

I feel overwhelmed to express my deep sense of gratitude to my supervisor and my mentor Professor P.W. Mashela, for his unconditional support, encouragement, expert guidance and his confidence in me during the duration of this study. His profound knowledge in research has made him a constant oasis of ideas and passion in plant sciences, which exceptionally inspired and enriched my growth as a PhD student. His constant dedication, optimist words and critical comments enabled me to do the best of my ability in completing this thesis work.

I whole-heartedly thank my co-supervisor, Dr. B. Nzanza, for his valuable guidance, unending support and encouragement in every aspect of my studies through his critical, scientific and logical inputs. His scientific guidance and statistical assistance were helpful in putting this thesis together. My sincere gratitude goes to Dr. K.M. Pofu, for her

unconditional assistance and mentoring in nematode aspects of this thesis. Her passion in scientific research remains an incredible motivator to me as an aspiring researcher.

I am over grateful to Dr. B. Okole and the staff at the African Biotechnologies Laboratory (ABL), Magoebaskloof, Tzaneen, South Africa, for their team-work-spirit and generosity. Dr. B. Okole unconditionally introduced and trained me in the exciting world of tissue culture. Ms Marika Van der Merwe of the Agricultural Research Council – Plant Protection Research Institute conducted viral-indexing on the two *Cucumis* species – which was a requirement prior to bringing the plant material into the ABL premises. I also owe my heartfelt gratitude to Professor R. Nikolova and Dr. M. Matla of the Department of Biodiversity, University of Limpopo, South Africa, for allowing me the opportunity to utilise their Plant Tissue Culture Laboratory space for my experiments and to train me in certain aspects of biology and technology that are related to *in vitro* propagation.

I am particularly indebted to Dr. C. Barker of the Electron Microscopy Unit, University of Limpopo, Medunsa Campus, for her guidance in seed morphological research studies. The valuable suggestions and assistance with sectioning, coating, identification, photographing of testa and providing technical assistance with the scanning electron microscopy, will forever remain part of my professional thoughts. I also extend my sincere thanks to the Director of Limpopo Agro-Food Technology Station (LATS), Dr. M.S. Mphosi and the entire staff for giving me the opportunity and support in-between my work schedules to pursue this doctoral study.

I am also indebted to the Department of Plant Production, Soil Science and Agricultural Engineering, the Land Bank Chair of Agriculture ─ University of Limpopo and LATS for sponsoring my trips during the study period to (1) Durban, where I presented two papers at the Combined Congress in 2012, (2) Uganda, where I presented two papers at the 11th African Crop Science Society Conference in 2013, (3) Malaysia, where I presented three papers at the International Conference on Advances in Plant Sciences in 2014 and Western Cape, George, where I presented two papers at the Combined Congress in 2015.

My warmest thanks go to my family, friends and loved ones, especially my loving mom Lillian, my sister Blondy and brothers, Peter, David and Tebogo, including their families for always believing in me and supporting me unconditionally in my decisions to read for a doctorate. I definitely would not be this far without their constant prayers and unwavering support. How can I forget to give all the gratitude to my dear husband Cecil, together with our three Cucurbits, 'Rado, 'Shibu and 'Gau, for sacrificing their needs of us being together as family and conceding me this lifetime opportunity of taking the family name to new and greater heights! Their constant love, patience, understanding, support and bearing of my absence when they needed me most during all these years of studying would never be forgotten. "Till this day, I still look forward to coming home just to spend quality time with you".

A special thank you to my beloved niece Rachel Mochaki, for constantly enquiring about the progress of my thesis writing. To my best buddies at the Green Technologies

Research Centre: Dr. Osvaldo Pelinganga, Martha Mamoepa, Pontsho Tseke, Zakheleni Dube and Apostle Kagiso Shadung, your constant motivation, prayers and fasting for me had unforgettable impact. You guys are all a “bunch of winners’’.

LIST OF TABLES

		Page
Table 3.1	Mean sum of squares for *in vitro* germination of leached-control (LC) and leached-scarified (LS) seeds of *Cucumis africanus.*	35
Table 3.2	Responses of germination percentage (GP), mean germination time (MGT), germination index (GI) and germination rate (GR) of leached-control (LC) and leached-scarified (LS) seeds of *Cucumis africanus in vitro.*	36
Table 3.3	*In vitro* optimum germination percentage (GP), mean germination time (MGT), germination index (GI) and germination rate (GR) of leached-control (LC) and leached-scarified (LS) seeds of *Cucumis africanus in vitro*.	38
Table 3.4	Mean sum of squares for *in vitro* germination of leached-scarified (LS) seeds of *Cucumis myriocarpus*.	39
Table 3.5	Responses of germination percentage (GP), mean germination time (MGT), germination index (GI) and germination rate (GR) of leached-scarified (LS) seeds of *Cucumis myriocarpus in vitro*.	39
Table 3.6	*In vitro* optimum germination percentage (GP), mean germination time (MGT), germination index (GI) and germination rate (GR) of leached-scarified (LS) seeds of	41

Cucumis myriocarpus.

Table 4.1 Comparison of the morphological indicators of testa in mature and dried seeds of *Cucumis africanus* and *Cucumis myriocarpus.* 55

Table 5.1 Mean sum of squares for five propagules of *Cucumis africanus* and *Cucumis myriocarpus* plantlets and six concentrations of BAP (µM) and their interactions in percentage tissue regeneration response and number of regenerated shoots *in vitro.* 69

Table 5.2 Effects of interaction in six concentrations of BAP (µM) and five propagules of *Cucumis africanus* and *Cucumis myriocarpus* seedlings in percentage tissue regeneration response *in vitro.* 70

Table 5.3 *In vitro* optimum BAP (µM) concentration (x) and percentage tissue regeneration response (y) in various propagules of *Cucumis africanus* and *Cucumis myriocarpus*. 73

Table 5.4 Effects of interaction in six concentrations of BAP (µM) and five propagules from *Cucumis africanus* and *Cucumis myriocarpus* seedlings in regeneration of shoots *in vitro.* 74

Table 5.5 *In vitro* optimum BAP (µM) concentration (x) and number of regenerated shoots (y) in nodal bud, shoot-tip and cotyledon explants of *Cucumis africanus* and *Cucumis myriocarpus*. 77

Table 5.6 Comparisons of performance of nodal bud and shoot-tip 78

explants in terms of shoot regeneration from *Cucumis africanus* and *Cucumis myriocarpus* over six levels of BAP *in vitro*.

Table 5.7 Mean sum of squares for number of shoots and vine length (cm) in shoot multiplication of *Cucumis africanus* shoot-tip explants and *Cucumis myriocarpus* nodal bud explants *in vitro.* 81

Table 5.8 Responses of *Cucumis africanus* shoot-tip explants and *Cucumis myriocarpus* nodal bud explants to concentrations of BAP (µM) during shoot multiplication stage *in vitro*. 82

Table 5.9 Integrated optimum level of BAP (µM) concentrations for *in vitro* shoot multiplication of *Cucumis africanus* shoot-tip explants and *Cucumis myriocarpus* nodal bud explants. 85

Table 5.10 Mean sum of squares for the number of roots and vine length (cm) in rooting stage of *Cucumis africanus* and *Cucumis myriocarpus in vitro.* 86

Table 5.11 Responses of shoots obtained from *Cucumis africanus* shoot-tip explants and *Cucumis myriocarpus* nodal bud explants to concentrations of IBA (µM) during root initiation stage *in vitro*. 88

Table 5.12 Mean optimum level of IBA (µM) concentrations for *in vitro* root initiation of shoots obtained from *Cucumis africanus* shoot-tip explants and *Cucumis myriocarpus* nodal bud explants. 90

Table 6.1 Mean sum of squares for stem diameter (SD), number of shoots (NS), dry shoot mass (DSM), dry root mass (DRM), vine length (VL) and chlorophyll content (CC) in *Cucumis africanus* and *Cucumis myriocarpus* plantlets at 56 days after transplanting *ex vitro.* 110

Table 6.2 Effects of potting media on stem diameter (mm), number of shoots, dry shoot mass (g) and dry root mass (g) of *Cucumis africanus* plantlets at 56 days after transplanting *ex vitro.* 111

Table 6.3 Effects of potting media on vine length (m) and chlorophyll content of *Cucumis africanus* plantlets at 56 days after transplanting *ex vitro.* 112

Table 6.4 Effects of potting media on stem diameter (mm), number of shoots, dry shoot mass (g) and dry root mass (g) of *Cucumis myriocarpus* plantlets at 56 days after transplanting *ex vitro.* 113

Table 6.5 Effects of potting media on vine length (m) and chlorophyll content of *Cucumis myriocarpus* plantlets at 56 days after transplanting *ex vitro.* 114

Table 7.1 Responses of final population densities (Pf) and the reproductive factor (RF) of *Meloidogyne incognita* race 2 in various levels of initial population densities (Pi) on *in vitro*-produced *Cucumis africanus* and *Cucumis myriocarpus* plantlets under greenhouse conditions. 127

Table 7.2 Responses of vine length (VL), number of shoots (NS), stem 128

diameter (SD), chlorophyll content (CC), dry shoot mass (DSM) and dry root mass (DRM) to various levels of initial population densities (Pi) of *Meloidogyne incognita* race 2 on *in vitro*-produced *Cucumis africanus* and *Cucumis myriocarpus* plants under greenhouse conditions.

LIST OF FIGURES

Page

Figure 3.1	(a) Mature seeds of wild *Cucumis* species. (b) *Cucumis africanus* and (c) *Cucumis myriocarpus* fruits. (d) Leaching *Cucumis* seeds under running tapwater and (e) disinfected prior scarification at the (f) chalaza region (CR) of the seed (seed image: S. Wood, RBG Kew).	29
Figure 3.2	*In vitro* responses of (a) germination percentage (GP), (b) mean germination time (MGT), (c) germination index (GI) and (d) germination rate (GR) of leached-control (LC) and leached-scarified (LS) seeds of *Cucumis africanus in vitro*.	37
Figure 3.3	*In vitro* responses of (a) germination percentage (GP), (b) mean germination time (MGT), (c) germination index (GI) and (d) germination rate (GR) of leached-scarified (LS) seeds of *Cucumis myriocarpus in vitro.*	40
Figure 4.1	Morphological structures in (a) *Cucumis africanus* and (b) *Cucumis myriocarpus* demonstrating the thickness of the testa layers.	53
Figure 4.2	Morphological structures of cell layers in the testa of (a, c) *Cucumis africanus* and (b, d) *Cucumis myriocapus*.	54
Figure 4.3	The micropyle canal (MC) in the testa of (a) *Cucumis africanus* and (b) *Cucumis myriocarpus* at the micropylar region of the seeds.	54
Figure 4.4	The chalaza canal (CL) in seeds of (a) *Cucumis africanus* and (b) *Cucumis myriocarpus* at the chalaza region of the seeds.	56

Figure 4.5 The cone-shape nucellar beak showing longitudinal sectioning of hypodermis cells (HC) at the micropylar region of (a) *Cucumis africanus* and (b) *Cucumis myriocarpus* seeds. 56

Figure 5.1 Responses of leaf, hypocotyl and cotyledon explants to six levels of BAP concentrations on percentage tissue regeneration response in *Cucumis africanus* (*Ca*) and *Cucumis myriocarpus* (*Cm*) *in vitro.* 72

Figure 5.2 Responses of nodal bud, shoot-tip and cotyledon explants to six levels of BAP (µM) concentrations in number of regenerated shoots in *Cucumis africanus* (*Ca*) and *Cucumis myriocarpus* (*Cm*) *in vitro*. 76

Figure 5.3 Various propagules used in the selection of suitable propagule: (a) leaves, (b) nodal buds, (c) shoot-tips, (d) hypocotyls, (e) cotyledons of *Cucumis africanus*, together with (f) leaves, (g) nodal buds, (h) shoot-tips, (i) hypocotyls and (j) cotyledons of *Cucumis myriocarpus in vitro.* 79

Figure 5.4 Responses of number of shoots and vine length to various concentrations of 6-benzyladeninepurine (BAP µM) during shoot multiplication stage of *Cucumis africanus* shoot-tip explants and *Cucumis myriocarpus* nodal bud explants *in vitro*. 84

Figure 5.5 Responses of number of roots and vine length to various concentrations of indole-3-butyric acid (IBA µM) during root 89

initiation of shoots obtained from *Cucumis africanus* shoot-tip explants and *Cucumis myriocarpus* nodal bud explants *in vitro*.

Figure 5.6 Various stages of *in vitro* propagation: (a) *Cucumis africanus* excised shoot-tip explants, (b) multiple shoots from shoot-tip explants, (c) *Cucumis myriocarpus* nodal bud explants and (d) multiple shoots from nodal bud explants. 91

Figure 6.1 Acclimatisation stages: (a) Rooted *Cucumis* species covered in unperforated plastic bags, (b) 7-day-old plantlets in perforated plastic bags, (c) 14-day-old acclimatised plantlets exposed to external conditions and (d) hardened *Cucumis* plantlets. 105

Figure 6.2 Greenhouse trials of (a) *Cucumis africanus* and (b) *Cucumis myriocarpus* plantlets in four various potting media. 107

Figure 7.1 *In vitro*-produced *Cucumis africanus* and *Cucumis myriocarpus* plantlets established for *Meloidogyne incognita* race 2 nematodes inoculation. 123

Figure 7.2 *Cucumis africanus* and *Cucumis myriocarpus* plants after infested with *Meloidogyne incognita* race 2 nematodes. 126

LIST OF APPENDICES

		Page
Appendix 3.1	Analysis of variance for germination percentage (GP) in leached-control (LC) and leached-scarified (LS) seeds of *Cucumis africanus in vitro*.	172
Appendix 3.2	Analysis of variance for mean germination time (MGT) in leached-control (LC) and leached-scarified (LS) seeds of *Cucumis africanus in vitro*.	173

Appendix 3.3	Analysis of variance for germination index (GI) in leached-control (LC) and leached-scarified (LS) seeds of *Cucumis africanus in vitro.*	174
Appendix 3.4	Analysis of variance for germination rate (GR) in leached-control (LC) and leached-scarified (LS) seeds of *Cucumis africanus in vitro.*	175
Appendix 3.5	Analysis of variance for germination percentage (GP) in leached-scarified (LS) seeds of *Cucumis myriocarpus in vitro*.	175
Appendix 3.6	Analysis of variance for mean germination time (MGT) in leached-scarified (LS) seeds of *Cucumis myriocarpus in vitro*.	176
Appendix 3.7	Analysis of variance for germination index (GI) in leached-scarified (LS) seeds of *Cucumis myriocarpus in vitro.*	176
Appendix 3.8	Analysis of variance for germination rate (GR) in leached-scarified (LS) seeds of *Cucumis myriocarpus in vitro*.	176
Appendix 5.1	Analysis of variance for percentage tissue regeneration response in five propagules of *Cucumis africanus* and *Cucumis myriocarpus* treated with six concentrations of BAP (µM) *in vitro*.	177
Appendix 5.2	Analysis of variance for shoot regeneration in five propagules of *Cucumis africanus* and *Cucumis myriocarpus* treated with six concentrations of BAP (µM) *in vitro*.	178

Appendix 5.3 Analysis of variance for number of shoots regenerated in *Cucumis africanus* and *Cucumis myriocarpus* at shoot multiplication stage *in vitro*. 179

Appendix 5.4 Analysis of variance for vine length (cm) in *Cucumis africanus* and *Cucumis myriocarpus* at shoot multiplication stage *in vitro*. 179

Appendix 5.5 Analysis of variance for number of roots in *Cucumis africanus* and *Cucumis myriocarpus* at rooting stage *in vitro*. 180

Appendix 5.6 Analysis of variance for vine length (cm) in *Cucumis africanus* and *Cucumis myriocarpus* at rooting stage *in vitro*. 180

Appendix 6.1 Analysis of variance for stem diameter (mm) of *in vitro* acclimatised *Cucumis africanus* and *Cucumis myriocarpus* plantlets transplanted in four various potting media. 181

Appendix 6.2 Analysis of variance for number of shoots for *in vitro* acclimatised *Cucumis africanus* and *Cucumis myriocarpus* plantlets transplanted in four various potting media. 182

Appendix 6.3 Analysis of variance for dry shoot mass (g) for *in vitro* acclimatised *Cucumis africanus* and *Cucumis myriocarpus* plantlets transplanted in four various potting media. 183

Appendix 6.4 Analysis of variance for dry root mass (g) for *in vitro* acclimatised *Cucumis africanus* and *Cucumis myriocarpus* plantlets transplanted in four various potting media. 184

Appendix 6.5 Analysis of variance for vine length (m) for *in vitro* 185

acclimatised *Cucumis africanus* and *Cucumis myriocarpus* plantlets transplanted in four various potting media.

Appendix 6.6 Analysis of variance for chlorophyll content of *in vitro* acclimatised *Cucumis africanus* and *Cucumis myriocarpus* plantlets transplanted in four various potting media. 186

ABSTRACT

Wild watermelon (*Cucumis africanus* LF.) and wild cucumber (*Cucumis myriocarpus* Naude.) are known for their ethnomedicine, ethnopesticide, ethnonematicide and nutritional properties, along with nematode resistance. The two *Cucumis* species were successfully used as inter-generic seedling rootstocks for watermelon (*Citrullus lanatus* Thunb.) cultivars, where nematode-resistant genotypes are not available. Also, the two *Cucumis* species are hardy and resilient to inland South Africa conditions, where temperatures are predicted to increase by 6°C in the year 2030. Seeds in the Cucurbitaceae Family contain high concentration of cucurbitacins, which induce auto-allelopathy that inherently inhibits plant growth and germination. Poor germination and non-uniform stands as a result of seed dormancy are a major challenge in sexual propagation of wild *Cucumis* species for various potential industries. Generally, true-to-type, uniform and disease-free plants in plant production are asexually-generated through *in vitro* propagation techniques. This study was therefore, initiated to address seed dormancy and related challenges of sexual propagation in the two wild *Cucumis* species by determining whether: (1) seed dormancy in *C. africanus* and *C. myiocarpus* would be ameliorated to allow for *in vitro* sexual propagation to establish pathogen-free parent stock, (2) the testa in *C. africanus* and *C. myiocarpus* seeds would possess structures, which interfere with imbibition and movement of water to the endosperm, (3) all organs of *C. africanus* and *C. myriocarpus* would be suitable for *in vitro* propagation, (4) suitable potting medium for *in vitro* propagated plantlets of *C. africanus* and *C. myriocarpus* would be available for acclimatisation of plantlets and (5) *in vitro*-produced

plantlets from nematode-resistant *C. africanus* and *C. myriocarpus* would retain their resistance to *Meloidogyne incognita* race 2 under greenhouse conditions. *In vitro* and *ex vitro* experiments were conducted to achieve the stated objectives, with treatments in the laboratory and the greenhouse being arranged in completely randomised and randomised complete block designs, respectively. Validity was primarily ensured through the use of factorial trials, while the reliability of data was ensured by using appropriate levels of statistical significance. Leaching alone in *C. africanus* improved germination, while in *C. myriocarpus* this treatment had no effect on germination. The optimum leaching time in leached-control seeds of *C. africanus* was achieved at 7.1 h, with a 25-day mean germination time (MGT) and 52% optimum germination percentage (GP). In the two *Cucumis* species, the combined effect of leaching seeds in running tapwater and physical scarification of seeds at the chalaza region escalated germination in both *Cucumis* species, suggesting that both chemical and physical seed dormancies were involved. In *C. africanus*, cucurbitacin B ($C_{32}H_{48}O_8$) was deposited exogenously to the testa, whereas in *C. myriocarpus* cucurbitacin A [cucumin ($C_{27}H_{40}0_9$) and leptodermin ($C_{27}H_{38}0_8$)], was deposited endogenously to the testa. The optimum leaching time in leached-scarified (LS) seeds of *C. africanus* was achieved at 5.7 h, with at least 40-day MGT and 89% optimum GP. In contrast, in *C*. *myriocarpus* LS seeds had the optimum leaching time of 6.3 h, with at least 41 days MGT and 93% optimum GP. Field emission SEM confirmed that there were two “water-gaps”, one at the micropylar region (hilum end) and the other at chalaza region (abaxial end) of seeds in both *Cucumis* species. Five distinct testa layers in seeds of *C. myriocarpus* were observed, namely, (i) epidermis, (ii) hypodermis, (iii) sclerenchyma, (iv) aerenchyma

and (v) chlorenchyma. In contrast, *C. africanus* seeds did not have the hypodermis between the micropylar and chalaza regions, but was present around both regions, which may provide some explanation of sporadic germination in non-leached and non-scarified seeds in this *Cucumis* species. The most suitable plant propagules for *in vitro* mass propagation of the two *Cucumis* species were nodal and apical buds. The optimum PGRs for shoot regeneration using both propagules in *C*. *africanus* and *C. myriocarpus* were at 0.80 and 0.35 µM 6-benzyladeninepurine (BAP), respectively. In contrast, the largest number of roots was regenerated at 0.31 and 0.44 µM indole-3-butyric acid (IBA) for *C. africanus* and *C. myriocarpus,* respectively. *In vitro*-produced plantlets were successfully acclimatised to *ex vitro* conditions, with sand + compost potting medium being the most suitable growing medium for weaning both *Cucumis* species. The *in vitro*-produced plantlets retained their resistance to *M. incognita* race 2. In conclusion, seeds of *C. africanus* and *C. myriocarpus* are structurally and chemically different, with strong evidence of chemical and physical dormancies. Structurally, *C. myriocarpus* seeds consist of five layers, four lignified and one non-lignified, whereas those of *C. africanus* have four layers, three lignified and one non-lignified. Evidence suggested that in *C. africanus* seeds, allelochemicals were primarily deposited outside the testa, whereas in *C. myriocarpus* they were deposited within the testa. The identified seed dormancies could successfully be ameliorated through combining leaching and scarification in both *Cucumis* species. The developed *in vitro* propagation protocols accord the two *Cucumis* species the potential for use as future crops in the context of climate-smart agriculture and research.

CHAPTER 1
GENERAL INTRODUCTION

1.1 Research problem

Indigenous *Cucumis* species, particularly the wild watermelon (*Cucumis africanus* LF.) and wild cucumber (*Cucumis myriocarpus* Naude.), have nutraceutical, pharmaceutical, cosmetical and pesticidal properties (Lee *et al*., 2010; Mashela *et al*., 2011; Thies *et al*., 2010; Van Wyk and Wink, 2012; Van Wyk *et al*., 2002). In addition to the stated uses, the two *Cucumis* species, indigenous to South Africa (Kristkova *et al*., 2003), are highly resistant to all three root-knot (*Meloidogyne* species) nematodes, which limit the introduction of future crops in many parts of South Africa (Pofu and Mashela, 2012; Pofu *et al*., 2010a, 2012a). Pofu and Mashela (2012) developed protocols for using the two *Cucumis* species in inter-generic grafting with commercially available watermelon (*Citrullus lanatus* Thunb.) cultivars, which are increasingly becoming an export agricultural commodity in South Africa (Anon., 2011). Incidentally, *Citrullus* cultivars do not have genotypes that are resistant to the root-knot nematodes (Thies *et al*., 2010), with yield reduced by these nematodes being as high as 50% to complete crop failure (Lamberti, 1979). The continued discovery of highly virulent biological races within *Meloidogyne* genus (Devran and Sogut, 2011; Robertson and Diez-Rojo, 2008), suggest that the development of sustainable management strategies for this nematode genus remains a top priority in crop husbandry. *Cucumis africanus* and *C. myriocarpus* fruits were used in the development of two phytonematicides, nemafric-BL and nemarioc-AL, respectively (Pelinganga, 2013), suggesting the need to produce large quantities of fruits from the two species. Also, the two *Cucumis* species are drought-tolerant, hardy and resilient to inland South Africa conditions whereby the year 2030,

maximum temperatures are predicted to increase by 6°C. The two *Cucumis* species are therefore, being considered to serve as future crops for managing *Meloidogyne* species in plant protection systems, along with serving various potential agro-food and agro-chemical industries in the context of climate-smart agriculture. However, the two plant species have non-uniform emergences, which result in high variation among the produced plant population densities.

1.2 Problem statement

Cultivation of the two *Cucumis* species as future crops is limited by poor germination and uneven emergences that result in uneven plant population densities ─ with seed dormancy being the primary suspect. The issue of seed dormancy, particularly in the Cucurbitaceae Family, is complex since the family contains cucurbitacins, which are groups of oxygenated tetracyclic triterpenes, which are renowned for their allelopathy and auto-allelopathy (Chen *et al*., 2005). Previous attempts to leach out cucurbitacins in seeds of both *Cucumis* species partially improved germination in *C. africanus*, but not in *C. myriocarpus* (Pofu, 2012). The observation was difficult to explain since the two *Cucumis* species contain water insoluble and soluble cucurbitacins, respectively (Chen *et al*., 2005). Currently, it is also not known whether it would be feasible to vegetatively propagate *C. africanus in vitro* since all organelles contain cucurbitacin B (Rimington, 1938).

Generally, non-uniformity in crop production is undesirable, as shown by the wide preferences of uniform hybrids, which enhance uniformity in production operations.

Erratic germination and high non-uniformity result in plants reaching maturity at various times, thus, increasing the production costs through different timeframes for various operations, including harvesting. Non-uniformity in sexually-produced plants had been attributed to genetic differences between the pollen and ovum wherein after fertilisation (Campbell, 1990) true-to-type characteristics of the parent plant are inherently lost (George, 1993). Most of the 125 genera in the Cucurbitaceae Family are self-infertile (Hartmann *et al*., 2010), with cross pollination escalating plant variability (Van Rooyen, 2001). Generally, the pollen grain and the ovum from different plants hardly confer true-to-type characteristics, due to their genetic variability. *In vitro* propagation produces uniform plantlets, which are true-to-type and have the capabilities of producing a large number of disease-free plantlets (George, 1993). Preliminary viral-indexing at the Agricultural Research Council (ARC) suggested that the two *Cucumis* species, which were deposited for quarantine prior to propagation in African Biotechnologies Laboratory, were virus-free (ARC Virology-Report Letter, 2011).

1.3 Motivation of the study

Successful *in vitro* mass propagation of *C. africanus* and *C. myriocarpus* will enable the two indigenous plant species to be used as future crops in nutraceutical, pharmaceutical, cosmetical and pesticidal industries. Also, should the plantlets retain their nematode resistance to *Meloidogyne* species; they will continue to be candidates for inter-generic grafting with nematode-susceptible commercial watermelon cultivars, which would accord the South African watermelon industry a competitive advantage in export markets (Pofu, 2012).

1.4 Aim and objectives

The aim of the study was to develop *in vitro* commercial protocols for vegetative mass propagation of seed dormancy-containing indigenous *C. africanus* and *C. myricarpus* for use as future crops in the context of climate-smart agriculture.

The objectives included:

1. To determine whether seed dormancy in *C. africanus* and *C. myiocarpus* would be ameliorated to allow for *in vitro* sexual propagation to establish pathogen-free parent stock.
2. To determine whether the testa in *C. africanus* and *C. myiocarpus* seeds would possess structures, which interfere with imbibition and movement of water to the endosperm.
3. To determine whether all organs of *C. africanus* and *C. myriocarpus* would be suitable for *in vitro* propagation.
4. To determine whether suitable potting medium for *in vitro* propagated plantlets of *C. africanus* and *C. myriocarpus* would be available for acclimatisation of plantlets.
5. To determine whether *in vitro*-produced plantlets from nematode-resistant *C. africanus* and *C. myriocarpus* would retain their resistance to *Meloidogyne incognita* race 2 under greenhouse conditions.

1.5 Reliability, validity and objectivity

Reliability, the extent to which a measuring instrument yields consistent results when the variable being measured repeatedly had not changed (Leedy and Ormrod, 2005), was ensured by using appropriate levels of statistical significance for mean separation and when evaluating the variance explained by models as measured by coefficients of

determination (R^2) (Berenson and Levine, 1996). Validity is described as an extent to which the instrument measures what was actually intended to be measured (Leedy and Ormrod, 2005). In empirical research, experiments are either replicated in time or space in order to increase the range of validity of conclusions drawn thereof (Little and Hills, 1981). A factorial set of treatments is another form of increasing the range of validity (Leedy and Ormrod, 2005). In this study, the factorial treatment option was preferred over other available options for improving the range of validity. Objectivity is striving as far as possible, to eliminate biases by relying on verifiable data (Leedy and Ormrod, 2005). Objectivity was achieved by discussing the findings on the basis of empirical evidence as shown by statistical analyses, with findings compared and contrasted with findings in other studies (Little and Hills, 1981).

1.6 Bias

Bias is defined as any influence, conditions or set of conditions that singly or altogether distort the data (Leedy and Ormrod, 2005). In this study, bias was reduced by ensuring that the experimental error in each experiment was minimised through increased replications and randomisation (Little and Hills, 1981).

1.7 Ethical considerations

Propagules from wild indigenous *Cucumis* species would be used to develop *in vitro* propagation protocols for use in commercial vegetative mass production of *Cucumis* plantlets. Commercial uses of the acquired protocols would be in accordance to the legal rights entered between the researcher and the Green Technologies Research

Centre, which was in line with the University of Limpopo research policies and the appropriate legislative framework in South Africa. Ethical considerations as outlined above would endure beyond the termination of the project.

1.8 Significance of the study

Successful *in vitro* propagation would help in the production of uniform, true-to-type, disease-free and nematode-resistant plantlets for use as future crops in various industries, namely, ethnomedicine, ethnopesticide, ethnonematicide and agro-food. Adoption of the indigenous *Cucumis* nematode-resistant seedling rootstocks in South Africa would inevitably accord the watermelon industry a competitive edge in export markets.

1.9 Format of thesis

Subsequent to the description and detailed outlining of the research problem (Chapter 1), work done and the related gaps on the research problem were reviewed (Chapter 2). Then, each of the five objectives would constitute a separate Chapter (Chapters 3-7). In the final chapter (Chapter 8), findings from all Chapters would be summarised and integrated to provide the significance of the findings and recommendations with respect to future research and then culminated in an overall conclusion of the study.

CHAPTER 2
LITERATURE REVIEW

2.1 Work done on the research problem

The wild watermelon (*Cucumis africanus* LF.) and wild cucumber (*Cucumis myriocarpus* Naude.) have the potential to serve as future crops for various industries in South Africa. However, sexual propagation of the two *Cucumis* species encounters challenges of seed dormancies with scant information on the causes of the observed dormancies. Also, it is not clear whether *in vitro* propagation would not result in the plantlets losing attributes such as nematode resistance, which were observed in sexually-produced plants (Pofu, 2012).

2.1.1 Seed dormancy

Seed dormancy is a temporary interference in a viable seed, with germination processes inhibited for a specific period in a particular set of environmental conditions, after which germination might proceed provided interference factors were ameliorated by either natural or artificial means (Baskin and Baskin, 2004; Simpson, 1990). Generally, germination is a complex chemical process triggered by imbibition of water after potential dormancy factors had been released by appropriate triggers (Campbell, 1990). Dormant seeds fail to germinate even when all conditions for germination are favourable (Hartmann *et al*., 2010).

2.1.2 Advantages of seed dormancy

Naturally, seed dormancy imposes an ecological advantage to different plant species whereby germination is deferred until favourable conditions for seedling survival are in place (Finch-Savage and Leubner-Metzger, 2006). In certain plant species, seeds may be shed in late summer or autumn, but although environmental conditions could be favourable for germination, it might not be the ideal conditions for seedling survival since adverse winter conditions would soon occur. Another advantage for seed dormancy is to create a seed bank, especially for seeds shed from plants that do not germinate easily for years due to dormancy (Hartmann *et al*., 2010). Also, Simpson (1990) noted that a seed bank ensured differential seed germination in a particular plant species within a single year, providing insurance against seedlings being simultaneously exposed to adverse factors like drought, fires, floods or chilly conditions that could destroy the entire generation of a plant species. Generally, seed banks also allow seedlings to grow during favourable years even if the mother plants failed to flower and produce seeds during the previous unfavourable seasons (Simpson, 1990).

2.1.3 General causes of seed dormancy

In dormant seeds, structural, chemical or both factors had been associated with the phenomenon of dormancy (Baskin and Baskin, 2001; Krugman *et al*., 1974). The necessary changes in the factors should gradually occur through interactive responses to aeration, moisture, temperature and light (Baskin and Baskin, 2001). In certain plants species, by mimicking certain factors that occur under natural conditions in laboratory or greenhouse conditions as dormancy-release treatments in the imbibed state of seeds,

dormancy was eliminated (Krugman *et al*., 1974). Broadly, the seed comprises an exterior part that consists of the testa that contains several layers, which could prevent water imbibition ─ an important element during storage, but a hindrance during seed germination (Campbell, 1990). Allelochemicals, which are secondary metabolites, when deposited on the exterior or interior parts of the testa, could also prevent germination, in what had been termed auto-allelopathy (Rice, 1984).

2.1.4 Forms of seed dormancy

Three forms of seed dormancy have been documented and are briefly reviewed below.

2.1.4.1 Chemical dormancy

Allelopathy is the production and release of chemical compounds, referred to as allelochemicals, by one plant species that inhibit germination, growth and development of other plant species (Rice, 1984; Shaukat *et al*., 2003). Auto-allelopathy occurs when inhibition is directed to the allelochemical-producing plant (Rice, 1984) and these chemicals were shown to play an important role in species sequences and dynamics during secondary succession (Rice, 1984). Allelochemicals also play a significant role in spatial pattern and interspecific associations among plant species (Shaukat *et al*., 2003). Generally, once allelochemicals are leached from plant organs into the soil, their efficacy is dependent upon their sorption, fixation, volatilisation and leaching capabilities, along with the existing microbial degradation rates (Blum, 1999; Inderjit *et al*., 1999).

Allelochemicals with capabilities to induce seed dormancies include coumerins, para-ascorbic acids, cucurbitacins, abscisic acids and hydrogen cyanides (Chen *et al*., 2005; Giraudat *et al.*, 1992; Murray *et al*., 1982; Nocito *et al*., 2002). Such allelochemicals had been mostly isolated from leaves, barks, roots, testa, endosperm and juices of fresh fruit (Akinnifesi *et al*., 2007; Maghembe, 1995). In seeds, allelochemicals can either be deposited in the testa or in the endosperm, suggesting that separation of testa from endosperm could not improve germination in all plant species (Barnea *et al*., 1993). The view that during seed maturation, some allelochemicals were stored either in the testa or the endosperm had been empirically verified (Chen *et al*., 2005). In seeds of some desert plants that contain allelochemicals, dormancy is eliminated through heavy rains that soak the soil sufficiently so that the germination process could establish before the soil dried out (Emery, 1987). Emery (1987) demonstrated that in the tested plants, allelochemicals were exterior to the testa layers. Generally, when the chemical inhibitor is situated on the exterior side of the testa, leaching seeds in running water for several hours eliminates the inhibitor, with improved germination (Emery, 1987). However, when the chemical inhibitor is interior to the testa in the endosperm, leaching fails to remove the inhibitors (Hartmann *et al*., 2010).

Chemical dormancy was also viewed as a challenge in germination of *Ecballium elaterium* (L) A. Rich., seeds, which is a minor crop in the Cucurbitaceae Family (Attard and Scicluna-Spiteri, 2003). Attard and Scicluna-Spiteri (2003) noted that the concentration of cucurbitacin in *E. elaterium* was the highest in fruit (3.84% w/w), followed by in stems (1.34% w/w) and then in leaves (0.34% w/w). Incidentally, although

the leaves contained low concentration of cucurbitacins or none at all, the role of cucurbitacins as antifeedants were important, with the plant being used as a protection barrier against several pests and diseases when planted adjacent to other cucurbits. Germination (73.8%) after 55 days was achieved in *E. elaterium* through washing seeds in distilled water, followed by physical scarification of seed coats and soaking overnight in distilled water, thereby leaching the cucurbitacins (Attard and Scicluna-Spiteri, 2003). This observation suggested that chemical and physical dormancies could be occurring concurrently in seeds of *E. elaterium*.

In wild *Cucumis* species, which are indigenous to the arid regions of South Africa (Kristkova *et al*., 2003), research demonstrated that crude extracts from *C. myriocarpus* fruit were allelopathic to a wide range of commercially available crops in agriculture (Mafeo, 2012), with strong auto-allelopathy whereby the allelochemicals could not be completely leached out, but could be leached out in *C. africanus* (Pofu, 2012). Mafeo (2006) demonstrated that exposure of *C. myriocarpus* seeds to 45°C improved germination. Apparently, the location of allelochemicals responsible for seed germination in the two *Cucumis* species differ as observed in other plant species (Bewley, 1997; Chen *et al*., 2005; Rice, 1984). The major locations of allelochemicals in seeds had been identified as exterior and interior to the testa (Emery, 1987; Kucera *et al.*, 2005). The observation by Pofu (2012) was unexplainable since *C. africanus* and *C. myriocarpus* seeds contain high concentrations of cucurbitacin B and cucurbitacin A, respectively (Chen *et al*., 2005), with cucurbitacin B and cucurbitacin A being insoluble and soluble in water, respectively (Chen *et al*., 2005).

2.1.4.2 Physical dormancy

Physical dormancy occurs primarily in angiosperms and is to date, unknown in gymnosperms (Baskin and Baskin, 1998). Physical dormancy specifically occurs in hard seeds as a result of the testas, which inherently inhibit water and gaseous exchanges (Baskin *et al*., 2000), that are indispensable for kick-starting the germination process (Campbell, 1990). Physical dormancy could occur as a result of the existence of one or more water-impermeable layers in the testa (Baskin and Baskin, 2004), along with the closed chalaza and/or micropylar ends (Salanenka *et al*., 2009). The chalaza region is the basal broad part of the seed, whereas the micropylar region is the pointed region that contains the hilum ─ a scar where there was a seed stalk (Campbell, 1990). During the maturation-drying period, cells in the different layers of the testa are differentiated to improve a stable environment in the endosperm by preventing imbibition and gaseous exchange (Baskin, 2003). During differentiation, existing “canals” which were formed during fertilisation (Campbell, 1990; Salenenka *et al*., 2009) are sealed with various cells, including cork (Baskin, 2003). The latter is impervious to water and gases (Campbell, 1990).

Physically dormant seeds hardly germinate until the testa is physically altered through either cracking or reduction in thickness to improve water permeability and gaseous exchange (Hartmann *et al*., 2010). Scarification is an artificial process, which is intended to break physical dormancy through altering the thickness of the testa (Hartmann *et al*., 2010). Commercial growers prefer acid scarification, where concentrated sulphuric acid is used (Emery, 1987), whereas vinegar is safer for plant species that do not have

extremely hard testa. In mechanical scarification, Emery (1987) demonstrated that testas could also be filed with a metal, rubbed with sandpaper, nicked with a knife or gently cracked with a hammer, all of which have the intended outcome of weakening the testa. Another popular method is hot-water scarification, where seeds are dropped into boiling water, with the container being removed from the heat source for cooling (Odo and Oleghe, 1988). Under natural conditions, fire, drying, freezing/thawing, high temperatures or microbial degradation modify the testa during winter, which have similar effects as passage through acids in the digestive tracts of various animals (Baskin, 2003). Once physical dormancy is eliminated using any of the described methods, imbibition and gaseous exchange are improved, with germination occurring unhindered over a wide range of environmental conditions (Emery, 1987).

The Cucurbitaceae Family, within the angiosperms, contains plant genera with seed testas that comprise a complex system of structurally distinct impermeable layers, with those in several commercial cultivars bearing lignified thickenings on radial walls (Baskin and Baskin, 2004; Esau, 1960). Lignified walls inherently restrict imbibition and the eventual exit of radicles (Baskin and Baskin, 1998). A morphological study in watermelon seeds demonstrated the existence of cutinised layers with nucellar tissues identified as being responsible for physical dormancy (Thornton, 1968). Improved germination in physically-dormant watermelon seeds was achieved by clipping the micropylar region with a razor blade, which constituted mechanical scarification (Thornton, 1968).

In *Cucumis sativas* (L.) and other commercial cucurbit seeds, between the testa and the endosperm is a thin membrane known as the perisperm-endosperm layer, which restricts the transport of solutes (Ramakrishna and Amritphale, 2005), with passage allowed only at the chalaza region (Salanenka *et al*., 2009). The perisperm-endosperm layer was confirmed in *Cucurbita maxima* (Duch.) seeds (Tao and Khan, 1974). The micropylar region in commercial *Cucumis* varieties also serves as a barrier for the exit of radicles, suggesting the existence of an unconfirmed layer (Salanenka *et al*., 2009). In Geraniaceae Family, the impermeable layer in the testa is a continuous layer of palisade cells, except in the chalaza region and is located below the outer polygonal and middle parenchyma layers of the outer integument (Meisert *et al*., 1999; Schulz *et al*., 1991). Generally, palisade cells are strongly lignified (Meisert *et al*., 2001), whereas the “chalaza canal” between the palisade cells of the chalaza region is closed by a suberised chalaza plug (cork), which enhanced impermeability (Boesewinkel and Been, 1979). Scarification of the palisade layer in affected seeds improved permeability and thereby imbibition and germination (Nell *et al*., 1981, Schulz *et al*., 1991).

After fruit harvest, certain seeds may not germinate because of a physiological process referred to as after-ripening (Campbell, 1990). The seed treatment to remove after-ripening involves imbibition, cold, hot, light, gibberellin (GA_3), smoke or nitric oxide stratification (Emery, 1987; Kucera *et al*., 2005). After-ripening should be distinguished from seed dormancy, where seeds do not germinate because of some structural and/or chemical inhibitory factors (Baskin and Baskin, 2004).

2.1.4.3 Physiological dormancy

Physiological dormancy is the commonest form of dormancy and occurs in seeds of both gymnosperms and all major angiosperms (Baskin and Baskin, 2004). In temperate seed banks, physiological dormancy is the most prevalent dormancy and the most abundant dormancy class (Baskin and Baskin, 2004). Seeds with physiological dormancy do not germinate until they have been subjected to a period of moist-chilling and/or moist-warm period referred to as stratification, with dormancy occurring at three degrees, namely, (1) deep, (2) intermediate and (3) non-deep (Baskin and Baskin, 2004). In deep physiological dormancy, excised embryos from seeds soon after harvest either do not grow or produce abnormal seedlings (Baskin *et al*., 2005, Finch-Savage *et al*., 1998). Generally, treatment with GA_3 does not break deep physiological dormancy, whereas 3-4 months exposure to cold or warm stratification is required to break this form of seed dormancy (Baskin *et al*., 2005). In contrast, in seeds with intermediate physiological dormancy, excised embryos from seeds soon after harvest produce normal seedlings, with GA_3 promoting germination (Finch-Savage *et al*., 1998). Although, cold stratification is a requirement for seeds with this degree of dormancy, dry storage referred to as after-ripening can shorten the stratification period (Finch-Savage *et al*., 1998). The majority of commercial seeds have non-deep physiological dormancy and embryos excised from seeds soon after harvest produce normal seedlings (Baskin and Baskin, 2004). Under natural conditions, physiological dormancy could be managed by storing seeds in moist soil over winter and/or could also be simulated by keeping seeds in a plastic bag containing a moist growing medium such as sand or vermiculite in the refrigerator for several months (Andersson *et al*., 1997). The optimum

temperature for stratification lies within the range of 1-5°C, which is the temperature range for most refrigerators. Generally, cold stratification involves mixing seeds with an equal volume of a moist growing medium in a closed container and then refrigerating (Baskin *et al*., 2005). Seeds need to be checked occasionally to ensure that the medium is moist, but should not be wet (Andersson *et al*., 1997). Also, similar conditions could be fulfilled naturally if seeds were placed in moist sandy soil outdoors in winter (Andersson *et al*., 1997).

Gibberellins are known to substitute for a chilling requirement in plants (Pinfield and Stobart, 1969; Webb and Dumbroff, 1969). Gibberellic acid escalates germination of seeds that require an after-ripening period for germination, as observed in cherries (*Prunus avium* L.) (Pillay and Edgerton, 1965). Brown and Van Staden (1973) demonstrated that germination of *Protea* and *Leucadendron* species could be improved by either stratification or application of GA_3. Germination of American hornbeam (*Carpinus caroliniana* Walt.) was increased by stratification, especially when it was followed by GA_3 treatment (Bretzloff and Pellett, 1979).

2.1.5 Morphology of testa in cucurbits

In most commercial cucurbit varieties, the testa imposes a physical barrier, which must be overcomed for the embryo to complete its germination. These covering layers, which may include testa and perisperm-endosperm layer, are known to display unique permeability properties in most seeds on account of the presence of semi-permeable layers, which allow water uptake and gaseous exchange, while restricting solute

transport (Ramakrishna and Amritphale, 2005). Incidentally, in certain seeds the testa maybe completely impermeable. The testa in plant species is dominantly formed by the outer integument layers (Campbell, 1990), which consist of lignified epidermis, hypodermis, several layers of sclerotic cells and an inner one-layered protective cover, which in mature seeds could be heavily lignified (Singh and Dathan, 2001).

Generally, the testa in cucurbit seeds comprises four layers: (1) epidermis, (2) hypodermis, (3) sclerenchyma and (4) aerenchyma, which surround the endosperm (Singh and Dathan, 1972). The epidermis comprises radially elongated cells, which have more or less thickened lignified walls (Singh and Dathan, 2001). However, cells could vary in length, with the longer ones typically occurring around the marginal bulge (Teppner, 2004). In contrast, the hypodermis has shortened cells, with slightly thickened lignified walls, where the thickenings form a reticulate structure (Esau, 1977; Lott, 1973). Sclerenchyma cells are dead thick-walled cells, highly variable in size and shape, giving rigidity to the seed (Esau, 1977; Metcalfe and Chalk, 1979). Due to their variability in size and shape, sclereids in seeds often characterise the plant species and are widely used in taxonomy (Barua and Dutta, 1959). Sclereid cells could be short, isodiametric brachysclereids (stone cells), elongated rod-like macrosclereids, bone-shaped, columnar osteosclereids, star shaped astrosclereids, long, slender filiform sclereids or branched trichoschlereids (Nicolson, 1960). Sclerenchyma cells, which permeate through the sclereid cells with heavily lignified cell walls (Singh and Dathan, 1972), give the seed coat its firmness (Metcalfe and Chalk, 1979). Lott (1973) described the tissues between the sclerenchymotuos layer and the cotyledons as a

spongy parenchyma, which is formed by irregular-shaped cells with numerous outgrowths. The aerenchyma comprises cells that are intertwined with enlarged intercellular spaces (Esau, 1977; Hather, 2000). Singh and Dathan (1972) reported that the wild cucurbit species have one or two layers of aerenchyma formed by small cells, whereas the domesticated cucurbit species have bigger cells and several layers.

Germination in physically dormant seeds, in most cases, occurs after a cork that closes the “water-gap” in the water-impermeable layers is removed or disrupted, thereby creating an entrance for water to the embryo (Baskin *et al*., 2000). In 12 of the 17 families that have physical dormancy, the existence of specialised “water-gaps”, which develops an opening for water entry have been discovered (Baskin and Baskin, 2001). The “water-gaps” had been identified as (1) the micropyle in Anacardiaceae; (2) the chalaza plug in Bixaceae, Cistaceae, Cochlospermaceae, Dipterocarpaceae, Malvaceae and Sarcolaenaceae; (3) the imbibition lid in Cannaceae; (4) the lens and hilar slit in Fabaceae (Baskin *et al*., 2000); (5) the micropyle-water-gap complex in Geraniaceae (Gama-Arachchige *et al*., 2011); a bulge-gap adjacent to the micropyle in Convolvulaceae (Jayasuriya *et al*., 2007) and a hilar-slit in Convolvulaceae (Jayasuriya *et al*., 2008).

2.1.6 Propagule selection for *in vitro* propagation and use of plant growth regulators

Selection of suitable propagule, composition of nutrient media, concentration of plant growth regulators (PGRs) and protocols used for *in vitro* propagation have significant

effects on shoot regeneration and rooting rates of propagules (Moreno and Riog, 1990). *In vitro* propagation begins with the selection of suitable propagules for use in suitable nutrient medium in order to promote the regeneration of new plantlets (George, 1993). Clean mother plants that are pathogen-free are important in the production of healthy plants. The use of *in vitro* propagation methods in mass production of commercial cucurbits is well-documented (Debeaujon and Branchard, 1992; Dong and Jia, 1991; Haque *et al*., 2008; Misra and Bhatnagar, 1995; Moreno and Riog, 1990). *In vitro* regeneration of commercial cucurbit species is dependent upon the genotype, propagule-part, propagule age and PGRs (Li *et al*., 2011). Conventionally, any part of the plant can be used as a propagule (Murashige, 1974). However, in *C. africanus*, cucurbitacin B is equally distributed in all organs of the plant (Jefrey, 1978, 1980), suggesting that *in vitro* propagation could not be feasible due to auto-allelopathy. To date, regeneration in watermelon (*Citrullus lanatus* Thunb.) cultivars had been successfully developed through organogenesis (Chen *et al*., 1998; Compton, 1999, 2000), but with shoot-tips (Alper *et al*., 1994; Compton *et al*., 1993), cotyledons (Adelberg *et al*., 1993; Compton, 1997), hypocotyls (Srivastava *et al*., 1989), nodal buds (Haque *et al*., 2008) and leaves (Sultana *et al*., 2004) being the preferred propagules.

In *Cucurbita maxima* (Duch.) the highest number of shoot regeneration was achieved from shoot-tip propagules when 6-benzyladeninepurine (BAP) was used (Mahzabin *et al*., 2008). In *Citrullus lanatus*, the use of cotyledon propagules was the most efficient method for plant regeneration since they contain parenchymatous cells, which respond faster to exogenous PGRs and enhance the regeneration of adventitious shoots

(Ananthakrishnan *et al*., 2003). Also, Islam *et al*. (1999) obtained high shoot regeneration from hypocotyl propagules in the medium that contained BAP and indole-3-acetic acid (IAA). In *Citrullus colocynthis* (L.) shoot regeneration was successfully achieved from nodal bud propagules in the medium that was supplemented with BAP (Ganasan and Huyop, 2010). In *Cucumis melo* (L.) leaf propagule induced maximum shoot regeneration (91%) when BAP was used alone in the regeneration medium. The superiority of BAP over other PGRs for shoot regeneration is extensively documented (Arulpragasem and Latiff, 1986; Amin *et al*., 1997; Benjamin *et al*., 1987).

Plant growth regulators play an important role in determining the developmental pathway of plant cells and tissues in culture media (Hartmann *et al*., 2010). Auxins, cytokinins and gibberellins are the most commonly used PGRs (George, 1993). The type and the concentration of PGRs used are dependent upon the plant species, the propagule cultured and the trial objective (Ting, 1982). Auxins and cytokinins are the most widely used PGRs in plant tissue culture and their concentrations determine the type of culture established or regenerated. High concentrations of auxin generally promote root formation, whereas those of cytokinin promote shoot regeneration (Hartmann *et al*., 2010). High cytokinin to auxin ratio promotes shoot proliferation, whereas high auxin to cytokinin ratio promotes root proliferation (Rout, 2004).

The type and concentration of PGRs in the growing medium are crucial factors in regenerations of Cucurbitaceae genera, with the presence of cytokinins being critical for shoot regeneration and auxins for root development (Li *et al*., 2011). High frequencies

for shoot regeneration from cotyledon propagules in *Citrullus lanatus* require concentrations of BAP alone (Li *et al*., 2011). However, when cotyledons are used as propagules in shoot regeneration, regenerated shoots should be transferred to elongation medium since prolonged culture on BAP medium not only stimulates callus formation, but also induces the proliferation of abnormal shoots (Dirks and Van Bugenum, 1989). The use of both cytokinins and auxins improve shoot regeneration (Compton and Gary, 1993; Hoque *et al*., 1995).

2.1.7 Suitable potting media for *in vitro* weaned plantlets

In vitro propagation provides an alternative method for rapid mass production of plants, but its ultimate success depends upon the successful transfer and establishment of the plants from *in vitro* to *ex vitro* conditions (Pospíšilová *et al*., 1999). Selection of suitable potting medium is important for the successful growth of *in vitro*-produced plantlets. Plants produced under *in vitro* conditions with controlled high relative humidity, diffused light and constant temperatures should be acclimatised prior to transfer from *in vitro* to *ex vitro* conditions in order to reduce shock and high mortalities (Pospíšilová *et al*., 2007). The type of potting mixture used during acclimatisation is one of the most important factors determining survival percentage of plantlets from *in vitro* to *ex vitro* conditions (Kaur *et al*., 2011).

The ideal potting medium for hardening weaned plantlets should provide rapid drainage with sufficient water retention to keep the root zone uniformly moist. Most container growers are convinced that "soilless" potting media work best (Bass, 1999). In addition

to providing excellent drainage, "soilless" potting media are lightweight (Bass, 1999). The potting medium should have adequate nutrients, good water holding capacity and drainage capabilities to ensure better growth of plantlets (Noble, 1993). Sand is a common amendment in propagation applications and is occasionally used in greenhouse mixes (Scholes, 1990). Sand improves drainage and adds mass to the pot to minimise blow-over in outdoor containers and provides neutral pH, without adding any nutrient element (Scholes, 1990). Occasionally, soil is used as a potting medium primarily because it is locally available.

Major considerations when using soil should be the presence of weed seed and residual chemicals. Ideally, in potting mixes soil should be a minor component or not used at all. Kurtar *et al.* (2010) reported the lowest survival rate (12.5–16.7%) of winter squash (*Cucurbita maxima*) and pumpkin (*Cucurbita moschata* Duch.) plantlets when cultivated in a mixture (v/v) of sand and soil. In *Tamarindus indica* (L.) soil substrate resulted in 43.5% plantlet survival rate, probably due to poor drainage that resulted in waterlogging (Kung'u *et al.,* 2008). Waterlogging is known to hamper gaseous exchange, which inhibits growth and ultimately leads to wilting due to physiological drought (Kung'u *et al*., 2008). The latter is a condition where plants wilt while in water due to the failure of cell membranes to maintain the process of osmosis (Salisbury and Ross. 1992). In contrast to other growing mixtures, vermiculite is sterile, light in mass and due to its plate-like structure, has high water-holding capacity along with cations such as K^+, Mg^{2+} and Ca^{2+} (Bunt, 1988). Notably, the expanded vermiculite should not be pressed or compacted during potting, especially when wet as this inherently destroys the desirable physical

properties (Bunt, 1988). Hygromix is a peat-based growing medium, manufactured in South Africa, which contains high quality nutrient supplements with adjusted macro- and micro-nutrients, thereby ensuring healthy growth of seedlings, plantlets and transplants (Bezuidenhout and Lamprecht, 2010). Commercialised plant species within the Cucurbitaceae Family require a loose and well-drained soil, with high organic matter content (Milind and Kaur, 2011). A good mix consists of one part each of potting soil, perlite, sphagnum peat moss, Hygromix and compost (Palada and Chang, 2003).

Compost is derived from the biological decomposition of organic matter and is accomplished through mixing and piling occasionally in order to promote aerobic and/or anaerobic decay. Composting eradicates disease-inducing micro-organisms, viable weed seeds and unpleasant odours (Dalzell, 1987). Compost can be used as a soil conditioner and/or a fertiliser (Nair *et al*., 2011). Also, Dalzell (1987) demonstrated that in some areas, compost provides a low-cost media amendment, but the consistency of the product and the particle size should always be considered. However, lack of consistent high-quality compost, prevents the widespread use of compost in organic potting mixes. Vermicompost, in combination with equal parts of sand and topsoil were most suitable for the production of good quality transplants in tomato (*Solanum lycopersicum* L.), eggplant (*Solanum melongena* L.) and chili pepper (*Capsicum annuum* L.) (Prasanna Kumar and Raheman, 2010).

2.1.8 Degree of nematode resistance in plants

Plants respond to attacks by pests through activating a number of active defences. Upon infection, plants re-direct cellular metabolism, activating or enhancing the activity of metabolic pathways to fight pests (Lattanzio *et al*., 2006). When a plant experiences attack by a pest, it responds with a systemic acquired response (SAR) where the "immune system" is primed to defend itself against the pathogen (Dangl and Jones, 2001). Plants synthesise a broad range of secondary metabolites including alkaloids and terpenoids that are toxic to pests. The secondary metabolites act as chemical defence against pathogens (Bell, 1980). In their natural environment, plants encounter numerous pests, with an appropriate response to attack by such pests leading to tolerant or resistant mechanisms that enable the plants to survive (Lattanzio *et al*., 2006; Roy and Kirchner, 2000). Resistant mechanisms in attacked plants refer to traits that inhibit or limit attack, whereas tolerant mechanisms do not limit attack, but offset consequences on plant fitness by adjusting its physiology to buffer the effect of attacks (Paul *et al*., 2000). Resistant mechanisms in plants that include physical and/or chemical isolation of pathogens from healthy cells are termed hypersensitive responses (Taylor *et al*., 2004). Generally, hypersensitive resistance is characterised by death of cells around the invading pests, whereas tolerance often involves some degree of compensation for disease damage (Seinhorst, 1967). Plants can, for instance, tolerate attacks by increasing chlorophyll content in leaves, increasing leaf size, advancing the timing of bud break, delaying senescence of infected tissues and by improving nutrient uptake (Dietrich *et al*., 2005).

Plants produce a wide range of secondary metabolites, either as part of their normal growth, development or in response to biotic or abiotic stress (Morrissey and Osbourn, 1999). Preformed secondary metabolites that occur constitutively in healthy plants are likely to represent inbuilt chemical barriers to pests and protect plants against attack by a wide range of potential pests (Lattanzio *et al*., 2006). In contrast, induced defence chemical compounds are synthesised in response to biotic stress as part of the plant defence response and are restricted to the damaged tissue (Purrington, 2000). Both tolerance and resistance traits require the reallocation of host resources, therefore, defensive chemical compounds are considered to be costly for plants because of the resources used in their biosynthesis or the ecological consequences of their accumulation (Wittstock and Gershenzon, 2002). Wittstock and Gershenzon (2002) suggested that plants could reduce their cost of chemical defence through the synthesis of defence compounds only after an initial damage by a pathogen. However, the strategy could be hazardous because the initial attack might be too rapid or too severe for an effective defence response. Koricheva *et al*. (2004) suggested that, plants which are likely to suffer frequent and/or serious damage may be better off capitalising mainly in constitutive defences, whereas plants that are hardly attacked might rely predominantly on induced defences.

Pofu and Mashela (2012) demonstrated that *C. africanus* and *C. myriocarpus* were compatible with selected nematode-susceptible watermelon cultivars. Thus, the host-status and host-sensitivity of *C. africanus* and *C. myriocarpus* to various root-knot (*Meloidogyne* species) nematodes were investigated under various conditions to

determine if they had the potential to serve as seedling rootstocks in suppression of nematodes in watermelon husbandry (Pofu, 2012). Results of the trials showed that *C. africanus* and *C. myriocarpus* were resistant to *M. incognita* races 2 and 4 and *M. javanica.* Possibly, the two nematode-resistant *Cucumis* species could serve as seedling rootstocks to *Citrullus lanatus* cultivars (Mofokeng, 2005; Pofu and Mashela, 2012), which have no genotypes that are resistant to the nematode (Pofu, 2012). However, under field conditions the two *Cucumis* species were tolerant to the spiral nematode (*Helicotylenchus dihystera*) and the ring nematode (*Criconema mutabile*), whereas the two were still highly resistant to *Meloidogyne* species (Pofu *et al*., 2012a). However, it is not known whether *Cucumis* plantlet rootstocks that had been produced *in vitro* could still retain their resistance to *Meloidogyne* species after exposure to various PGRs. Others demonstrated that nematode resistance is lost under high temperature (Dropkin, 1969), cyclic salinity (Mashela *et al*., 1992) and attack by honey-dew-inducing insects such as the greenhouse whiteflies (*Trialeurode vaporariorum* Westwood) (Pofu *et al*., 2011).

2.2 Research gaps

The feasibility of *in vitro* propagation to improve uniformity of plant population densities in *C. africanus* and *C. myriocarpus* remains undocumented. *In vitro* propagation of the two *Cucumis* species would require detailed studies for eliminating seed dormancy, which could possibly encompasses chemical and physical dormancies. Should physical dormancy exists, the morphology of testa and the possibility of the existence of “water gaps” in seeds would have to be investigated. Also, whether the two *Cucumis* species

would retain nematode resistance after undergoing *in vitro* propagation would require additional investigations, alongside the appropriate growing media for weaning under greenhouse conditions.

CHAPTER 3
SEED DORMANCY AND *IN VITRO* SEEDLING PERFORMANCE OF TWO INDIGENOUS *CUCUMIS* SPECIES

3.1 Introduction

Seed dormancy in wild watermelon (*Cucumis africanus* LF.) and wild cucumber (*Cucumis myriocarpus* Naude.) is suspected to contribute to poor establishment or high variation in population density of seedlings, thereby limiting the potential cultivation of the two *Cucumis* species as future crops for potential uses in various industries. Generally, dormancy as a result of hard testa, referred to as physical dormancy, had been identified in certain plant species within the Cucurbitaceae Family (Baskin, 2003; Baskin and Baskin, 2005). The testa in the family comprises a complex system of structurally distinct layers, some with evidence of lignified thickenings on the radial walls, which restrict imbibition (Esau, 1965). Mafeo (2014) is of the opinion that chemical dormancy attributed to allelochemicals, could also contribute to uneven stands in *C. myriocarpus.* In the Cucurbitaceae Family, seeds contain high concentrations of cucurbitacin, which are produced as secondary metabolites through the mevalonic acid pathway (Érzek and Kiraly, 1986; Inderjit and Malik, 2002). Although allelochemicals

supress growth of other plant species, auto-allelopathy in certain plant species abounds (Rice, 1984). Since *C. africanus* and *C. myriocarpus* seeds contain high concentrations of cucurbitacin B and cucurbitacin A, respectively (Chen *et al*., 2005), it is probable that the two chemical compounds could also contribute towards erratic germination and therefore, uneven emergence. The objective of this study was to determine whether seed dormancy in *C. africanus* and *C. myriocarpus* would be ameliorated to allow for *in vitro* sexual propagation to establish pathogen-free parent stock.

3.2 Materials and methods

3.2.1 Location and seed preparation

In vitro germination studies were conducted at African Biotechnologies PTY (Ltd) Commercial Plant Tissue Culture Laboratory, Tzaneen (23°51'50.08'' S, 30°0'30.37'' E) from summer (October-December) to early autumn (January-March). Seeds from mature fruits of the two *Cucumis* species collected from cultivated plants at the University of Limpopo, Turfloop campus (23°53'10'' S, 29°44'15'' E) were separated from the fruit-carpus of each *Cucumis* species and separately shade-dried at room temperature for 7 days (Mafeo and Mashela, 2009). Unless otherwise stated, all chemicals used in this study were of analytical grade (Duchefa Laboratories, Harlem, Netherlands). Forty seeds/group of each *Cucumis* species were wrapped in light bonnets and leached for 0, 2, 4, 6, 8, 10 and 12 h in running tapwater and then sterilised in a mixture of 40% Domestos solution [5.2% (v/v) NaOCl active ingredient] plus 3 drops of Tween-20 [0.1% (v/v)] for 15 min during continuous agitation. After rinsing thrice in pasteurised distilled water, seed coats of half the seeds/group were aseptically severed at the chalaza region with separate sterile scalpel blade to

constitute non-leached-scarified (NLS) and leached-scarified (LS), whereas the other half served as non-leached control (NLC) and leached-control (LC) seeds (Figure 3.1).

Figure 3.1 (a) Mature seeds of wild *Cucumis* species. (b) *Cucumis africanus* and (c) *Cucumis myriocarpus* fruits. (d) Leaching *Cucumis* seeds under running tapwater and (e) disinfected prior scarification at the (f) chalaza region (CR) of the seed (seed image: S. Wood, RBG Kew).

3.2.2 Culture conditions and experimental design

The Murashige and Skoog (MS) medium that contained 30 g sucrose/L distilled water without plant growth regulators (PGRs) was prepared, adjusted to pH 5.8 and then gelled with 6.5 g micro agar/L distilled water, followed by autoclaving at 121°C for 20 min (Murashige and Skoog, 1962). After cooling the medium for 12 h, all seeds of each *Cucumis* species were cultured, with the seven leaching treatments laid out in a completely randomised design (CRD), at five replications (n = 35). In order to produce etiolated seedlings, the cultures were incubated in darkness within a growth chamber at 25°C and 50-60% relative humidity (RH) for 7 weeks. Etiolated cultures were then moved to a 12 h light/dark cycle on racks, fitted with cool white fluorescent tubes (Phillips, South Africa) of 40 µM/m^2/s irradiance at 25°C and 50-60% RH for 4 weeks.

3.2.3 Data collection

Germinated seeds in both *Cucumis* species were counted daily and expressed as germination percentage (GP) using the relation (AOSA 1990): $GP = \frac{\text{Germinated seeds}}{\text{Total seeds}} \times 100$. Three seedling performance tests, namely, mean germination time (MGT), germination index (GI) and germination rate (GR), were calculated using the Ellis and Roberts (1981) method, where: $MGT = \Sigma Dn/\Sigma n$, with *n* being the number of seeds that germinated on day D, where D was the number of days from initiation to the completion of the germination process,

$GI = \frac{\text{No. of germinated seedlings}}{\text{Days of first count}} + \ldots + \frac{\text{No. of germinated seedlings}}{\text{Days of final count}}$ and $GR = \frac{\text{Number of all germinated seeds}}{\text{Total experiment duration time}}$.

Data collection ceased at day 49 after the initial count.

3.2.4 Data analysis

Germination and seedling performance data were subjected to analysis of variance (ANOVA) using SAS software (SAS Institute, Inc. 2008). Discrete data were transformed through $\log_{10}(x + 1)$ to homogenise the variances (Gomez and Gomez, 1984), but untransformed means were reported. Waller-Duncan multiple range test was used to separate the means at the probability level of 5%. Seedling performance variables from LC and LS seeds with significant ($P \leq 0.05$) treatment means were subjected to lines of the best fit using seedling responses to increasing leaching time. Generated relationships were further modelled by the regression curve estimates from the quadratic equation ($Y = b_2x^2 + b_1x + a$), where Y = seedling performance response and x being the optimum leaching time, which was derived from $x = – b_1/2b_2$ (Mamphiswana *et al*., 2010). Unless otherwise stated, only treatment means significant at 5% level of probability were discussed (Appendices 3.1-3.8).

3.3 Results

3.3.1 *Cucumis africanus* seeds

The NLC seeds did not germinate (data not shown), whereas the LC, NLS and LS seeds germinated.

3.3.1.1 Germination percentage in *Cucumis africanus*

Treatments had significant ($P \leq 0.05$) effects in GP of LC and LS seeds of *C. africanus* (Appendix 3.1). Leaching treatment, contributed 41% of the total treatment variation (TTV) in GP of LC seeds (Table 3.1). Similarly, in LS seeds, leaching treatments contributed 72% of TTV in GP (Table 3.1). Mean performance variables of seedlings

were presented both in tabular and graphic formats. In tabular format (Table 3.2), NLC seeds did not germinate completely and assessment was done relative to 2-h-leaching time. Relative to 2-h-leaching time, in LC seeds, GP increased at low leaching times, which was followed by a decrease at high leaching times, whereas, in LS seeds, mean germination performance values in GP increased by 35-360% (Table 3.2).

The mean performance variables (y-axis), when regressed against the increasing leaching time (x-axis), had quadratic relationships (Figure 3.2), which allowed the computation of the optimum leaching time from $x = -b_1/2b_2$ (Table 3.3). The models explained 75% and 80% TTV in *C. africanus* LC and LS seeds, respectively (Figure 3.2a). Optimum GPs for LC and LS seeds were achieved at 6.6 h (52% germinated seeds) and 5.6 h (89% germinated seeds) leaching times, respectively (Table 3.3). The optimum leaching time for GP in LS seeds was lower than that in LC seeds, but had much higher optimum GP value of 89%.

3.3.1.2 Seedling performance in *Cucumis africanus*

Treatments had significant ($P \leq 0.05$) effects in LC and LS seeds of *C. africanus* (Appendix 3.2-3.4). Leaching treatment had slight significant ($P \leq 0.10$) effect in MGT of LC seeds, contributing 35% of the TTV in MGT of LC seeds (Table 3.1). However, in GI and GR of LC seeds, leaching treatment had significant ($P \leq 0.05$) effects, contributing 44% and 40% of TTV in GI and GR, respectively. In LS seeds, treatments also had significant ($P \leq 0.05$) effects, contributing 68%, 65% and 70% of TTV in MGT, GI and GR, respectively (Table 3.1).

Relative to 2-h-leaching time, MGT increased at low leaching times, which was followed by a decrease at high leaching times. However, in GI and GR the trend was similar in both cases as they increased by 7-378% and 5-168%, respectively (Table 3.2). In contrast, mean germination performance of LS seeds had much high magnitudes as shown by their respective relative values to the NLS seeds. In LS seeds, relative to untreated control, all MGT values decreased at an increasing rate from –17% to –4%. However, GI and GR performance values increased by 363-675% and 250-300%, respectively (Table 3.2).

In all mean seedling performance variables, increase in MGT, GI and GR over increasing leaching times also exhibited quadratic relationships (Table 3.3). In LC seeds, quadratic relationships were explained by 82%, 62% and 89% of TTV in MGT, GI and GR, respectively (Figure 3.2b,c,d), with the optimum leaching times and optimum performance values being 7.1 h and 25 days for MGT, 6.2 h and 0.50 seedlings/day for GI and 6.7 h and 0.99 seedlings/day for GR, respectively (Table 3.3).

In contrast, in LS seeds, quadratic relationships were explained by 67%, 67% and 80% of TTV in MGT, GI and GR, respectively (Figure 3.2b,c,d), with the optimum leaching times and optimum performance values being 5.7 h and 40 days for MGT, 6.4 h and 0.60 seedlings/day for GI and 7.7 h and 1.70 seedlings/day for GR, respectively. In LS seeds, the optimum leaching times and optimum performance values in GI and GR were much higher than those in LC seeds (Table 3.3).

3.3.2 *Cucumis myriocarpus* seeds

All the NLC and LC seeds did not germinate (data not shown). However, the NLS and LS seeds germinated.

3.3.2.1 Germination percentage in *Cucumis myriocarpus*

Treatments had significant ($P \leq 0.05$) effects in GP of LS seeds in *C. myriocarpus* (Appendix 3.5). Leaching treatment, contributed 76% of the TTV in GP of LS seeds (Table 3.4). The mean performance variables of seedlings were also presented both in tabular and graphic formats. Relative to NLC seed, leaching increased GP of LS seeds by 200-400% (Table 3.5). Increase in GP over increasing leaching times also exhibited quadratic relationships. The models explained 90% in LS seeds for *C. myriocarpus* (Figure 3.3a) with the optimum GP achieved at 6.7 h (93% germinated seeds) leaching times (Table 3.6).

3.3.2.2 Seedling performance in *Cucumis myriocarpus*

In all seedling performance variables, LS treatment effects were significant at 5% level of probability, except for MGT where effects were observed at 10% level of probability (Appendix 3.6-3.8). Treatment effects of LS seeds contributed 5%, 54% and 61% of TTV in MGT, GI and GR, respectively (Table 3.4). Relative to NLC seed, leaching increased MGT, GI and GR of LS seeds by 5-14%, 32-145% and 100-150% (Table 3.5), respectively, with seedling performance variables on increasing leaching times having

strong quadratic relationships (Figure 3.3). The relationship explained 86%, 75% and 89% of TTV in MGT, GI and GR, respectively (Figure 3.3b,c,d), with the optimum leaching times and optimum performance values being 6.3 h and 41 days for MGT, 9.5 h and 0.90 seedlings/day for GI and 9.0 h and 0.20 seedlings/day for GR, respectively (Table 3.6).

Table 3.1 Mean sum of squares for *in vitro* germination of leached-control (LC) and leached-scarified (LS) seeds of *Cucumis africanus*.

Source	DF	GP[z]		MGT		GI		GR	
		SS	%	SS	%	SS	%	SS	%
LC seeds									
Treatment	6	1017.1	41**	6595.8	35*	0.0154	44**	0.004	40**
Error	28	1440.0	59	12037.7	65	0.0200	56	0.006	60
Total	34	2457.1	100	18633.5	100	0.0354	100	0.010	100
LS seeds									
Treatment	6	17166.7	72**	197.05	68**	1.3036	65**	0.0646	70**
Error	28	6793.3	28	92.34	32	0.7035	35	0.0274	30
Total	34	23960.0	100	289.39	100	2.0069	100	0.0921	100

* Significant at P ≤ 0.10, ** significant at P ≤ 0.05.

[z]GP = germination percentage, MGT = mean germination time, GI = germination index and GR = germination rate.

Table 3.2 Responses of germination percentage (GP), mean germination time (MGT), germination index (GI) and

germination rate (GR) of leached-control (LC) and leached-scarified (LS) seeds of *Cucumis africanus in vitro*.

Leaching	GP		MGT		GI		GR	
(h)	Variable[y]	%[z]	Variable	%	Variable	%	Variable	%
LC seeds								
0	0[c]	–	0.00[b]	–	0.00[c]	–	0.00[c]	–
2	45[a]	–	18.96[a]	–	0.14[b]	–	0.38[b]	–
4	46[a]	2	22.64[a]	19	0.60[a]	328	1.00[a]	163
6	54[a]	20	22.46[a]	18	0.67[a]	378	1.02[a]	168
8	40[ab]	– 11	27.42[a]	45	0.30[ab]	114	0.76[ab]	100
10	35[bc]	– 22	16.53[a]	– 13	0.15[b]	7	0.80[ab]	111
12	30[bc]	– 33	17.78[a]	– 6	0.18[b]	29	0.40[b]	5
LS seeds								
0	20[c]	–	47.28[a]	–	0.08[c]	–	0.40[b]	–
2	88[a]	340	39.24[c]	– 17	0.54[ab]	575	1.40[a]	250
4	92[a]	360	40.84[bc]	– 14	0.62[a]	675	1.60[a]	300
6	84[a]	320	41.92[bc]	– 11	0.60[a]	650	1.50[a]	275
8	66[a]	230	41.80[bc]	– 11	0.48[ab]	500	1.60[a]	300
10	53[ab]	165	43.80[b]	– 7	0.37[b]	363	1.50[a]	275
12	27[b]	35	45.45[a]	– 4	0.40[b]	400	1.50[a]	275

[y]Column means with the same letter were not different (P ≤ 0.05) according to Waller-Duncan multiple range test.
[z]Impact = [(variable/control – 1) x 100].

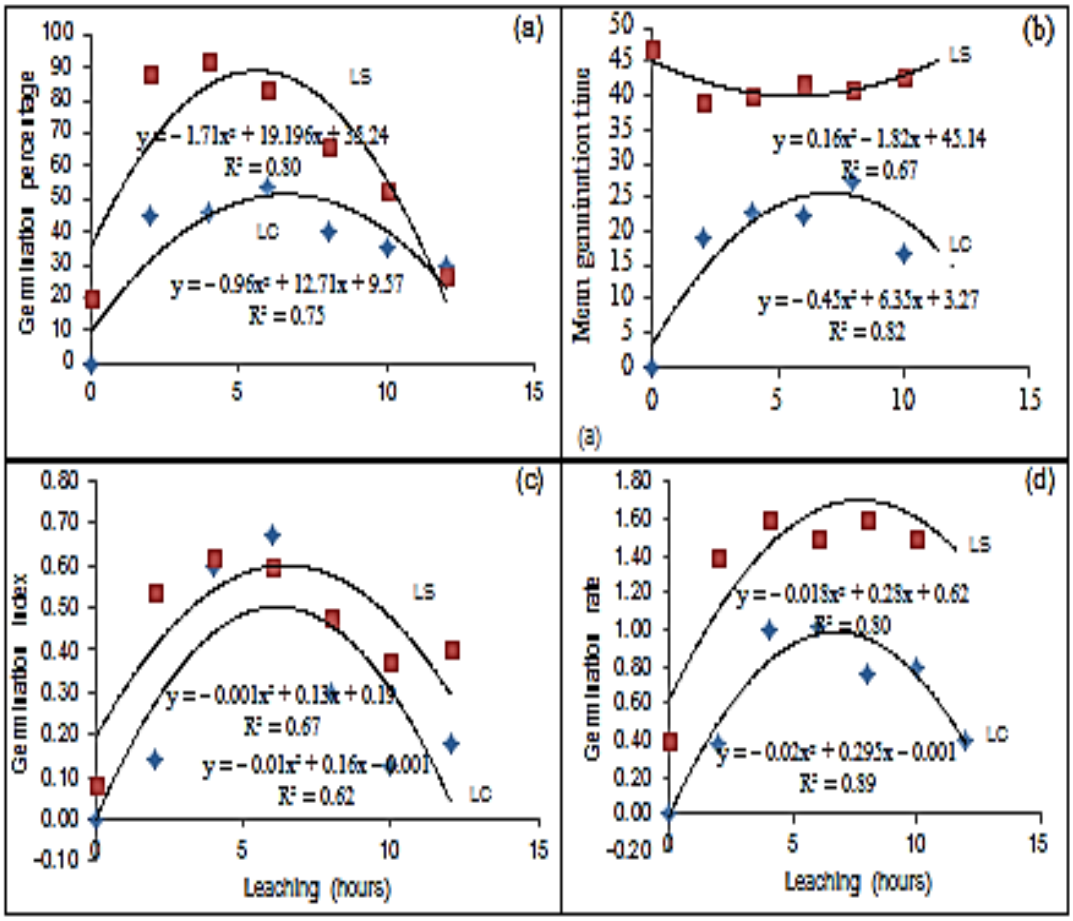

Figure 3.2 *In vitro* responses of (a) germination percentage (GP), (b) mean germination time (MGT), (c) germination index (GI) and (d) germination rate (GR) of leached-control (LC) and leached-scarified (LS) seeds of *Cucumis africanus in vitro*.

Table 3.3 *In vitro* optimum germination percentage (GP), mean germination time (MGT), germination index (GI) and germination rate (GR) of leached-control (LC) and leached-scarified (LS) seeds of *Cucumis africanus in vitro*.

Plant variables	**Quadratic relationship**	**R^2**	**x^z**	**y^z**	**P ≤**
	LC seeds				
Germination percentage	$Y = -0.96x^2 + 12.71x + 9.57$	0.75	6.6	52	0.05
Mean germination time	$Y = -0.45x^2 + 6.35x + 3.27$	0.82	7.1	25	0.10
Germination index	$Y = -0.01x^2 + 0.16x - 0.001$	0.62	6.2	0.50	0.05
Germination rate	$Y = -0.02x^2 + 0.295x - 0.001$	0.89	6.7	0.99	0.05
	LS seeds				
Germination percentage	$Y = -1.71x^2 + 19.196x + 35.24$	0.80	5.6	89	0.05
Mean germination time	$Y = 0.16x^2 - 1.82x + 45.14$	0.67	5.7	40	0.05
Germination index	$Y = -0.01x^2 + 0.13x + 0.19$	0.67	6.4	0.60	0.05
Germination rate	$Y = -0.018x^2 + 0.28x + 0.62$	0.80	7.7	1.70	0.05

[z]Calculated optimum response concentration $x = -b_1/2b_2$, where for germination percentage, $b_1 = 12.71$ and $b_2 = -0.96$, respectively.

Table 3.4 Mean sum of squares for *in vitro* germination of leached-scarified (LS) seeds of *Cucumis myriocarpus*.

Source	DF	GP		MGT		GI		GR	
		SS	%	SS	%	SS	%	SS	%
Treatment	6	26068.6	76**	91.94	5*	1.2949	54**	0.0574	61**
Error	28	8360.0	24	1800.65	95	1.0920	45	0.0374	39
Total	34	34428.6	100	1892.59	100	2.3869	100	0.0949	100

* Significant at P ≤ 0.10, ** significant at P ≤ 0.05.

Table 3.5 Responses of germination percentage (GP), mean germination time (MGT), germination index (GI) and germination rate (GR) of leached-scarified (LS) seeds of *Cucumis myriocarpus in vitro*.

Leaching	GP		MGT		GI		GR	
(h)	Variable[y]	%[z]	Variable	%	Variable	%	Variable	%
0	20.00[c]	–	36.22[a]	–	0.44[c]	–	0.08[b]	–
2	90.00[ab]	350	41.20[a]	14	0.58[bc]	32	0.18[a]	125
4	98.00[a]	390	40.52[a]	12	0.78[abc]	77	0.19[a]	138
6	82.00[ab]	310	38.16[a]	5	0.70[abc]	59	0.16[a]	100
8	100.00[a]	400	38.40[a]	6	1.08[a]	145	0.20[a]	150
10	60.00[b]	200	40.60[a]	12	0.82[abc]	86	0.20[a]	150
12	100.00[a]	400	39.50[a]	9	0.88[ab]	100	0.20[a]	150

[y]Column means with the same letter were not different (P ≤ 0.05) according to Waller-Duncan multiple range test.

[z]Impact = [(variable/control – 1) x 100].

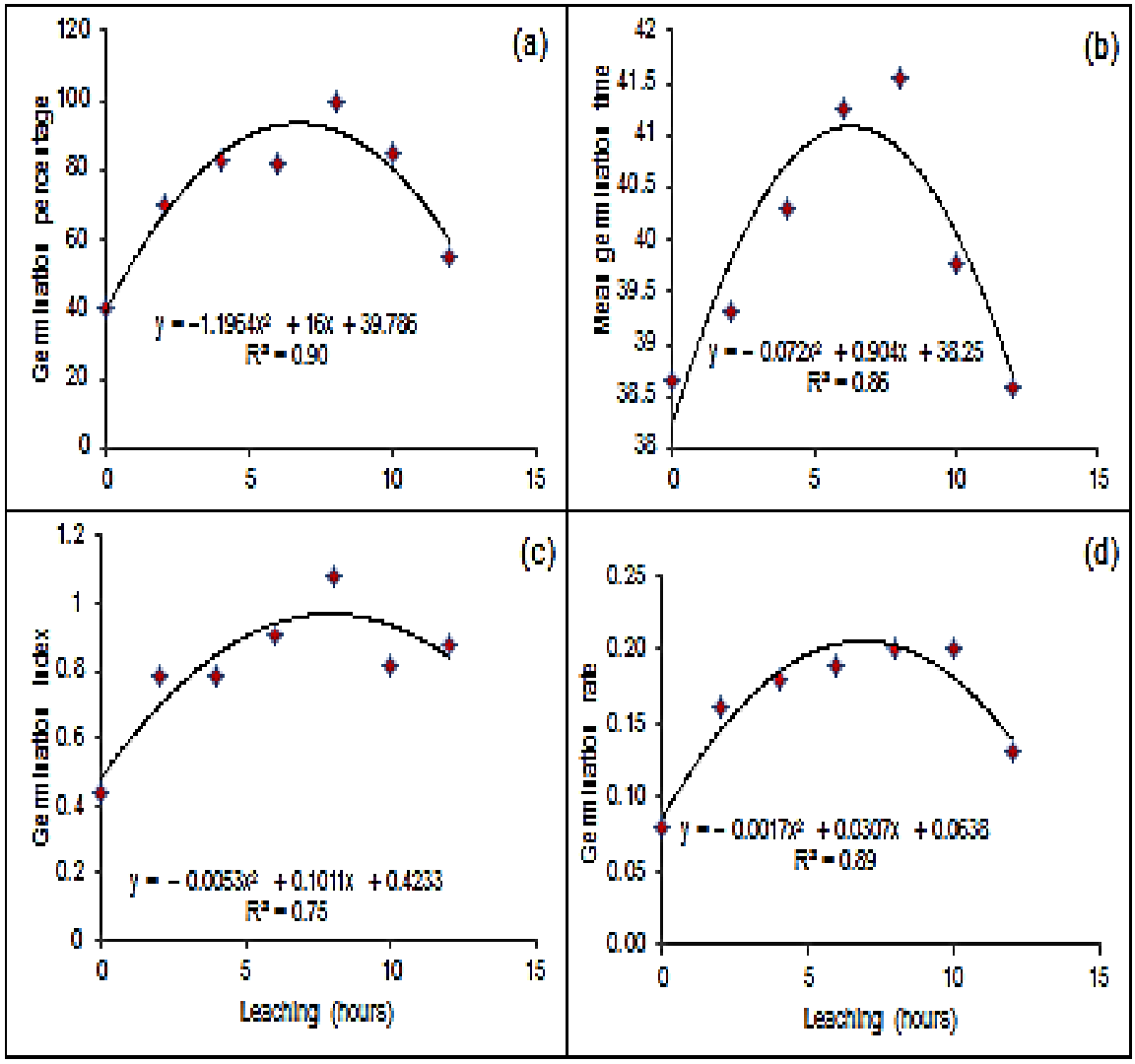

Figure 3.3 *In vitro* responses of (a) germination percentage (GP), (b) mean germination time (MGT), (c) germination index (GI) and (d) germination rate (GR) of leached-scarified (LS) seeds of *Cucumis myriocarpus in vitro*.

Table 3.6 *In vitro* optimum germination percentage (GP), mean germination time (MGT), germination index (GI) and germination rate (GR) of leached-scarified (LS) seeds of *Cucumis myriocarpus*.

Plant variable	**Quadratic relationship**	**R^2**	**x^z**	**y^z**	**P ≤**
Germination percentage	$Y = -1.1964x^2 + 16x + 39.786$	0.90	6.7	93	0.05
Mean germination time	$Y = -0.072x^2 + 0.904x + 38.25$	0.86	6.3	41	0.10
Germination index	$Y = -0.0053x^2 + 0.101x + 0.423$	0.75	9.5	0.9	0.05
Germination rate	$Y = -0.0017x^2 + 0.031x + 0.064$	0.89	9.0	0.2	0.05

[z]Calculated optimum leaching time $x = -b_1/2b_2$, where for germination percentage, $b_1 = 16$ and $b_2 = -1.1964$, respectively.

3.4 Discussion

3.4.1 Seed germination

In both *Cucumis* species, the NLC seeds failed to germinate completely *in vitro*, where growing conditions for germination were optimal. Germination, initiated by successful imbibition of water, is complete when the radicle raptures the testa (Hartmann *et al.*, 2010). Generally, when environmental conditions are favourable, germination could be restricted by the hard testa, which inhibits imbibition and/or the exit of the radicle, which is then referred to as physical seed dormancy (Baskin *et al*., 2000) and/or allelochemicals – referred to as chemical seed dormancy (Kobayashi, 2004). In both *C. africanus* and *C. myriocarpus*, substantial evidence existed that both chemical and physical seed dormancies are in place. However, with the allelochemicals deposited at different locations of the seed.

3.4.2 Existence of chemical dormancy

The distance between the x-axis and LC seeds in *C. africanus* and failure to germinate of LC seeds alone in *C. myriocarpus*, along with failure of NLC seeds to germinate under *in vitro* conditions in both *Cucumis* species, demonstrated the existence of chemical dormancy. In *C. africanus* and *C. myriocarpus,* the allelochemicals cucurbitacin B ($C_{32}H_{48}O_8$) and cucurbitacin A [cucumin ($C_{27}H_{40}0_9$) and leptodermin ($C_{27}H_{38}0_8$)], respectively (Chen *et al*., 2005), have the attributes to inhibit seed germination. Cucurbitacins have been extensively cited as being responsible for chemical dormancy in various plant species within the Cucurbitaceae Family (Chen *et al*., 2005; Martin and Blackburn, 2003; Rice, 1984).

In the two *Cucumis* species, chemical dormancy as a result of cucurbitacins appeared to be located on the outer layers of the testa in *C. africanus*, whereas in *C. myriocarpus* the cucurbitacins are deposited within the testa (Rice, 1984). In *C. africanus,* chemical dormancy was eliminated through leaching alone in running tapwater, whereas in *C. myriocarpus* the treatment had no effect, thereby resulting in failure to germinate. The observations agree with those of Emery (1987), who noted that exposure to running water was effective in eliminating chemical dormancy only when the chemicals were deposited outside the testa.

Generally, under natural conditions, once allelochemicals have entered the growing medium at bioactive concentrations, their bioactivities are limited by adsorption, leaching and microbial activities (Blum, 1999; Inderjit *et al*., 1999), which could then enhance germination. However, when allelochemicals are not sufficiently leached out by water or metabolised by soil microbial organisms in the rhizosphere (Rice, 1984), germination is checked, ensuring that germination does not occur until sufficient rainfall to leach out allelochemicals has occurred, which serves as a survival mechanism in various plant species (Rice, 1984). In contrast, when allelochemicals occur inside the impervious testa, especially when the concentrations are high, activation of metabolism in the embryo could be blocked (Eira and Caldas, 2000).

Das (1985) demonstrated that in *Citrullus colocynthis*, gibberellic acid (GA_3) which is required for seed germination was inhibited as a result of seeds containing considerable quantities of cucurbitacin B. Gibberellic acid stimulates the cells of germinating seeds to produce mRNA molecules that are programmed for hydrolytic

enzymes (Hartmann *et al*., 2010). In *Citrullus colocynthis*, a small amount (9 µg ml/L concentration) of cucurbitacin B in the embryo reduced GA_3 by 50% (Guha and Sen, 1973). Generally, seed germination is a chemical process that is initiated by imbibition, followed by the release of GA_3, which stimulates the aleurone layer to release alpha-amylase, which hydrolyses starch in the endosperm to glucose molecules, which are absorbed by the cotyledons, resulting in growth of the embryo, starting with the radicle (Campbell, 1990). Upon the emergence of the radicle from the testa, germination is said to have occurred (Campbell, 1990). Notably, allelochemicals within the testas are difficult to leach out, unless the testas had been scarified, as observed in the current study.

3.4.3 Existence of physical dormancy

In *C. africanus* seeds, the distance between the curves of LC and LS seeds demonstrated the feasibility of the existence of physical dormancy. Similarly, the failure of NLC seeds to germinate during *in vitro* propagation unless the seeds are leached and scarified was also evidence that physical dormancy existed in this *Cucumis* species. Notably, the NLC seeds under *in vitro* conditions did not germinate, which could be explained in terms of the absence of microbial organisms which reduce the thickness of the hard testas under soil conditions (Blum, 1999; Inderjit *et al*., 1999).

Physical dormancy in hard-coated seeds is associated with several main mechanical layers of testa, which could be palisade of radially-elongated cells that inhibit water movement to embryos (Egley, 1989; Werker, 1997), with the sclerenchyma having the most heavily lignified thickened cell walls (Campbell, 1990; Singh and Dathan,

1972, 1998). The testa in seeds are heavily lignified with sclereid cells, tightly packed together and impregnated with water-repellent features, including suberin, cutin and lignin (Baskin, 2003; Rolston, 1978). In most Cucurbitaceae species, heavily lignified microsclerid or malpighian cells also restrict water movement through the palisade cells and as a result, seeds are prevented from germinating until physical dormancy is broken through scarification (Baskin and Baskin, 1998; Egley, 1989; Offord and Meagher, 2009), as observed in seeds of *C. africanus* and *C. myriocarpus*.

In the two *Cucumis* species, higher germination percentages observed in LS seeds, suggested that the existence of physical dormancy was eliminated through physical scarification at the chalaza region, which in addition to allowing imbibition, enabled exit of the radicle. Physical dormancy had also been shown to be a limiting factor in germination performance of economically-cultivated *Cucumis* species (Baskin and Baskin, 2005; Shifriss and George, 1965). In *Ecballium elaterium* (L.) A. Rich., a minor crop in the Cucurbitaceae Family, which contains 3.8% total cucurbitacin content in seeds, physical dormancy was eliminated through cracking the seed coat slightly and immersing seeds in water overnight (Attard and Scicluna-Spiteri, 2003; Costich, 1995; Kirschman and Suber, 1989). In *Citrullus colocynthis* (L.) decoating and/or abrasion of the seed coat by sandpaper eliminated dormancy (Koller *et al*., 1963; Saberi *et al*., 2011). In *Cucumis melo* (L.), weakening of the testa enhanced imbibition, germination and the associated seedling emergence (Welbaum *et al*., 1995), whereas in *Citrullus lanatus* (Thunb.) cultivars complete decoating of seeds enhanced germination and the resultant emergence (Krug *et al*., 2005).

Under natural conditions, physical dormancy can be broken by various factors, including high temperatures, fluctuating high and cold temperatures, fire, freezing/thawing, drying or passage through the digestive tract of animals (Baskin and Baskin, 1998). Mechanically, physical dormancy can be broken down through microbial degradation and scarification, all relying on the reduction of the testa layers (Emery, 1987; Hartmann *et al*., 2010; Mafeo, 2014; Schmidt, 1980). Mechanical scarification is a technique for overcoming the effect of an impermeable testa and can be achieved through rubbing seeds between two pieces of sandpaper (Koller *et al*., 1963), mixing seeds with sand and vigorously shaken in a jar (Schmidt, 1980) or rupturing the hard seed coat through any physical structure or through immersing seeds in hot water (Emery, 1987).

3.4.4 Seedling performance

Seedling performance is described using various indices, but in this study the variable was limited to MGT, GI and GR. Mean germination time (MGT) in a seed lot is the average time taken in a seed to germinate (in days), from start of imbibition to radicle emergence (Ellis and Roberts, 1980). High MGT in a seed lot is an indication of delayed germination, which is associated with seed dormancy (Chen *et al*., 2006), low seed vigour (Matthews and Powell, 2012), ageing (Osbourne, 1983), deterioration (Basak *et al*., 2006) and germination inhibitors (Baskin *et al*., 2000). In *C. africanus* seeds, low optimum MGT (25 days) occurred in LC seeds, although it was expected in LS seeds, which had optimum MGT of 40 days. The reason for this was not clear, since in *C. myriocarpus* LC seeds also had optimum MGT of 41 days. Similar observations were noted in *Panicum virgatum* (L.) germination trials, whereby elimination of inhibitory features through scarification did not reduce MGT (Zhang

and Maun, 1989). The high MGT in LS seeds should probably be viewed alongside the rapid removal of allelochemicals, which are known to have stimulating effects on biological activities at low concentrations (Liu *et al*., 2003; Mashela *et al*., 2013; Pelinganga *et al*., 2013). Apparently, after the stimulating effect has been lost due to the leaching of seeds, on average, seed lots take a much longer period from imbibition to the time when the radicle raptures the testa through the scarified region. Germination index (GI), which is associated with seed vigour (Tanveer *et al*., 2013) was high in LS seeds of both *Cucumis* species. High GI is an indicative of the seed lots that were highly vigorous (Demir *et al*., 2008). Vigorous seeds produce more uniform and larger seedlings (Matthews and Powell, 2012), as observed in the LS seeds of the current study.

Germination rate in a seed lot measures the length of time it takes for a seed to germinate (Tanveer *et al*., 2013). In *C. africanus*, the differences observed in GR in LC and LS seeds suggested that leaching eliminated the possibility of auto-allelopathy from exogenous cucurbitacin B, which could have had an impact on growth of the radicle from the seed. However, due to the mechanical strength exhibited by the hard testa in this plant species (Baskin *et al.,* 2001), GR in LC was lower than in LS seeds. When physical scarification was applied on the testa of leached seeds, GR escalated. In contrast, in *C. myriocarpus* LS seeds, it is probable that low GR resulted from the existence of high levels of endogenous cucurbitacin A in seeds (Jeffrey, 1978), which might have interfered with the process of germination when cucurbitacin A was leached out during imbibition. The auto-allelopathic effect of endogenous cucurbitacin A (Chen *et al.*, 2005) during leaching probably exhibited a suppressive effect on germination processes and radicle growth, which resulted in

low GR. Suppressive effects of cucurbitacin A were also reported in germination of *Cucurbita moschata* (Duch), *Citrullus lanatus* and *Solanum lycopersicum* (L.) (Mafeo and Mashela, 2009) and emergence of ten dicotyledonous seeds (Mafeo and Mashela, 2010) when crude extracts of *C. myriocarpus* fruit were applied as pre-emergence bio-nematicide.

In *Trichosanthes cucumerina* (L.) var. *cucumerina* seeds, which contain 3.5% total cucurbitacin content, dormancy was overcome by treating seeds at 45°C in tray dryers and thereafter, soaking de-coated seeds in water overnight, with 89% seeds germinating after 35 days (Devendra *et al*., 2011). The ability of *C. myriocarpus* leached-seeds to germinate under *ex vitro* conditions when leached for 8 h (Mafeo, 2014), could be attributed to the presence of microbial activities in soil, which degraded the hard testa (Blum, 1999; Inderjit *et al*., 1999), enabling water imbibition to stimulate germination processes inside seed after an extended period. However, inhibited germination *in vitro,* suggested that no microbial activity took place inside the MS medium since all inputs were aseptic.

3.5 Conclusion

Both chemical and physical dormancies appeared to be responsible for inhibited germination and subsequent performance of seedlings in *C. africanus* and *C. myriocarpus*. The successful leaching out of cucurbitacin B in *C. africanus* seeds suggested that most of the cucurbitacins were deposited on the testa, where it were easily washed off by running tapwater, even though they are insoluble in water (Chen *et al*., 2005). However, the water-soluble cucurbitacin A in *C. myriocarpus* seeds appeared to be trapped within the testas, which were impermeable to water and thereby, resulting in the complete failure of seed germination in both leached

and NLC seeds. The successful removal of dormancy in seeds of the two *Cucumis* species through leaching and scarification for *in vitro* propagation opens numerous avenues for the two *Cucumis* species as future crops for use in various industries, including medicine, agriculture and olericulture. The observed findings make it essential that the morphology of testas in seeds of the two *Cucumis* species be investigated to establish the degree of impermeability of this structure.

CHAPTER 4
MORPHOLOGY OF SEED TESTA IN INDIGENOUS *CUCUMIS* SPECIES

4.1 Introduction

Physical dormancy, which is due to the hard testa, appeared to be strong in wild watermelon (*Cucumis africanus* LF.) and wild cucumber (*Cucumis myriocarpus* Naude.) (Chapter 3). Generally, water-impermeability of testas is caused by various layers, which could be impregnated with water-repellent chemical compounds such as lignin, suberin-cutin matrix and waxes (Baskin, 2003; Baskin and Baskin, 2001; Morrison *et al*., 1998). Poor germination in leached seeds of *C. africanus* and complete failure in germination of leached *C. myriocarpus* seeds (Chapter 3) were probably due to the existence of impermeable layers in seed testas. Seeds of the two *Cucumis* species have thick and hard, whitish to ochre testas (Teppner, 2000), which when physically scarified to release seeds from physical dormancy, allowed germination (Chapter 3). However, the morphology of the testa and/or the presence of “water-gaps” in the two *Cucumis* species are not documented. The objective of this study, therefore, was to determine whether the testas in *C. africanus* and *C. myiocarpus* seeds would possess structures, which interfere with imbibition and movement of water to the endosperm.

4.2 Materials and methods

4.2.1 Location

The study was conducted at the electron microscopy unit of University of Limpopo-Medunsa campus (25°3'8" S, 28°1'22" E). Seeds from mature fruits of the two *Cucumis* species were collected from cultivated plants at the University of Limpopo, Turfloop campus (23°53'10" S, 29°44'15" E) and prepared (Chapter 3).

4.2.2 Seed preparation

Seeds were cut into longitudinal and transverse sections with a microtome blade S35 (Feather safety razor, Japan). The transverse sections were obtained from the micropyle, middle and the chalaza region of seeds. The cut sections were mounted on aluminium stubs with double-sided tape (Agar Scientific) and carbon-coated with a Q150T sputter/carbon coater (Quorum ES).

4.2.3 Identification of layers

The sections were then examined, measured and photographed under a field emission electron microscope (FE-SEM) (Carl Zeiss, Germany) at 3.00 kV. Micrographs were taken and the testa of *C. africanus* and *C. myriocarpus* seeds delineated for comparisons.

4.3 Results

The testa of *C. africanus* and *C. myriocarpus* were 0.21 mm and 0.22 mm thick, respectively (Figure 4.1a,b). In *C. africanus* four layers (Figure 4.2a,c) were observed, but the second layer of the hypodermis (H) was missing or undeveloped at the middle part of the seed, whereas in *C. myriocarpus*, five distinct layers (Figure 4.2b,d) of the testa were observed in all parts of the seed: (1) The epidermis (E) layer, which marked the outside of the testa consisted of radially, small flattened epidermal cells, tightly packed together with cellulose-thickening in transversal

sections. (2) The hypodermis (H) layer comprised one or two layers of large rectangular hypodermal cells, horizontally arranged with thick cell walls separated by lignin. (3) The (S) sclerenchyma layer, which occurred as the most thickened layer, comprised heavily, lignified, thick-walled sclereid cells, tightly interlocked into each other and arranged in transversal section. (4) The aerenchyma (A) layer comprised thickened cells of the spongy parenchyma with large intercellular spaces that differed greatly in size and shape. (5) The chlorenchyma (C) was the innermost layer, which was naturally parenchymatous, comprising numerous intercellular spaces. The discussed results were also represented in a tabular form (Table 4.1).

In the two *Cucumis* species, a “canal” was observed at the micropylar region extending towards the cone-shape nucellar beak of the seed coat (Figure 4.3a,b). Another opening was observed at the chalaza region of the seed coat through the tegument to the endosperm (Figure 4.4a,b). The undeveloped layer of the hypodermis at the middle and chalaza region of *C. africanus* seeds was distinctly developed at the micropylar region in the cone-shape nucellar beak of the seed coat (Figure 4.5a,b).

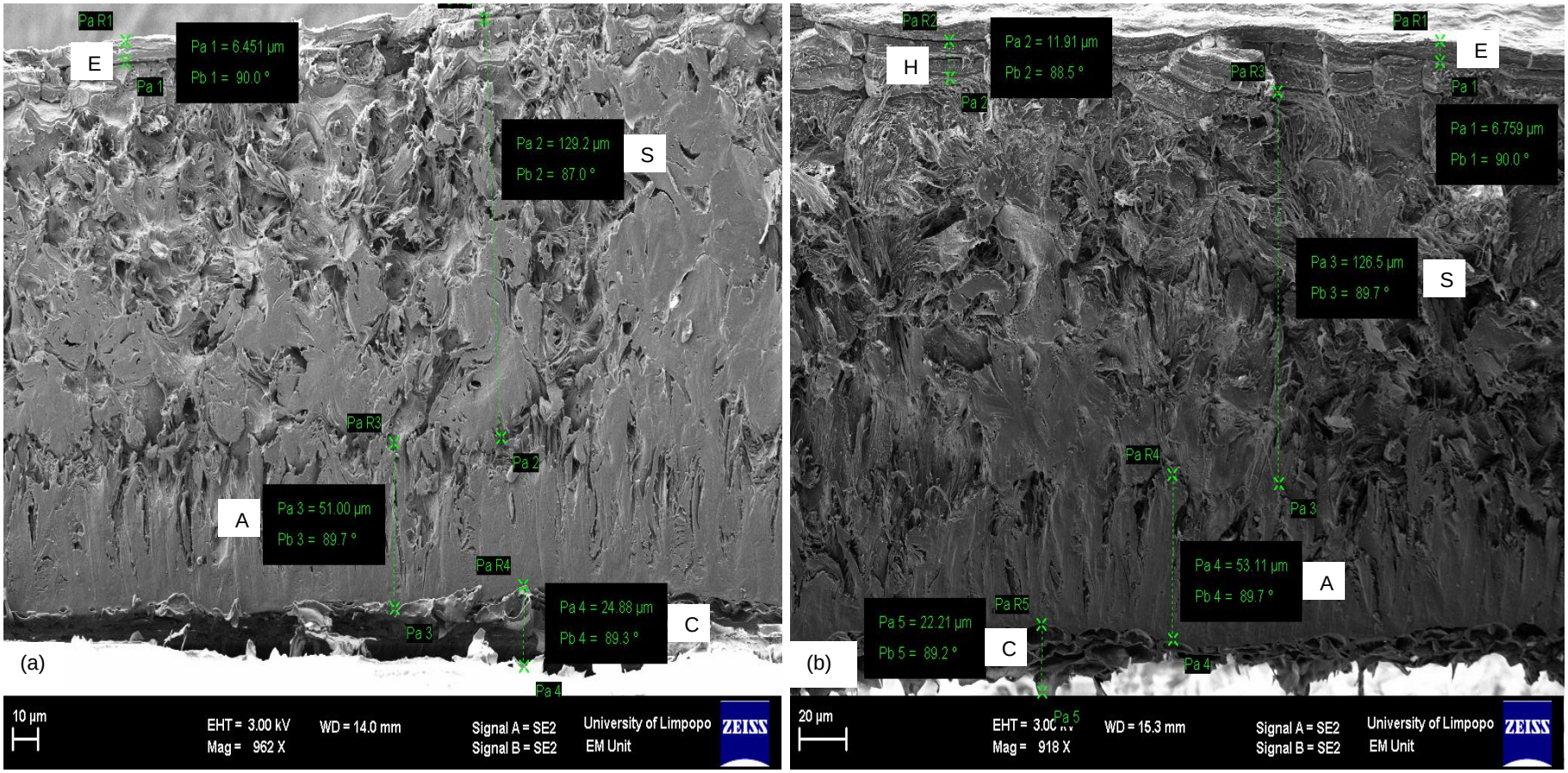

Figure 4.1 Morphological structures in (a) *Cucumis africanus* and (b) *Cucumis myriocarpus* demonstrating the thickness of the testa layers. E = epidermis, H = hypodermis, S = sclerenchyma, A = aerenchyma and C = chlorenchyma.

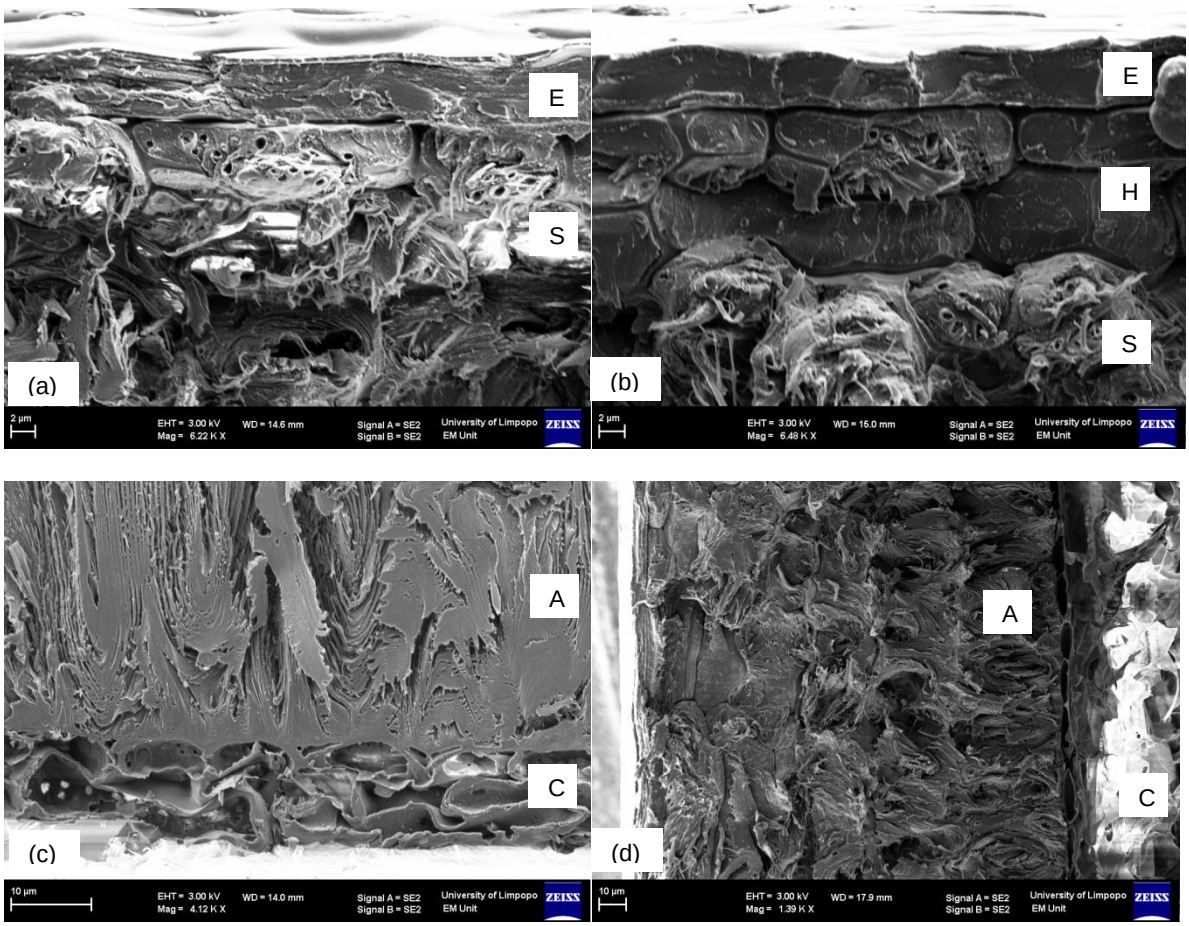

Figure 4.2 Morphological structures of cell layers in the testa of (a, c) *Cucumis africanus* and (b, d) *Cucumis myriocarpus*. E = epidermis, H = hypodermis, S = sclerenchyma, A = aerenchyma and C = chlorenchyma.

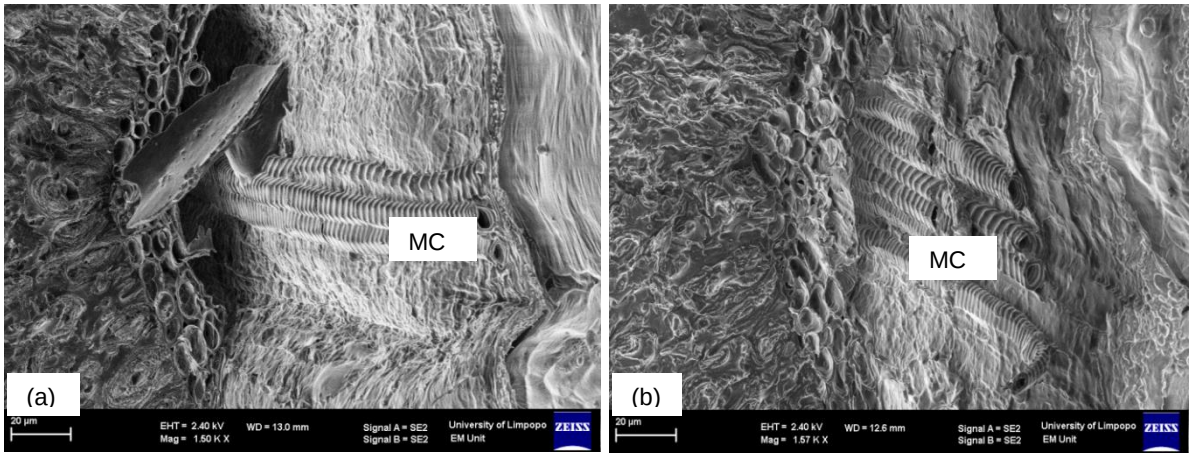

Figure 4.3 The micropyle canal (MC) in the testa of (a) *Cucumis africanus* and (b) *Cucumis myriocarpus* at the micropylar region of the seeds.

Table 4.1 Comparison of the morphological indicators of testa in mature and dried seeds of *Cucumis africanus* and *Cucumis myriocarpus*.

Cell layer	Morphological indicator	Figure	Morphological indicator	Figure
	Cucumis africanus		*Cucumis myriocarpus*	
Epidermis	An exterior layer comprising small, flat, rectangular epidermal cells with thickened cell walls.	4.2a	An exterior layer comprising small, flat, rectangular epidermal cells with thickened cell walls.	4.2b
Hypodermis	Undeveloped between the micropylar and chalaza region of the seed, however, visible at the micropylar region of the seed and arranged in a palisade.	4.2a, 4.5a	Two layers of wide rectangular cells separated from each other by thickened lignified walls, where thickening forms a clear line in between the cell walls.	4.2b
Sclerenchyma	A layer of sclereid cells, which are irregular in shape with thickened, hard and lignified cell walls.	4.2a	A layer of sclereid cells, which are irregular in shape with thickened, hard and lignified cell walls.	4.2b
Aerenchyma	A layer of parenchyma cells with enlarged intercellular spaces.	4.2c	A layer of parenchyma cells with enlarged intercellular spaces.	4.2d
Chlorenchyma	Innermost one to three layers of irregular arranged parenchymatous cells comprising of numerous intercellular spaces.	4.2c	Innermost one to three layers of irregular arranged parenchymatous cells comprising of numerous intercellular spaces.	4.2d

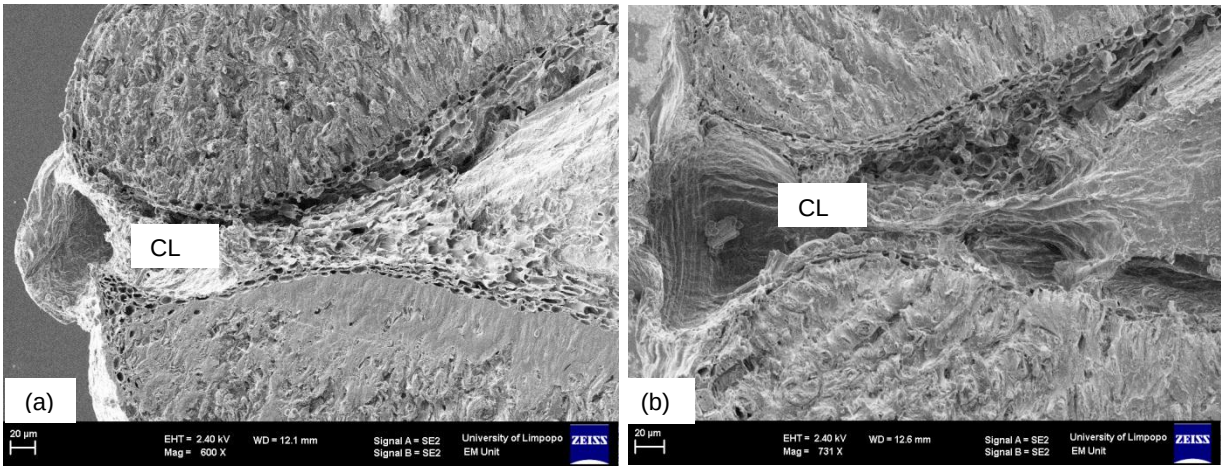

Figure 4.4 The chalaza canal (CL) in seeds of (a) *Cucumis africanus* and (b) *Cucumis myriocarpus* at the chalaza region of the seeds.

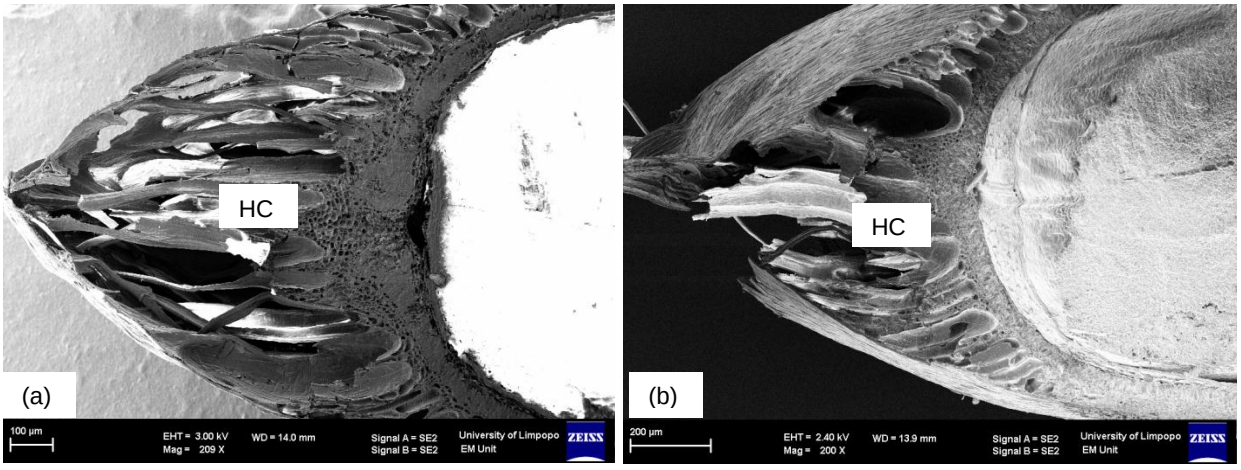

Figure 4.5 The cone-shape nucellar beak showing longitudinal sectioning of hypodermis cells (HC) at the micropylar region of (a) *Cucumis africanus* and (b) *Cucumis myriocarpus* seeds.

4.4 Discussion

4.4.1 Variation in testa thickness

In the two *Cucumis* species, variation in size of the testas suggested that the plant species were structurally different in terms of the physical arrangement of individual layers. *Cucumis africanus*, which contains insoluble cucurbitacin B exogenous to the testa (Chapter 3), comprised a thinner testa than *C. myriocarpus*, which contains water-soluble cucurbitacin A deposited within the testa, due to the undeveloped second layer of the hypodermis between the micropylar (adaxial end) and the chalaza (abaxial end) region. Sporadic germination in leached seeds of *C. africanus* (Chapter 3) might probably be the result of the undeveloped hypodermal layer.

4.4.2 Epidermal layer

In both *Cucumis* species, the exterior layer of the epidermis marks the outside of the seed, which certainly represent the first line of defence against harsh external factors from entering the seed (Radchuk and Borisjuk, 2014). In the two *Cucumis* species, the epidermis was observed as a single cell layer, with epidermal cells tightly connected to each other providing mechanical strength and protection to the enclosed endosperm (Singh and Dathan, 1972, 2001). Yeung and Cavey (1990) reported that at the final stage of seed development cells in the testa withers, whereas the structure of the epidermal cells is preserved by the mucilage, epidermal and sub-epidermal cell walls, with the layers crushing as the endosperm proliferates. Cutin, which is found in the epidermal cells, minimise water entry into the seed and water loss from within the seed due to the presents of wax that gives the surface of the seed a whitish colour (Raven *et*

al., 1981, Teppner, 2000). The deposition of a wax layer in the epidermis protects seeds from harsh external conditions caused by sunlight and wind, also acting as a barrier to moisture from entering the seed (Baskin, 2003).

4.4.3 Hypodermal layer

In the two *Cucumis* species, a distinct line separates the epidermis and hypodermis layers, which divides once or twice periclinary in *C. myriocarpus*. In contrast, in *C. africanus*, the hypodermal layer coincided with the border of the third layer ─ the sclerenchyma layer, but with the hypodermis cells being more developed in the micropylar region than in the middle bulge of the seed. Lema *et al*. (2008) noticed that in seeds of *Cucurbita maxima* (Duch.) var. 'Maxima' and *Cucurbita maxima* var. 'Andreana' the hypodermis-epidermis division was a regular line as observed in *C. myriocarpus*, whereas in *Cucurbita ficifolia* (Bouche), the division appeared irregular and occurring in "waves", but coinciding with the border of the epidermal cells. In the two *Cucumis* species, the hypodermal layer appeared to be thick-walled and lignified with large rectangular cells in *C. myriocarpus*, whereas in *C. africanus* the cells were shaped into a palisade-like structure towards the micropylar region. The presence of lignin in the hypodermis cells inhibited imbibition and gaseous exchange in seeds of the two *Cucumis* species, as had been usually the case in other plants species (Salanenka *et al*., 2009; Stuart and Loy, 1983).

4.4.4 Sclerenchyma layer

The heavily, lignified sclerenchyma layer in the two *Cucumis* species appeared to be the hardest due to the presence of sclereid cells, as observed in other seeds (Singh, 1967). Generally, the sclerenchyma layer comprised sclereid and fibre cells, both with secondary walls, which in most seeds wither as the seed matured (Campbell, 1990). Usually, sclereid cells are impregnated with lignin, which had been shown in various plant species to accord water-impermeable attributes (Campbell, 1990). Lignification in the sclerenchyma layer generally occurs as a result of the accumulation of lignin in the cell walls (Stuart and Loy, 1983). Quantitative analysis of the sclerenchyma layer demonstrated that the domesticated cucurbits (*Cucurbita moschata* Duch.) comprised the shortest sclereid cells than their wild counterpart in *Cucurbita maxima* var. 'Andreana' (Lema *et al*., 2008).

4.4.5 Aerenchyma layer

In the two *Cucumis* species, the aerenchyma layer comprised the spongy parenchyma with numerous intercellular spaces, differing greatly in size and shape as observed in other cucurbits (Gunawardena *et al*., 2001). Generally, the aerenchyma layer is a parenchyma tissue comprising large intercellular spaces, with functional roles of storage and transportation of oxygen to the living tissues of seeds (Campbell, 1990). In the two *Cucumis* species, the aerenchyma layer lacked both suberin and lignin, thereby enhancing gaseous exchange to and from the embryo.

4.4.6 Chlorenchyma layer

In *C. africanus* and *C. myriocarpus* seeds, the chlorenchyma layer formed the innermost tissue of the testa, as was the case in most seeds (Singh, 1967; Wardrop, 1969). In dry mature seeds, the chlorenchyma layer could be separated as a thin green membrane of the testa and generally forms the tegument (Singh, 1967). Cells in the chlorenchyma layer have metabolic capabilities and might contain chloroplasts and starch granules (Campbell, 1990). Generally, cell walls in chlorenchyma cells contain cellulose and pectin compounds, with a high proportion of water (Wardrop, 1969), with elongation capabilities during plant growth (Majumdar and Preston, 1941). The plasticity of chlorenchyma in seeds had been associated with tensile-strength comparable to the sclerenchyma fibres and the combination of strength and plasticity makes the chlorenchyma cells effective as a strengthening tissue (Leroux, 2012).

4.4.7 Existence of “water gaps”

In the two *Cucumis* species, the two anatomical openings, namely, the micropylar and chalaza regions are positioned in opposite directions and are known to provide specialised functions in the permeability of the testa as observed in other cucurbit seeds (Tillman, 1906). The micropyle is a “canal” located in the cone-shaped structure of the testa, formed during fertilisation by the passage of the pollen tube to the embryo (Salanenka *et al*., 2009). At maturity, the “micropyle canal” had been viewed as a minute opening for imbibition and the subsequent exit of the radicle when physical scarification had been applied. In the two *Cucumis* species, the role of the “micropyle canal” in water permeability could be inhibited by the presence of thick-walled suberised

cells as suggested in other cucurbit seeds (Salanenka *et al*., 2009). The inhibition of imbibition at the micropylar region had been extensively cited (Groot and Karssen, 1987; Ikuma and Thimann, 1963; Leubner-Metzger *et al*., 1996; Watkins *et al*., 1985).

Another anatomical opening was also observed at the chalaza region of the two *Cucumis* species, referred to as the “chalaza canal” in other plant species (Salanenka *et al*., 2009). The chalaza region had been viewed as the base of an ovule bearing an embryo sac surrounded by integuments (Campbell, 1990). The observed “canal” at the chalaza region functioned in the transportation of nutrients from maternal tissues into the developing embryo during seed development (Tillman, 1906). In the two *Cucumis* species, the observed “canal” appeared to be blocked with a suberised stopper known as a “chalaza plug” in other plant species (Boesewinkel and Been, 1979). In *Cucumis sativas* (L.), sectioning at the chalaza region showed numerous layers of suberised chalaza cells, which acted as a barrier for water imbibition (Salanenka *et al*., 2009).

4.4.8 Elimination of physical dormancy

In *C. africanus* and *C. myriocarpus*, the observation of the two “canals” suggested that both the micropylar and chalaza region could serve as a point of water entry into the seed through physical scarification and/or microbial degradation in the soil. The latter is not available for *in vitro*-propagated seeds. Generally, elimination of physical dormancy involved the disruption of the coverings of the specialised “water-gap” structures, which acted as environmental “signal detectors” for germination (Baskin *et al*., 2000). Once the closed “water-gap” structures were broken, rapid imbibition would then takes place,

with seeds germinating under a wide range of environmental conditions (Baskin and Baskin, 2001; Baskin and Baskin, 1998; Baskin *et al*., 2000; Bewley *et al*., 2006). In this study, physical scarification at the chalaza region rendered the testa permeable to water and gases, resulting in enhanced germination of the two *Cucumis* species (Chapter 3), as previously observed in other plant species (Nell *et al*., 1981; Schulz *et al*., 1991).

4.5 Conclusion

The undertaken morphological study of the testa layers through the electron microscope enhanced the observed variations in testa thickness and the two “water-gap” structures located in the micropyle and chalaza region of seeds in the two *Cucumis* species. Both the micropyle and chalaza “canal” could probably serve as a point of water entry in *C. africanus* and *C. myriocarpus* seeds, when physical scarification was applied. In the two *Cucumis* species, physical scarification at the chalaza region dislodged the chalaza plug, improving imbibition and the subsequent germination. Enhanced germination would enable the availability of mother stocks for subsequent *in vitro*-propagation of *C. africanus* and *C. myriocarpus* for use as future crops for various industries.

CHAPTER 5
OPTIMUM PLANT GROWTH REGULATOR CONCENTRATIONS FOR *IN VITRO* PROPAGATION OF TWO INDIGENOUS *CUCUMIS* SPECIES

5.1 Introduction

Both physical and chemical seed dormancies in wild watermelon (*Cucumis africanus* LF.) and wild cucumber (*Cucumis myriocarpus* Naude.) were successfully eliminated through simultaneous leaching and scarification (Chapter 3), to allow for the establishment of mother stocks for vegetative propagules. *Cucumis africanus* and *C. myriocarpus* contain large concentrations of cucurbitacin B ($C_{32}H_{46}O_8$) and cucurbitacin A [cucumin ($C_{27}H_{40}O_9$), leptodermin ($C_{27}H_{38}O_8$)], respectively (Rimington, 1938). Cucurbitacin A is the only water-soluble cucurbitacin among the 12 identified cucurbitacins (Chen *et al*., 2005) and is restricted to roots and seeds (Jeffrey, 1978). In contrast, cucurbitacin B is distributed equally in all organs of *C. africanus* plants (Jeffrey, 1978). According to the distribution of cucurbitacins within the two *Cucumis* species, *C. africanus* might not have suitable vegetative propagules for *in vitro* propagation, whereas in *C. myriocarpus* all organs except the radicle might be suitable. Generally, different plant species require specific concentrations of cytokinin and auxin for shoot regeneration and root initiation, respectively (George, 1993; Hartmann *et al*., 2010). The objective of this study therefore, was to determine whether all organs of *C. africanus* and *C. myriocarpus* would be suitable for *in vitro* propagation.

5.2 Materials and methods

5.2.1 *In vitro* propagule selection

5.2.1.1 Location and source of explants for propagule selection

In vitro propagule selection trials were conducted at African Biotechnologies PTY (Ltd) Commercial Plant Tissue Culture Laboratory, Tzaneen (23°51'50.08" S, 30°0'30.37" E) from the beginning of winter (May-July) to the middle of spring (August-October). Two-week-old *C. africanus* and *C. myriocarpus* seedlings (Chapter 3) were used as mother stock for explant materials.

5.2.1.2 Preparation of medium

Murashige and Skoog medium containing 30 g sucrose/L distilled water was prepared as described previously (Chapter 3). Six concentrations, namely, 0.00, 0.05, 0.10, 0.25, 0.50 and 0.75 µM 6-benzyladeninepurine (BAP), were used. The media were cooled for 12 h.

5.2.1.3 Preparation of explants and culturing

Explant materials were prepared as follows: (1) fully expanded leaves with a small petiole were aseptically excised from two-week-old-*in-vitro*-raised *C. africanus* and *C. myriocarpus* seedlings and then placed with the abaxial-side being in contact with the MS medium (Kathal *et al*., 1988), (2-3) approximately 20-25 mm and 5-6 mm long nodal bud and shoot-tip explants, respectively, were excised from seedlings and cultured by inserting them in upright position in the MS medium (Agarwal and Kamal, 2004; Ahmad and Anis, 2005; Vasudevan *et al*., 2004), (4) hypocotyls of approximately 1.0 cm long

were dissected from the basal part of seedlings and horizontally placed into the medium (Novak and Dolezeloa, 1982; Selveraj *et al*., 2006), and (5) cotyledon explants were gently flicked back with the blunt-end of sterile scalpel blade to separate them from the hypocotyls, with edges slightly trimmed and divided into two halves prior to culturing in MS medium with the abaxial-side being in direct contact with the MS media (Compton, 1999). After culturing, all experimental units were then incubated in a 12 h light/dark cycle on racks, fitted with cool white fluorescent tubes of 40 µM/m^2/s irradiance at 25°C and 50-60% relative humidity (Chapter 3).

5.2.1.4 Experimental design, data collection and statistical analysis

The experiment was a 5 x 6 factorial laid out in a completely randomised design (CRD), with 5 replications (n = 150). The first and second factors comprised the five explants and six concentrations of BAP, respectively. At 56 days after initiating the treatments, percentage (%) tissue regeneration responses and number of shoots were recorded. Data were subjected to factorial analysis of variance using SAS software (SAS Institute, Inc. 2008). Discrete data were transformed through $\log_{10}(x + 1)$ to homogenise the variances (Gomez and Gomez, 1984), but untransformed means were reported. Treatment means were separated using Tukey's honestly significant difference (HSD) all-pairwise comparison test at the probability level of 5%. Unless otherwise stated, only treatment means significant at 5% level of probability were discussed (Appendices 5.1-5.2).

5.2.2 Optimum plant growth regulator concentrations for shoot regeneration and root initiation

5.2.2.1 Location and source of explant material for optimisation of plant growth regulators

Trials were conducted at the Plant Tissue Culture Laboratory, Department of Biodiversity, University of Limpopo, South Africa (23°53'10" S, 29°44'15" E). Using information derived during propagule selection, *s*hoot-tip and nodal bud explants were used from *C. africanus* and *C. myriocarpus*, respectively. Explants were aseptically excised from 2-week-old-*in-vitro*-raised seedlings using protocols described in propagule selection.

5.2.2.2 Shoot multiplication stage and culture conditions

Using optimum values for shoot regeneration in *C. africanus* (0.44 µM BAP) and *C. myriocarpus* (0.38 µM BAP) during propagule selection, a decision was made that the concentrations for shoot multiplication be above and below the derived optima. Thus, the excised explants were cultured in MS medium containing six treatments, namely, 0.00, 0.20, 0.40, 0.60, 0.80 and 1.00 µM BAP for each *Cucumis* species and arranged in CRD, with 5 replications (n = 30). After three weeks of incubation under light conditions similar to those described for the selection of the suitable propagules, vine length and number of shoots were recorded.

5.2.2.3 *In vitro* rooting of regenerated shoots

Preliminary trials suggested that indole-3-butyric acid (IBA) at similar concentrations with BAP used in shoot multiplication were already in the inhibition concentrations. The suitable concentration for root initiation was viewed as being below 0.50 µM IBA. In each *Cucumis* species, regenerated shoots for rooting were aseptically excised from 3-week-old plantlets from the shoot multiplication stage and transferred into the MS medium, which contained 0.00, 0.05, 0.10, 0.25, 0.50 and 0.75 µM IBA, arranged in CRD, with 5 replications (n = 30). After a 3-week-incubation period in conditions similar to those of shoot multiplication, vine length, root length and number of roots were recorded.

5.2.2.4 Data analysis

Data were separately subjected to ANOVA through the SAS software (SAS Institute, Inc. 2008). Discrete data were transformed through $\log_{10}(x + 1)$ to homogenise the variances (Gomez and Gomez, 1984), but untransformed means were reported. Fisher's least significant difference test was used to separate the means at the probability level of 5%. Plant variables with significant treatment means were subjected to lines of the best fit using plant growth responses to increasing PGR concentrations. The generated relationships were modelled by the regression curve estimates from the quadratic equation ($Y = b_2x^2 + b_1x + a$), where Y = explant growth response and x being the optimum PGR concentration, which was derived from $x = – b_1/2b_2$ (Mamphiswana *et*

al., 2010). Unless otherwise stated, only treatment means significant at 5% level of probability were discussed (Appendices 5.3-5.4).

5.3 Results

5.3.1 *In vitro* propagule selection

Organ x concentration interaction, significantly ($P \leq 0.05$) affected % tissue regeneration response and number of shoots (Appendix 5.1-5.2), contributing 57% and 61% of total treatment variation (TTV) in *C. africanus*, respectively (Table 5.1). In *C. myriocarpus*, the interaction significantly ($P \leq 0.05$) affected % tissue regeneration response and number of shoots, each contributing 20% of TTV, respectively (Table 5.1). In *C. africanus*, % tissue regeneration response in all treatments was 100% in nodal bud and shoot-tip explants (Table 5.2). However, in leaf, hypocotyl and cotyledon explants, tissue regeneration was reduced by 6-100%, 58-100% and 22-100%, respectively. In the cotyledon explants, tissue regeneration was initially increased by 127% at the lowest BAP (µM) concentration, prior to reduction (Table 5.2). In *C. myriocarpus*, similar trends were observed, with nodal bud and shoot-tip explants having 100% tissue regeneration response each, whereas in leaf, hypocotyl and cotyledon explants the variable was reduced by 2-84%, 42-98% and 7-100%, respectively. In both *Cucumis* species, the variable was not inhibited in untreated control for leaf, nodal bud, shoot-tip and hypocotyl explants (Table 5.2). However, in *C. africanus* and *C. myriocarpus*, the variable in cotyledon explants was reduced by 56% and 18%, respectively, in untreated control, suggesting that endogenous chemicals were already in operation in this organ, whereas in other organs endogenous chemicals were still inactive or had negligent

concentrations. In both *Cucumis* species, the increasing concentrations of BAP had an inverse relationship in percentage response in tissue regeneration of leaf, hypocotyl and cotyledon explants (Table 5.2).

Table 5.1 Mean sum of squares for five propagules of *Cucumis africanus* and *Cucumis myriocarpus* plantlets and six concentrations of BAP (µM) and their interactions in percentage tissue regeneration response and number of regenerated shoots *in vitro.*

Source	**DF**	**% response**		**No. of shoots**	
		SS	**%**	**SS**	**%**
Cucumis africanus					
Replication	4	217	0*	2.352	1*
Concentration	5	48134	19**	29.792	7**
Organ	4	148191	57**	252.072	61**
Concentration × organ	20	47529	18**	97.585	24**
Error	116	16503	6	30.676	7
Total	149	260574	100	412.478	100
Cucumis myriocarpus					
Replication	4	463	0*	3.465	1*
Concentration	5	59384	25**	33.438	7**
Organ	4	116203	48**	297.407	61**
Concentration × organ	20	47629	20**	98.683	20**
Error	116	17977	7	50.783	11
Total	149	241656	100	483.776	100

* Significant at P ≤ 0.10, ** significant at P ≤ 0.05.

Table 5.2 Effects of interaction in six concentrations of BAP (µM) and five propagules of *Cucumis africanus* and *Cucumis myriocarpus* seedlings in percentage tissue regeneration response *in vitro.*

BAP (µM)	Leaf % response[y]	Nodal bud % response	Shoot-tip % response	Hypocotyl % response	Cotyledon % response
			Cucumis africanus		
0.00	100^{a}	100^{a}	100^{a}	100^{a}	44^{b}
0.05	94^{a}	100^{a}	100^{a}	42^{b}	100^{a}
0.10	0^{c}	100^{a}	100^{a}	38^{b}	34^{b}
0.25	38^{b}	100^{a}	100^{a}	34^{b}	18^{bc}
0.50	30^{b}	100^{a}	100^{a}	0^{c}	0^{c}
0.75	20^{bc}	100^{a}	100^{a}	0^{c}	0^{c}
			Standard error = 7.54		
			Cucumis myriocarpus		
0.00	100^{a}	100^{a}	100^{a}	100^{a}	82^{abc}
0.05	98^{a}	100^{a}	100^{a}	58^{bcd}	88^{ab}
0.10	48de	100^{a}	100^{a}	56^{cd}	32^{defg}
0.25	16^{fgh}	100^{a}	100^{a}	34^{def}	14^{fgh}
0.50	30^{defgh}	100^{a}	100^{a}	2^{gh}	8^{fgh}
0.75	20^{efgh}	100^{a}	100^{a}	6^{fgh}	0^{h}
			Standard error = 7.87		

[y]Tukey HSD all-pairwise comparison test in percentage tissue regeneration response for BAP (µM) concentration x organ interaction.

Percentage tissue regeneration and the increasing concentration of BAP had density-dependent growth (DDG) patterns, with the model for *C. africanus* explaining 44%, 80% and 63% of TTV in leaf, hypocotyl and cotyledon explants, respectively (Figure 5.1). In *C. myriocarpus* the models for the respective organs were explained by 81%, 94% and 85% (Figure 5.1). In *C. africanus*, the optimum BAP concentrations (x-axis) for leaf, hypocotyl and cotyledon explants for the variable were 0.52, 0.59 and 0.63 µM, respectively, whereas the optimum percentage tissue regeneration responses (y-axis) were 12.97%, –3.47% and –2.60%, respectively. In *C. myriocarpus*, the optimum BAP concentrations for leaf, hypocotyl and cotyledon explants were 0.52, 0.59 and 0.56 µM, respectively, whereas the optimum percentage tissue regeneration responses were 8.97%, 0.24% and –6.64%, respectively (Table 5.3).

In both *Cucumis* species, leaf and hypocotyl explants completely failed to initiate shoots (Table 5.4). However, relative to untreated control, organ x concentration interaction increased shoot regeneration in *C. africanus* nodal bud and shoot-tip explants by 133-560% and 410-995%, respectively. However, in cotyledon explants the interaction reduced shoot regeneration by 43-100% (Table 5.4). Similarly in *C. myriocarpus,* relative to untreated control, organ x concentration interaction in shoot regeneration, registered similar trends in nodal bud and shoot-tip explants. Shoot regeneration was increased by 60-460% and 243-443% in nodal bud and shoot-tip explants, respectively, whereas in cotyledon explants regeneration of shoots was reduced by 57-100% (Table 5.4).

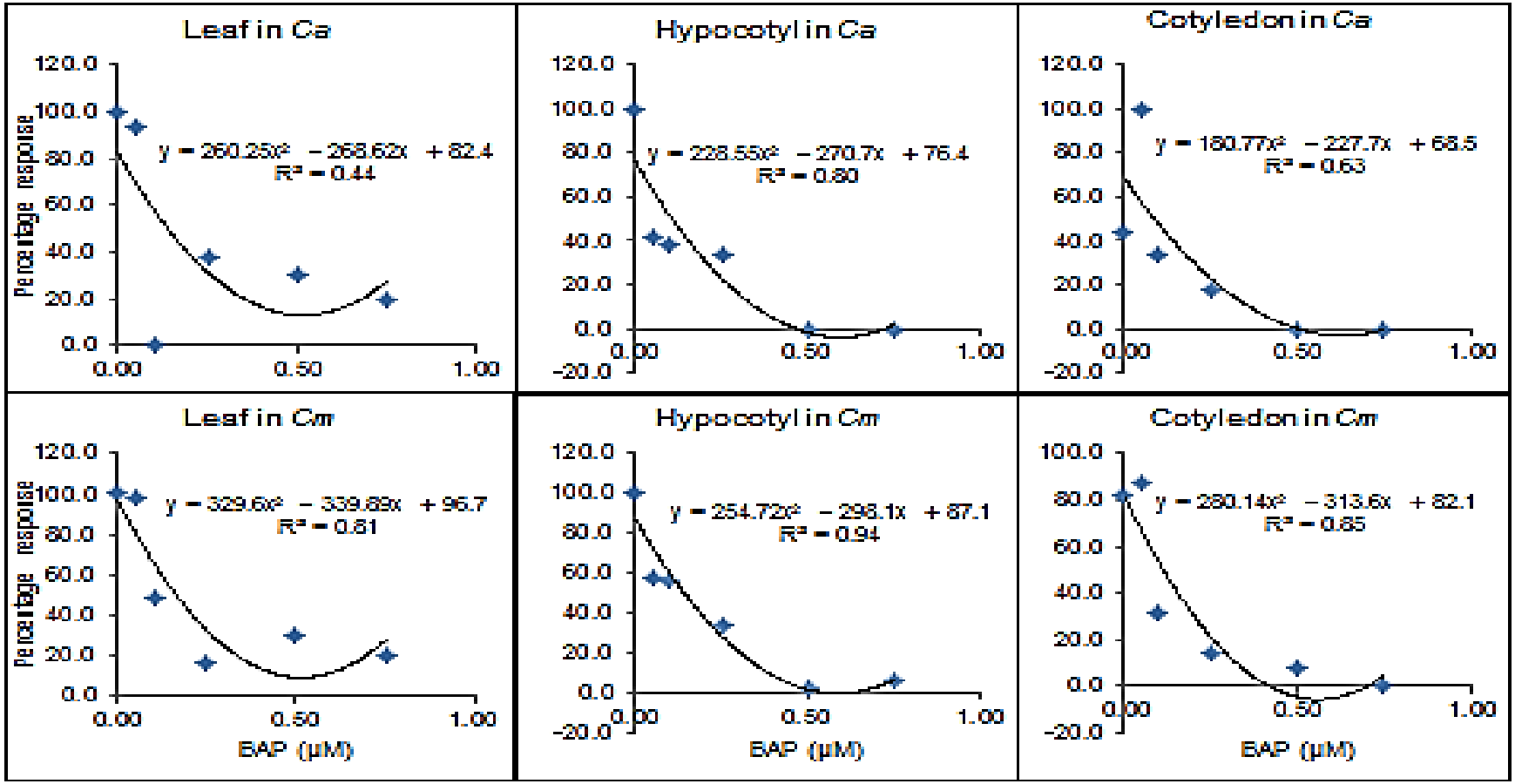

Figure 5.1 Responses of leaf, hypocotyl and cotyledon explants to six levels of BAP (µM) concentrations on percentage tissue regeneration response in *Cucumis africanus* (*Ca*) and *C. myriocarpus* (*Cm*) *in vitro*.

Table 5.3 *In vitro* optimum BAP (µM) concentration (x) and percentage tissue regeneration response (y) in various propagules of *Cucumis africanus* and *Cucumis myriocarpus*.

Plant variable	**Quadratic relationship**	**R^2**	**x^z**	**y^z**	**P ≤**
	Cucumis africanus				
Leaf	$Y = 260.25x^2 – 268.62x + 82.4$	0.44	0.52	12.97	0.01
Hypocotyl	$Y = 228.55x^2 – 270.86x + 76.4$	0.80	0.59	– 3.47	0.01
Cotyledon	$Y = 180.77x^2 – 227.71x + 68.6$	0.63	0.63	– 2.60	0.01
	Cucumis myriocarpus				
Leaf	$Y = 329.6x^2 – 339.89x + 96.7$	0.81	0.52	8.97	0.01
Hypocotyl	$Y = 254.72x^2 – 298.1x + 87.1$	0.94	0.59	0.24	0.01
Cotyledon	$Y = 280.14x^2 – 313.6x + 82.1$	0.85	0.56	– 6.64	0.01

[z]Calculated optimum BAP concentration $x = – b_1/2b_2$, where for *Cucumis africanus* nodal buds, $b_1 = – 268.62$ and $b_2 = 260.25$, respectively.

Table 5.4 Effects of interaction in six concentrations of BAP (µM) and five propagules from *Cucumis africanus* and *Cucumis myriocarpus* seedlings in regeneration of shoots *in vitro.*

BAP (µm)	Leaf		Nodal bud		Shoot-tip		Hypocotyl		Cotyledon	
	Variable[y]	%[z]	Variable	%	Variable	%	Variable	%	Variable	%
Cucumis africanus										
0.00	0.00^{f}	–	0.60^{ef}	–	0.40^{f}	–	0.00^{f}	–	1.66^{cde}	–
0.05	0.00^{f}	0	1.40^{de}	133	2.04^{cd}	410	0.00^{f}	0	0.94^{def}	– 43
0.10	0.00^{f}	0	3.90^{ab}	550	3.96^{ab}	890	0.00^{f}	0	0.68^{ef}	– 59
0.25	0.00^{f}	0	3.96^{ab}	560	4.38^{a}	995	0.00^{f}	0	0.00^{f}	– 100
0.50	0.00^{f}	0	3.72^{ab}	520	3.44^{ab}	760	0.00^{f}	0	0.00^{f}	– 100
0.75	0.00^{f}	0	2.74^{bc}	357	3.40^{ab}	750	0.00^{f}	0	0.00^{f}	– 100
Standard error = 0.33										
Cucumis myriocarpus										
0.00	0.00^{g}	–	1.00^{fg}	–	0.70^{g}	–	0.00^{g}	–	1.40^{efg}	–
0.05	0.00^{g}	0	3.20^{bcd}	220	2.40^{cdef}	243	0.00^{g}	0	0.40^{g}	– 71
0.10	0.00^{g}	0	3.90^{bc}	290	3.80^{bc}	443	0.00^{g}	0	0.60^{g}	– 57
0.25	0.00^{g}	0	4.60^{ab}	360	3.28^{bc}	369	0.00^{g}	0	0.00^{g}	–100
0.50	0.00^{g}	0	5.60^{a}	460	3.42^{bc}	389	0.00^{g}	0	0.00^{g}	– 100
0.75	0.00^{g}	0	1.60^{defg}	60	2.64^{cde}	277	0.00^{g}	0	0.00^{g}	– 100
Standard error = 0.42										

[y]Tukey HSD all-pairwise comparison test in regeneration of shoots for BAP (µM) concentration x organ interaction.
[z]Impact = [(variable/control – 1) x 100].

The number of shoots regenerated in nodal bud, shoot-tip and cotyledon explants in both *Cucumis* species (y-axis), when regressed against the increasing concentration of BAP (x-axis), had quadratic relationships (Figure 5.2), which allowed the computation of the optimum BAP concentration (µM) from $x = – b_1/2b_2$, along with the respective optimum number of regenerated shoots as predicted by the quadratic relationship (Table 5.5). In *C. africanus*, quadratic relationships were explained by 72% and 62% of TTV in nodal bud and shoot-tip explants, whereas in *C. myriocarpus* the relationships were explained by 91% and 57% of TTV in the respective explants (Table 5.5). In contrast, the production of shoots in both *C. africanus* and *C. myriocarpus* had negative quadratic relationships, which were explained by 91% and 77% to TTV in cotyledon explants, respectively (Figure 5.2).

In *C. africanus*, the optimum BAP concentration for nodal bud and shoot-tip explants were 0.42 and 0.44 µM, respectively, whereas the optimum number of regenerated shoots were 4.1 and 4.4 shoots per explant, respectively (Table 5.5). In *C. myriocarpus,* the computed optimum BAP concentration for nodal bud and shoot-tip explants were 0.38 and 0.42 µM, respectively, whereas the respective optimum number of shoots regenerated were 5.9 and 5.7 shoots per explant. However, in both *Cucumis* species the optimum values for regeneration of shoots in cotyledon explants were negative (Table 5.5). Using the two-way t-test within each *Cucumis* species, nodal bud and shoot-t tip explants were not significantly ($P \leq 0.05$) different (Table 5.6), which allowed the use of any of the two explants for *in vitro* propagation. The discussed results were graphically represented (Figure 5.3).

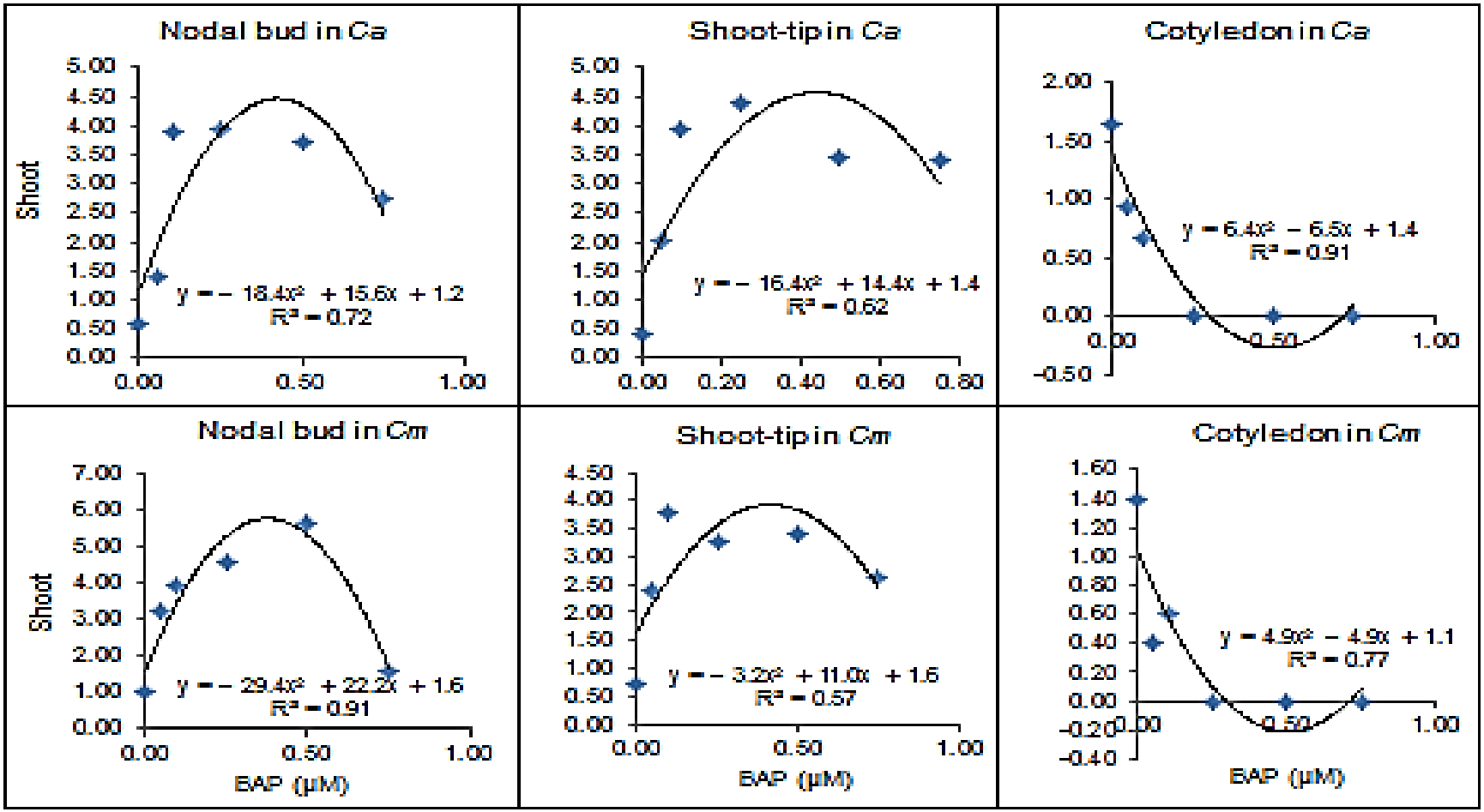

Figure 5.2 Responses of nodal bud, shoot-tip and cotyledon explants to six levels of BAP (µM) concentrations in number of regenerated shoots in *Cucumis africanus* (*Ca*) and *Cucumis myriocarpus* (*Cm*) *in vitro*.

Table 5.5 *In vitro* optimum BAP (µM) concentration (x) and number of regenerated shoots (y) in nodal bud, shoot-tip and cotyledon explants of *Cucumis africanus* and *Cucumis myriocarpus*.

Plant variable	Quadratic relationship	R^2	x^z	y^z	P ≤
	Cucumis africanus				
Nodal bud	$Y = -18.4x^2 + 15.6x + 1.2$	0.72	0.42	4.1	0.01
Shoot-tip	$Y = -16.4x^2 + 14.4x + 1.4$	0.62	0.44	4.4	0.01
Cotyledon	$Y = 6.4x^2 - 6.53x + 1.4$	0.91	0.51	– 0.3	0.01
	Cucumis myriocarpus				
Nodal bud	$Y = -29.4x^2 + 22.2x + 1.6$	0.91	0.38	5.9	0.01
Shoot-tip	$Y = -3.2x^2 + 11.00x + 1.6$	0.57	0.42	5.7	0.01
Cotyledon	$Y = 4.9x^2 - 4.9x + 1.0$	0.77	0.51	– 2.3	0.01

[z]Calculated optimum BAP concentration $x = -b_1/2b_2$, where for *Cucumis africanus* nodal buds, $b_1 = 15.6$ and $b_2 = -18.4$, respectively.

Table 5.6 Comparisons of performance of nodal bud and shoot-tip explants in terms of shoot regeneration from *Cucumis africanus* and *Cucumis myriocarpus* over six levels of BAP *in vitro*.

BAP (µM)	***Cucumis africanus***			***Cucumis myriocarpus***	
	Nodal bud	**Shoot-tip**		**Nodal bud**	**Shoot-tip**
0.00	0.60	0.40		1.00	0.70
0.05	1.40	2.04		3.20	2.40
0.10	3.90	3.96		3.90	3.80
0.25	3.96	4.38		4.60	3.28
0.50	3.72	3.44		5.60	3.42
0.75	2.74	3.40		1.60	2.64
Mean	$2.72^{ns} \pm 0.58$	$2.94^{ns} \pm 0.60$		$3.32^{ns} \pm 0.72$	$2.57^{ns} \pm 0.41$

^{ns} Implies that mean shoot regeneration within each *Cucumis* species was not different ($P \leq 0.05$) according to two-sample t-test.

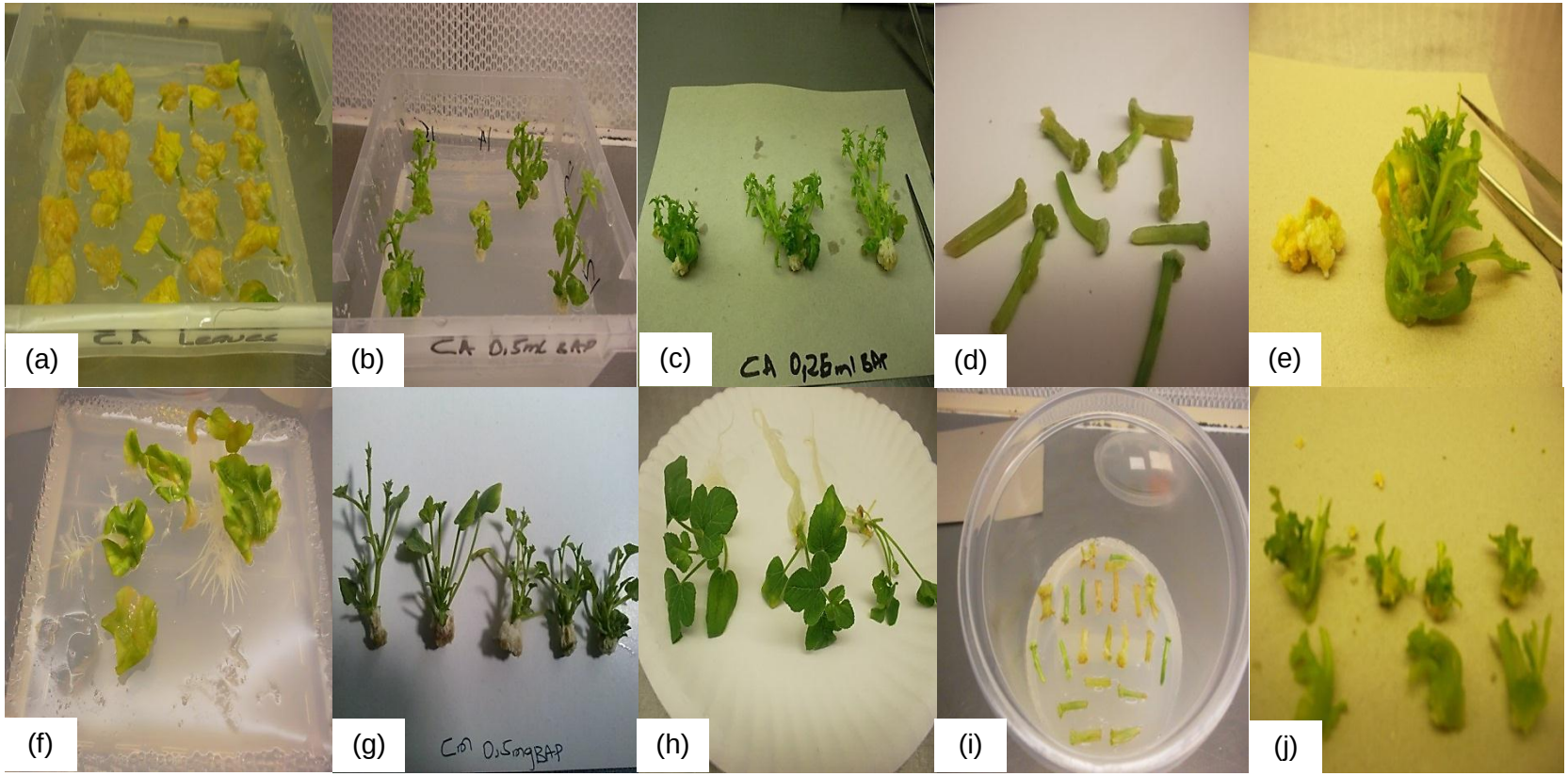

Figure 5.3 Various propagules used in the selection of suitable propagule: (a) leaves, (b) nodal buds, (c) shoot-tips, (d) hypocotyls, (e) cotyledons of *Cucumis africanus*, together with (f) leaves, (g) nodal buds, (h) shoot-tips, (i) hypocotyls and (j) cotyledons of *Cucumis myriocarpus in vitro.*

5.3.2 Selection of optimum plant growth regulator concentrations

5.3.2.1 Shoot multiplication stage

In both the *Cucumis* species, increasing BAP concentrations had highly significant ($P \leq 0.05$) effects in their respective variables (Appendix 5.3-5.4). In *C. africanus* shoot-tip explants, the effect of BAP contributed 43% and 91% to TTV in number of shoots and vine length, whereas in *C. myriocarpus* nodal bud explants, the effect of BAP contributed 74% and 65% to TTV in number of shoots and vine length, respectively (Table 5.7).

Relative to untreated control, BAP treatment increased number of shoots by 34-92%, but decreased vine length by 41-71% in *C. africanus* shoot-tip explants. Similarly, treatment increased number of shoots by 1315-2090% in *C. myriocarpus* nodal bud explants, respectively. However, in contrast, vine length in *C. myriocarpus* was initially increased by 2-19% and started decreasing by 27-47% as concentrations of BAP was increased (Table 5.8). The number of shoots (3.77 shoots per explant) produced in *C. africanus* shoot-tip explants, were the highest at 0.80 µM BAP. In *C. myriocarpus*, maximum number of shoots (4.38 shoots per explant) were produced at 0.40 µM BAP in nodal bud explants, respectively (Table 5.8).

Table 5.7 Mean sum of squares for number of shoots and vine length (cm) in shoot multiplication of *Cucumis africanus* shoot-tip explants and *Cucumis myriocarpus* nodal bud explants *in vitro.*

Source	**DF**	**No. of shoots**		**Vine length (cm)**		**No. of shoots**		**Vine length (cm)**	
		SS	**%**	**SS**	**%**	**SS**	**%**	**SS**	**%**
		***Cucumis africanus* shoot-tip**				***Cucumis myriocarpus* nodal bud**			
Treatment	5	11.78	43**	250.64	91**	52.08	74**	65.95	65**
Error	24	15.66	57	24.08	9	18.66	26	36.135	35
Total	29	27.44	100	274.72	100	70.71	100	102.135	100

* Significant at P ≤ 0.10, ** significant at P ≤ 0.05.

Table 5.8 Responses of *Cucumis africanus* shoot-tip explants and *Cucumis myriocarpus* nodal bud explants to concentrations of BAP (µM) during shoot multiplication stage *in vitro*.

BAP (µM)	No. of shoots		Vine length (cm)		No. of shoots		Vine length (cm)	
	Variable[y]	%[z]	Variable	%	Variable	%	Variable	%
	***Cucumis africanus* shoot-tip**				***Cucumis myriocarpus* nodal bud**			
0.00	1.96[b]	–	12.20[a]	–	0.20[b]	–	5.98[ab]	–
0.20	2.88[ab]	47	7.22[b]	– 41	3.06[a]	1430	7.12[a]	19
0.40	3.00[ab]	34	4.51[c]	– 63	4.38[a]	2090	6.09[ab]	2
0.60	3.66[ac]	53	5.13[c]	– 58	3.24[a]	1520	4.36[bc]	– 27
0.80	3.77[a]	92	4.83[c]	– 60	3.79[a]	1795	3.19[c]	– 47
1.00	3.32[ab]	69	3.50[c]	– 71	2.83[a]	1315	3.31[c]	– 45

[y]Column means with the same letter were not different (P ≤ 0.05) according to Fisher's least significant difference test.

[z]Impact = [(variable/control – 1) x 100].

The number of shoots over increasing concentrations of BAP had DDG patterns, characterised by quadratic relationships, which explained 82% and 84% of the model in *C. africanus* and *C. myriocarpus*, respectively (Figure 5.4). In contrast, vine length and increasing BAP had negative quadratic relationship, which explained 96% and 81% of the model in the two *Cucumis* species (Figure 5.4). According to the relation $x = -b_1/2b_2$, in *C. africanus* shoot-tip explants, the number of shoots were optimised at 0.81 µM BAP, whereas vine length was optimised at 0.79 µM BAP. The mean optimum BAP concentration for shoot multiplication stage was 0.80 µM, with the number of shoots and vine length being 3.55 shoots per clump and 3.75 cm in *C. africanus* shoot-tip explants (Table 5.9). In contrast, the number of shoots in *C. myriocarpus* nodal bud explants, were optimised at 0.60 µM BAP and vine length was optimised at –0.26 µM BAP. The mean optimum BAP concentration for shoot multiplication stage was 0.35 µM BAP, with the number of shoots and vine length being 4.17 shoots per clump and 1.96 cm in *C. myriocarpus* nodal bud explants, respectively (Table 5.9).

5.3.2.2 Root initiation stage

Increasing IBA concentrations had no significant ($P \leq 0.05$) effects in root length of the two *Cucumis* species (data not shown). Highly significant ($P \leq 0.05$) effects (Appendix 5.5-5.6) were observed in number of roots and vine length of both the *Cucumis* species, contributing 84% and 73% to TTV in *C. africanus* shoot-tip explants, whereas in *C. myriocarpus* nodal bud explants, IBA concentrations contributed 69% and 36% to TTV in their respected variables (Table 5.10).

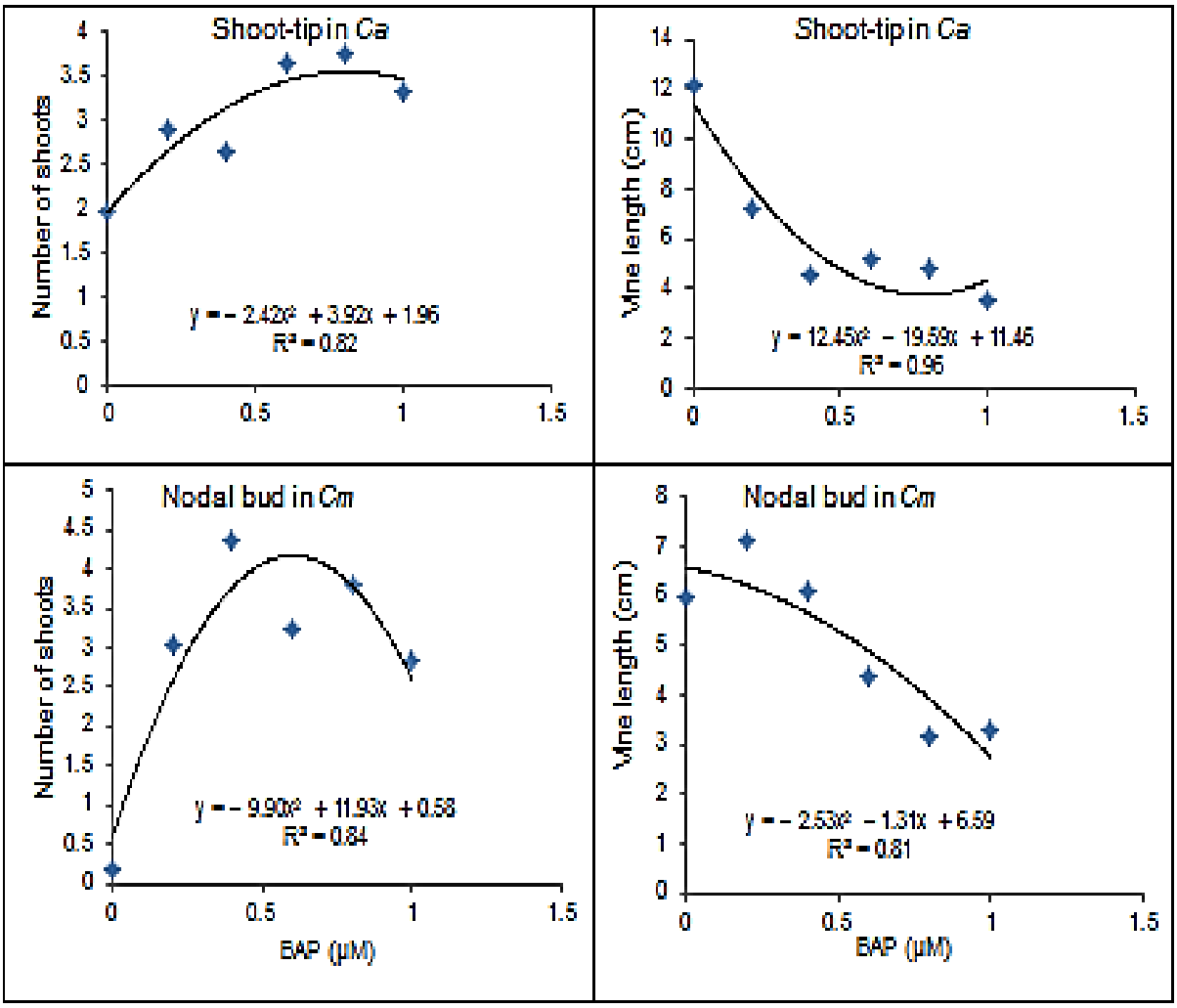

Figure 5.4 Responses of number of shoots and vine length to various concentrations of 6-benzyladeninepurine (BAP µM) during shoot multiplication stage of *Cucumis africanus* shoot-tip explants and *Cucumis myriocarpus* nodal bud explants *in vitro*.

Table 5.9 Integrated optimum level of BAP (µM) concentrations for *in vitro* shoot multiplication of *Cucumis africanus* shoot-tip explants and *Cucumis myriocarpus* nodal bud explants.

***Cucumis* species**	**Plant variable**	**Quadratic relationship**	**R^2**	**x^z**	**y^z**	**P ≤**
Cucumis africanus	Number of shoots	$Y = -2.42x^2 + 3.92x + 1.96$	0.82	0.81	3.55	0.05
	Vine length (cm)	$Y = 12.45x^2 - 19.59x + 11.46$	0.96	0.79	3.75	0.05
	Mean optimum level of BAP (µM)			0.80		
Cucumis myriocarpus	Number of shoots	$Y = -9.90x^2 + 11.93x + 0.58$	0.84	0.60	4.17	0.05
	Vine length (cm)	$Y = -2.523x^2 - 1.311x + 6.59$	0.81	– 0.26	1.97	0.05
	Mean optimum level of BAP (µM)			0.35		

[z]Calculated optimum response concentration $x = -b_1/2b_2$, where for the number of shoots, $b_1 = 3.92$ and $b_2 = -2.42$, respectively.

Table 5.10 Mean sum of squares for the number of roots and vine length (cm) in rooting stage of *Cucumis africanus* and *Cucumis myriocarpus in vitro.*

Source	**DF**	**No. of roots**		**Vine length (cm)**		**No. of roots**		**Vine length (cm)**	
		SS	**%**	**SS**	**%**	**SS**	**%**	**SS**	**%**
		Cucumis africanus				***Cucumis myriocarpus***			
Treatment	5	227.02	84**	327.84	73**	559.20	69**	25.23	36**
Error	24	43.58	16	121.97	27	247.10	31	45.52	64
Total	29	270.61	100	449.81	100	806.30	100	70.74	100

* Significant at P ≤ 0.10 level, ** significant at P ≤ 0.05.

In *C. africanus*, relative to untreated control, IBA treatment increased the number of roots and vine length by 198-284% and 29-40%, except at 0.75 µM, where the variables were reduced by 69% and 58% in shoot-tip explants. In contrast, the number of roots was increased by 139-275%, whereas vine length was reduced by 15-30% at all levels of IBA in *C. myriocarpus* nodal bud explants (Table 5.11). In *C. africanus* the number of roots produced (7.84 roots per vine) were the highest at 0.50 µM IBA. However, in *C. myriocarpus*, maximum number of roots (18.90 roots per vine), were produced at a lower IBA concentration of 0.05 µM, respectively (Table 5.11). All variables tested and increasing concentrations of IBA had DDG patterns in the two *Cucumis* species, characterised by quadratic curves, with number of roots and vine length explaining 80% and 96% of the model in *C. africanus* shoot-tip explants and 83% and 79% in *C. myriocarpus* nodal bud explants, respectively (Figure 5.5).

In *C. africanus,* the number of roots were optimised at 0.33 µM IBA concentration, whereas vine length was optimised at 0.28 µM IBA. The mean optimum IBA concentration for rooting stage was 0.31 µM IBA, with the number of roots and vine length being 8.39 roots per vine and 14.51 cm in *C. africanus* shoot-tip explants, respectively. In contrast, the number of roots was optimised at 0.46 µM IBA and vine length optimised at 0.42 µM IBA in *C. myriocarpus* nodal bud explants, respectively. The mean optimum IBA concentration for rooting stage was 0.44 µM, with the number of roots and vine length being 16.21 roots per vine and 6.01 cm in *C. myriocarpus*, respectively (Table 5.12). The discussed results are graphically demonstrated (Figure 5.6).

Table 5.11 Responses of shoots obtained from *Cucumis africanus* shoot-tip explants and *Cucumis myriocarpus* nodal bud explants to concentrations of IBA (µM) during root initiation stage *in vitro*.

IBA (µM)	No. of roots		Vine length (cm)		No. of roots		Vine length (cm)	
	Variable[y]	%[z]	Variable	%	Variable	%	Variable	%
	***Cucumis africanus* shoot-tip explants**				***Cucumis myriocarpus* nodal bud explants**			
0.00	2.04[c]	−	9.54[b]	−	5.04[c]	−	9.39[a]	−
0.05	7.08[ab]	247	12.66[a]	33	18.90[a]	275	7.48[ab]	− 20
0.10	6.08[b]	198	13.16[a]	38	15.04[ab]	198	7.18[ab]	− 24
0.25	7.04[ab]	245	13.40[a]	40	12.06[b]	139	6.54[b]	− 30
0.50	7.84[a]	284	12.34[ab]	29	15.72[ab]	212	6.94[ab]	− 26
0.75	0.64[c]	− 69	4.03[c]	− 58	14.84[ab]	194	7.92[ab]	− 15

[y]Column means with the same letter were not different (P ≤ 0.05) according to Fisher's least significant difference test.

[z] Impact = [(variable/control – 1) x 100].

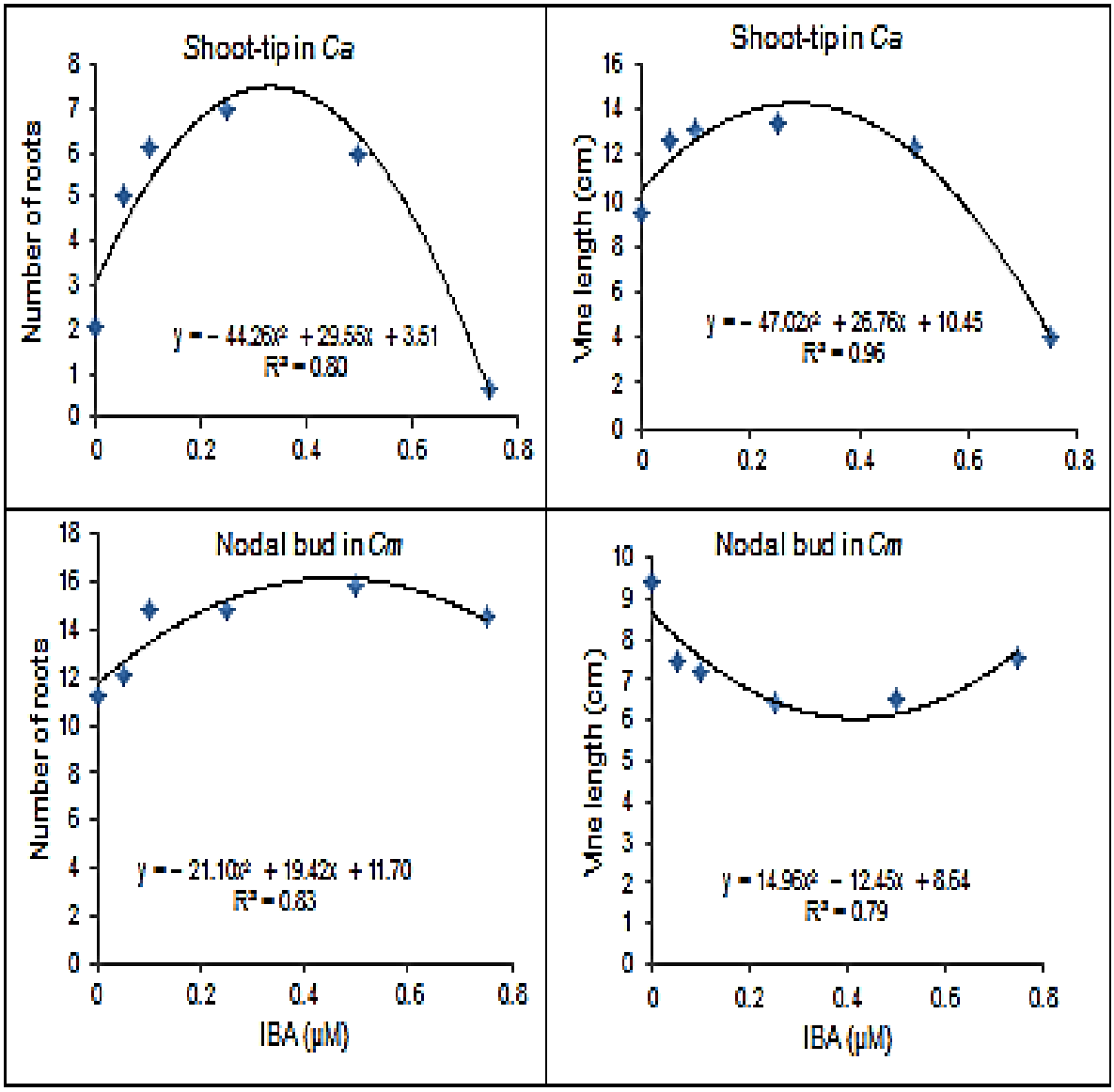

Figure 5.5 Responses of number of roots and vine length to various concentrations of indole-3-butyric acid (IBA µM) during root initiation of shoots obtained from *Cucumis africanus* shoot-tip explants and *Cucumis myriocarpus* nodal bud explants *in vitro*.

Table 5.12 Mean optimum level of IBA (µM) concentrations for *in vitro* root initiation of shoots obtained from *Cucumis africanus* shoot-tip explants and *Cucumis myriocarpus* nodal bud explants.

***Cucumis* species**	**Plant variable**	**Quadratic relationship**	**R^2**	**x^z**	**y^z**	**P ≤**
Cucumis africanus	Number of roots	$Y = -44.26x^2 + 29.55x + 3.51$	0.80	0.33	8.39	0.05
	Vine length (cm)	$Y = -47.02x^2 + 26.76x + 10.45$	0.96	0.28	14.51	0.05
	Mean optimum level of IBA (µM)			0.31		
Cucumis myriocarpus	Number of roots	$Y = -21.10x^2 + 19.42 + 11.70$	0.83	0.46	16.21	0.05
	Vine length (cm)	$Y = 14.96x^2 - 12.45 + 8.64$	0.79	0.42	6.01	0.05
	Mean optimum level of IBA (µM)			0.44		

[z]Calculated optimum response concentration $x = -b_1/2b_2$, where for the number of shoots, $b_1 = 29.55$ and $b_2 = -44.26$, respectively.

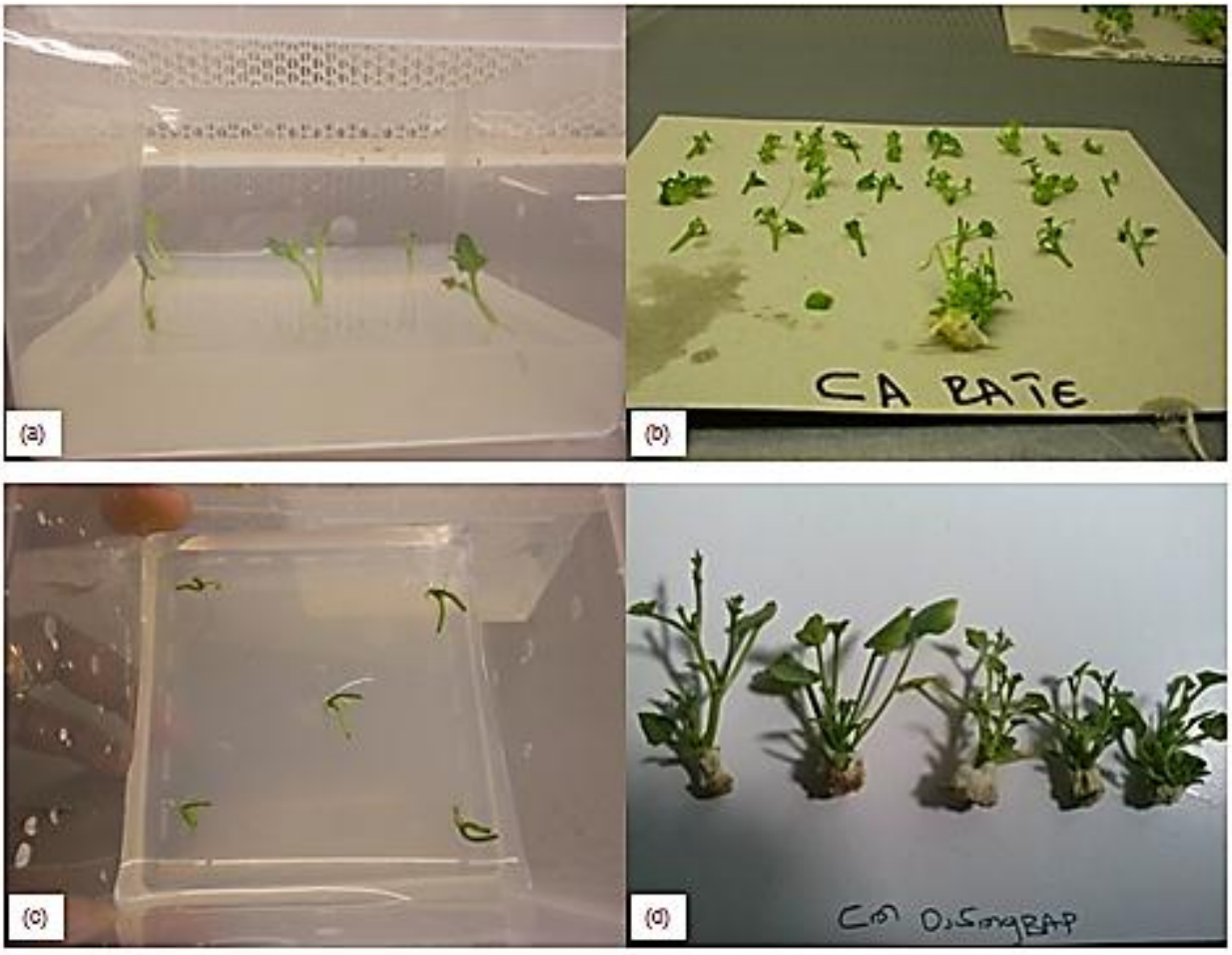

Figure 5.6 Various stages of *in vitro* propagation: (a) *Cucumis africanus* excised shoot-tip explants, (b) multiple shoots from shoot-tip explants, (c) *Cucumis myriocarpus* nodal bud explants and (d) multiple shoots from nodal bud explants.

5.4 Discussion

5.4.1 Selection of *in vitro* propagule

5.4.1.1 Regeneration of plant tissues from various organs

Complete tissue regeneration in all plant organs except for cotyledon explants in the two *Cucumis* species at untreated control (0 µM BAP) suggested that the concentration of cucurbitacins in the organs was in the neutral phase or was less than the stimulation

threshold, whereas the cotyledon explants already had accumulated inhibitory concentrations of cucurbitacin (Chen *et al*., 2005). Generally, soon after seedling emergence, the cotyledons remain the oldest chloroplast-containing aerial organ with the highest metabolite rates (Pogson and Albrecht, 2011), where biosynthesis of secondary metabolites (Érzek and Kiraly, 1986; Inderjit and Malik, 2002), like cucurbitacins in the Cucurbitaceae Family (Tallamy and Krischik, 1989), is highly probable. Although for most allelochemicals the exact physiological role in plants are not clear, literature is replete with evidence that these chemical compounds are used in plant defence system (Chen *et al*., 2005; Miller and Feeney, 1983) and that in the Cucurbitaceae Family, the materials are essential in the cotyledons for providing protection against herbivorous insects (Tallamy and Krischik, 1989). Thus cotyledons in both *Cucumis* species were not suitable to serve as propagules for *in vitro* propagation.

In *C. africanus,* cucurbitacin B is equally distributed in the entire plant (Rimington, 1938), whereas in *C. myriocarpus* cucurbitacin A is localised in fruits and roots (Jeffrey, 1978; Rimington, 1938). Consequently, it was expected that all organs of *C. africanus* would not be suitable for *in vitro* use as propagules. However, results of this study showed that in *C. africanus* cucurbitacin B was not uniformly distributed in all organs of *in vitro*-produced seedlings, but as in *C. myriocarpus in vitro* seedlings, the chemical compounds were localised in the cotyledons. Studies on herbivorous behaviour of *Diabrotica* beetles suggested that the accumulation of cucurbitacins in the cotyledons were important for use in defence (Chambliss and Jones, 1966; Ferguson *et al*., 1983; Howe *et al*., 1976; Metcalf *et al*., 1980, 1982; Metcalf, 1985; Sharma and Hall, 1973).

Generally, the concentration of cucurbitacins in the Cucurbitaceae Family changes rapidly during seedling growth (Jaworski *et al*., 1985), especially when plants are in nutrient-rich environments (Tallamy and Krischik, 1989). Due to the nutrient-rich nature of the MS growing medium for *in vitro* trials, growth rates in younger organs were high, which in *Cucurbita pebo* (L.) were associated with lower concentrations of cucurbitacins (Tallamy and Krischik, 1989). Literature is replete with evidence that cucurbitacin accumulation in various organs is directly proportional to the degree of environmental stresses (Browning and Hodges, 2004; Ho *et al*., 2014; Shah *et al*., 2010).

Tissue regeneration and the increasing concentration of BAP followed the density-dependent growth (DDG) patterns, which were characterised by quadratic relationships. Generally, when living entities are exposed to increasing levels of biotic or abiotic factors, they respond to the pressures through DDG patterns (Salisbury and Ross, 1992). The DDG patterns have three growth stages, namely, stimulating, neutral and inhibitory phases, which had been used for various practical applications (Pelinganga, 2013; Pofu and Mashela, 2014). In both *Cucumis* species, percentage tissue regeneration responses and the increasing BAP concentrations from the nodal bud and shoot-tip explants had neutral effects, whereas in the other organs the patterns suggested inhibitory effects. Similar DDG responses had been reported widely when tomato (*Solanum lycopersicum* L.) and rough lemon (*Citrus jambhiri* Lush.) seedlings were exposed to increasing concentrations of cucurbitacin in the form of phytonematicides (Maila and Mashela, 2013; Pelinganga and Mashela, 2012; Pelinganga *et al*., 2012; Pelinganga *et al*., 2013). The neutral effects of BAP

concentrations on trial tissue regeneration from nodal bud and shoot-tip explants, suggested that the concentrations used in this study were neither stimulatory nor inhibitory and the two propagules were therefore, suitable for *in vitro* propagation of the two *Cucumis* species.

5.4.1.2 Shoot regeneration from various organs

The nodal bud and shoot-tip explants successfully resulted in shoot regeneration in the two *Cucumis* species, whereas in other organs regeneration completely failed. The observation on shoot regeneration in the two explants confirmed findings with those in tissue regeneration. The effectiveness of BAP for shoot regeneration in nodal bud and shoot-tip explants of the two *Cucumis* species was consistent with observations in other genera species within the Cucurbitaceae Family. In nodal bud explants, multiple shoot regeneration was reported in *Cucumis sativas* (L.) (Ahmad and Anis, 2005), *Cucurbita maxima* (Duch.) and *Benincasa hispida* (Thunb.) cv. 'Ash gourd' (Haque *et al*., 2008). In shoot-tip explants, shoot regeneration was also observed in *Cucurbita maxima* (Mahzabin *et al*., 2008), *Cucumis sativas* (Kathal *et al*., 1988) and *Cucurbita* interspecific hybrid cultivar 'Shintoza' (Sarowar *et al*., 2003). Enhanced responses to shoot regeneration could be attributed to the successful tissue regeneration and the subsequent high growth rates of the two explants in the nutrient-rich MS medium, as suggested by the resource-availability hypotheses (Coley *et al*., 1985; Janzen, 1974, 1979). According to the resource-availability hypotheses, the accumulation of cucurbitacins in an organ was inversely proportional to the growth rates of an organ (Tallamy and Krischik, 1989). The nodal and apical buds in plants comprise

meristematic cells (Campbell, 1990), which were renowned for their rapid growth rates and therefore the two explants in MS medium, could be viewed as being compliant with the requirements of the resource-availability hypotheses.

Generally, as BAP concentrations increase, the cotyledon explants from the two *Cucumis* species turned yellowish and withered in the medium as observed *in vitro* organogenesis of *Citrullus lanatus* (Thunb.) (Krug *et al*., 2005). Reduced shoot regeneration from cotyledon explants in *Cucumis* species in the current study was consistent with observations in other genera within the Cucurbitaceae Family. At low BAP concentration, cotyledon explants swelled and increased in size, with low rates of shoot regeneration occurring as clusters instead of individual buds (Krug *et al*., 2005; Ugandhar *et al*., 2011). Generally, response to PGRs was dependent upon the combined concentrations of different PGRs (Campbell, 1990). For instance, in *Cucumis sativas* cv. 'Poinsett 76' and cv. 'Sultan' cotyledon explants, when cultured in BAP medium alone, each responded as observed in *C. africanus* and *C. myriocarpus*. However, enhanced shoot regeneration occurred when BAP (cytokinin) was combined with 2,4-D (auxin) (Abu-Romman *et al*., 2013; Selvaraj *et al*., 2006). BAP is an adenine-type cytokinin, which is synthesised in roots and transported to shoots through the xylem to induce bud breaking by the activation of meristems, promotion of shoot proliferation and independently regulates outgrowth of axillary buds (George *et al*., 2008; Hartmann *et al*., 2010; Murashige, 1974). In contrast, 2,4-D is biosynthesised in meristematic tissues of above ground plant organs and then translocated to roots

through the phloem, where it primarily regulates root growth (Campbell, 1990; Hartmann *et al*., 2010).

The DDG patterns, early observed in % tissue regeneration response, were also observed in shoot regeneration and increasing BAP concentrations from nodal bud, shoot-tip and cotyledon explants in both *Cucumis* species. Optimum numbers of shoots regenerated from nodal bud and shoot-tip explants in *C. africanus* were 4.1 and 4.4 shoots, respectively, which occurred at concentrations of 0.42 and 0.44 µM BAP, respectively. Similarly, optimum number of shoots per explant from nodal bud and shoot-tip explants in *C. myriocarpus* were 5.9 and 5.7 shoots, respectively, which occurred at concentrations 0.38 and 0.42 µM BAP, respectively. According to the DDG principles, shoot regeneration and increasing BAP concentrations in nodal bud and shoot-tip explants for both *Cucumis* species encompassed all three stages, namely, stimulation, neutral and inhibition. However, for the cotyledon explant, the concentrations were already in the inhibition phase. The quantitative observations in this study are incomparable with those in other studies within the Cucurbitaceae Family since shoot regeneration values versus increasing BAP concentrations were not optimised and thereby rendering the observations qualitative (Ahmad and Anis, 2005; Haque *et al*., 2008; Kathal *et al*., 1988; Mahzabin *et al*., 2008; Sarowar *et al*., 2003; Shrivastava and Roy, 2011). Generally, at lower concentrations, BAP stimulated shoot regeneration in the two *Cucumis* species, whereas at higher concentration the PGR inhibited regeneration of shoots in nodal bud and shoot-tip explants (George, 1993). In the two *Cucumis*, high BAP concentrations induced massive callus structure with total

failure of shoot regeneration as observed in *Cucumis sativas* (Kathal *et al*., 1988, 1994; Misra and Bhatnagar, 1995).

5.4.1.3 Suitable propagule

In the two *Cucumis* species, nodal bud and shoot-tip explants were suitable for use as propagules. Generally, in the Cucurbitaceae Family, *in vitro* propagules have been reported either directly from nodal bud (Haque *et al*., 2008) or shoot-tip explants (Alper *et al*., 1994; Compton *et al*., 1993) or indirectly through callus that developed on hypocotyls (Pal *et al*., 2007), cotyledons (Dennis Thomas and Sreejesh, 2004; Ganasan and Huyop, 2010) and leaves (Sultana *et al*., 2004). Direct shoot regeneration, technically referred to as organogenesis (Mohammadi and Sivritepe, 2007), is less time consuming with minimal abnormalities in the regenerants (Monohiuddin *et al*., 1997). Also, organogenesis from nodal bud and shoot-tip explants (Mohammadi and Sivritepe, 2007), was the preferred *in vitro* technique in *Citrullus lanatus* commercial production systems (Ganasan and Huyop, 2010).

5.4.2 Optimum plant growth regulator concentrations

The optimum shoot multiplication in *C*. *africanus* at 0.80 µM BAP, was twice that required for *Cucumis sativas* at 0.40 µM BAP (Mohammadi and Sivritepe, 2007), whereas that required by *C. myriocarpus* was 0.35 µM BAP, respectively. The effectiveness of BAP in stimulating multiple number of shoots in the two *Cucumis* species, was in conformity with the conclusion made in *Cucumis melo* (L.), *Cucumis*

sativas and *Lagenaria siceraria* (Mol.), reporting BAP as an essential PGR for shoot bud formation and shoot regeneration (Misra and Bhatnagar, 1995; Mohammadi and Sivritepe, 2007; Saha *et al*., 2007). In this study, multiple number of shoots were optimised in MS medium supplemented with 0.80 µM BAP in *C. africanus* and 0.35 µM BAP in *C. myriocarpus,* respectively.

The response of a plant organ to PGRs is density-dependent, as depicted by the observed DDG patterns, which are characterised by quadratic curves (Salisbury and Ross, 1992). Generally, growth of plants responds to increasing concentrations of PGRs and allelochemicals in DDG patterns (Pofu *et al*., 2010a; Mafeo and Mphosi, 2012). In principle, PGRs can stimulate one process in an organ, while inhibiting other processes within the same organs (Hartmann *et al*., 2010). During *in vitro* shoot multiplication of the *Cucumis* species, the simultaneous stimulation and inhibition effects of BAP were observed in shoots and vine length, respectively, suggesting that the former was achieved through reduced leaf growth and the latter through hyperhydricity. Gradual increasing in BAP concentrations reduced vine length in *Cucumis* species. In other plant species, as BAP concentrations increase, hyperhydricity was induced due to failure of internode elongation, resulting in a rosette structure (George, 1993). At 1.00 µM BAP, vine length of *C*. *africanus* was already reduced by 71%, which implied that it would be undesirable to go beyond the empirically-derived level of 0.80 µM BAP. In *Cucumis sativas*, at high levels, BAP induced massive callus structure with total failure of shoot regeneration (Kathal *et al*., 1988, 1994; Misra and Bhatnagar, 1995; Mahzabin *et al*., 2008). Similar callus structures were observed in *Cucurbita* interspecific hybrid cv. ‘Shintoza’ (Sarowar *et al*., 2003). In contrast, shoot multiplication of *Cucumis maxima*

and *Benincasa hispida* cv. 'Ash gourd' from nodal explants had the highest number of shoots per explants at 2.00 µM BAP, beyond this level, callused shoots emerged (Haque *et al*., 2008; Mahzabin *et al*., 2008).

The stimulation of roots regeneration in the two *Cucumis* species at 0.31 µM IBA and 0.44 µM IBA, agreed with observations in *Cucumis melo,* where IBA was required for maximum root initiation (Haque *et al*., 1984; Singh *et al*., 1990; Khalekuzzaman *et al*., 2003). In this study, optimum number of roots in *C. africanus* (8.7 per vine), optimised at 0.31 µM IBA, was exactly the same with that in *Cucurbita* m*axima* (8.7 per vine), optimised at 1.00 µM IBA (Mahzabin *et al*., 2008). In contrast, maximum number of 16.2 roots per plant were produced in *C. myriocarpus* at the lowest concentration of 0.44 µM IBA, whereas maximum number of roots (11.7 per vine) were produced in *Cucumis melo* at 2.0 µM IBA (Dabauza *et al*., 1997).

In biological systems, quadratic relationships are an indication of DDG patterns (Salisbury and Ross, 1992; Pofu *et al*., 2010a). Generally, DDG patterns suggest that there was, depending on the concentration, stimulation growth phase, followed by saturation or no response phase and then the inhibition growth phase (Salisbury and Ross, 1992). In this study, quadratic relationships of all plant variables tested demonstrated existence of phases of the DDG patterns except for vine length at shoot multiplication stage in *C. africanus* and at rooting stage in *C. myriocarpus,* in which concentrations of PGRs were already in inhibition growth phase at the lowest PGR

concentration used. Apparently, concentrations of BAP and IBA were already in excess for stimulating elongation in both the *Cucumis* species at different stages.

5.5 Conclusion

In the two *Cucumis* species, tissue and shoots regenerations were successful when nodal bud and shoot-tip explants were used. Comparative analysis of tissues and shoot regeneration within *Cucumis* species suggested that each of the explants could be chosen as a propagule for *in vitro* mass propagation. The quantity of BAP required to produce optimum number of shoots in *C*. *africanus* and *C. myriocarpus* were 0.80 and 0.35 µM BAP, whereas that required for the optimum number of roots were 0.31 and 0.44 µM IBA, respectively. The optimised concentrations are within the range required in some of plant species in the Cucurbitaceae Family. The optimum concentrations of the two PGRs were validated and found suitable for rapid shoot multiplication and rooting stages of these *Cucumis* species during *in vitro* propagation. The developed protocol was efficient and could be used for mass propagation of *C. africanus* and *C. myriocarpus* seedling rootstocks for use in inter-generic grafting technology in large-scale commercial watermelon production.

CHAPTER 6
ACCLIMATISATION OF *IN VITRO* PROPAGATED *CUCUMIS* PLANTLETS UNDER GREENHOUSE CONDITIONS

6.1 Introduction

A substantial number of *in vitro* propagated plantlets do not survive transition to *ex vitro* conditions (Pospíšilová *et al*., 1999). *Ex vitro* conditions have lower relative humidity, higher light intensity and septic conditions that are stressful to plants originated *in vitro* (Ziv, 1986). The benefits of *in vitro* propagated systems can be fully realised by the successful transfer of plantlets from tissue-culture vessels to moderate *ex vitro* ambient conditions. Most plant species raised under *in vitro* conditions require an acclimatisation process in order to ensure that sufficient number of plants survive and grow vigorously when transferred to appropriate potting media (Bolar *et al*., 1998; Kadleček, 1997; Preece and Sutter, 1991).

The transplantation stage in a suitable potting media, which follows the acclimatisation stage, continues to be a major challenge in plants propagated *in vitro* (Broome and Zimmerman, 1978; Conner and Thomas, 1981; Earle and Langhans, 1975; Ziv, 1986), with *Cucumis* species not being an exemption (Ziv, 1992). *In vitro*-propagated plants are continuously exposed to a unique micro-environment that has been selected to provide

minimal stress and optimum conditions for plant growth (Ziv, 1991). *In vitro*-propagated plantlets are developed within the culture vessels under low level of light, aseptic conditions, on a medium containing considerable amount of sugar and nutrients to allow for heterotrophic growth and in an atmosphere with high level of humidity (Desjardins, 1995; Kozai and Smith, 1995). The favourable conditions of *in vitro* culture, contribute a culture-induced phenotype that might not survive the *ex vitro* conditions when directly placed in the greenhouse or field conditions. The physiological and anatomical characteristics of *in vitro*-propagated plantlets necessitate that they gradually be acclimatised to *ex vitro* conditions through exposure of adverse conditions and use of suitable potting media (Hazarika, 2003).

Almost always, soil from field conditions is not suitable for use as potting media for acclimatised plantlets because the potted soil results in conditions that are completely different from those under natural conditions. Soil from field conditions could induce restricted volume, imbalance of soil microorganisms, impaired nutrition, drainage and aeration challenges (Miller and Jones, 1995). Such conditions could be unfavourable for healthy root growth and subsequent plant development (Hartmann *et al*., 2010). Field surveys of tropical and sub-tropical areas of Africa, Asia and Latin America have inherently poor field growth conditions for seedlings, with poor root development being common when such soils are used as soil-based media in nurseries (Miller and Jones, 1995). Alternatives to soil-based media are organic-based or ‘artificial’-based, whereas the use of steam pasteurised soil is limited to a small percentage of the mixture with other ingredients (Lindsay, 1995; Miller and Jones, 1995).

Generally, potting media are materials other than soils, which offer physical support, supply essential mineral nutrients, maintain good aeration and adequate porosity to absorb and store water required by the growing plants (Vaughn *et al*., 2011). Potting media include (1) organic materials such as peat, compost, tree bark and coconut (*Cocos nucifera* L.) coir, (2) selected inorganic materials, namely, sand, perlite and vermiculite and (3) a mixture of organic and inorganic materials such as Hygromix (Grunert *et al*., 2008). The acclimatising suitable potting medium for transplanting *in vitro*-propagated plantlets of *C. africanus* and *C. myriocarpus* species is not documented. The objective of this study therefore, was to determine whether suitable potting medium for *in vitro* propagated plantlets of *C. africanus* and *C. myriocarpus* would be available for acclimatisation of plantlets.

6.2 Materials and methods

6.2.1 Acclimatisation and greenhouse conditions

Trials on acclimatisation of *in vitro*-propagated *Cucumis* species were conducted at the Department of Biodiversity, University of Limpopo, South Africa (23°53'10" S, 29°44'15" E) in summer (October), 2013. Growth conditions in chambers were similar to those used for shoot multiplication and rooting stages (Chapter 5), but at a 16-h-photoperiod. Selection of suitable potting medium trials was conducted at the Green Technologies Research Centre, University of Limpopo, South Africa (23°53'10" S, 29°44'15" E) in summer (November, 2013-January, 2014). Ambient day/night temperatures in the greenhouse averaged 25/16°C, with maximum temperatures controlled using

thermostatically-activated fans, whereas minimum temperatures depended upon the greenhouse effect. Other greenhouse conditions, namely, relative humidity (RH), solar radiation and photosynthetically active radiation were not measured.

6.2.2 Acclimatisation of *in vitro* plantlets

In vitro C. africanus shoot multiplication and rooted propagules were obtained at the optimum BAP and IBA concentrations of 0.80 µM and 0.31 µM, respectively, (Chapter 5), whereas in *C. myriocarpus*, propagules were obtained at the optimum BAP and IBA concentrations 0.35 µM and 0.44 µM, respectively (Chapter 5). At the end of the rooting stage, 50 g vermiculite growing medium was added to the culture vessels, while the 300 plantlets were still rooted in the Murashige and Skoog (MS) medium. The medium was slightly irrigated with tapwater to increase RH, thereby avoiding the drying up of the medium. Approximately 80-90% RH was maintained by removing the lids of each culture vessel containing plantlets and covered with clear plastic bags (Figure 6.1a). Plantlets were then incubated at 16-h-photoperiod for 7 days *ex vitro* under light intensity and temperature conditions similar to those explained for shoot multiplication stage (Chapter 5). Plantlets were gradually acclimatised to *ex vitro* conditions by perforating the plastic bags (Figure 6.1b) while still covering the culture vessels and incubated for additional 7 days. Irrigation was done with 50 ml tapwater/culture vessel to avoid wilting (Figure 6.1c). At 14 days, plastic bags were completely removed from the plantlets (Figure 6.1d) and kept uncovered for 5 days to acclimatise to stronger illumination (≥ 40 µM/m^2/s) under indoor conditions.

Figure 6.1 Acclimatisation stages: (a) Rooted *Cucumis* species covered in unperforated plastic bags, (b) 7-day-old plantlets in perforated plastic bags, (c) 14-day-old acclimatised plantlets exposed to external conditions and (d) hardened *Cucumis* plantlets.

6.2.3 Experimental design and cultural practises

Sand was collected from Magatle village (24°27'19" S, 29°23'39" E), loam soil was obtained from the Green Technologies Research Centre, University of Limpopo, South Africa (23°53'10" S, 29°44'15" E), vermiculite, Hygromix and compost media were purchased (Hygrotech®, West Pretoria, South Africa). Four-week-old healthy plantlets

measuring 15 cm in height with at least 5-8 leaves and well-developed root systems were carefully removed from the acclimatisation conditions and transplanted into 15-cm-diameter steam pasteurised (300°C for 1 h) plastic pots without washing or trimming of roots. Pots were filled with four mixtures of steam pasteurised growing media, *viz*. sand + loam, sand + vermiculite, sand + Hygromix and sand + compost, each at 3:1 (v/v). The four treatments (Figure 6.2a,b) were arranged in a randomised complete block design (RCBD), with 10 replications (n = 40). Each plant was fertilised with 0.63 g Multifeed P (Plaaskem®, Witfield) to provide 0.12 g N, 0.05 g P, 0.10 g K, 0.59 mg Mg, 0.23 mg Zn, 0.65 mg B, 0.05 mg Mol, 0.05 mg Fe, 0.20 mg Mn and 0.50 mg Cu per ml water. Each plantlet was irrigated with 250 ml tapwater/pot every other day.

6.2.4 Data collection

At harvest, 56 days after transplanting, percentage survival of the acclimatised plantlets was determined by counting the total number of survived plantlets. Stem diameter was measured at 3 cm above soil surface using a digital vernier caliper. Four leaves towards the tip of the vine were selected and measured for chlorophyll content using chlorophyll meter (SPAD 502). Vine length and number of shoots were also measured. Vines were severed at the soil surface using scalpel blade, with root systems removed from pots, washed in a bucket with tapwater to remove excess soil particles and blotted dry. Both shoots and roots were oven-dried at 70°C for 72 h. Data on dry shoot mass and dry root mass were recorded.

Figure 6.2 Greenhouse trials of (a) *Cucumis africanus* and (b) *Cucumis myriocarpus* plantlets in four various potting media.

6.2.5 Statistical analysis

Data for each trial were subjected to analysis of variance through the SAS software (SAS Institute, Inc. 2008). Discrete data were transformed through $\log_{10}(x + 1)$ to homogenise the variances (Gomez and Gomez, 1984), but untransformed data were reported. Fisher's least significant difference test was used to separate the means at the probability level of 5%. Sand + loam were used as reference potting medium, whereas data from sand + vermiculite, sand + Hygromix and sand + compost were expressed relative to the reference potting medium.

6.3 Results

6.3.1 Acclimatisation

Plantlets developed new leaves and elongated inside the clear plastic bags with evidence of more root development in the agar-vermiculite medium. Generally, rooted plantlets acclimatised well under *ex vitro* conditions, with 95-100% survival rate at 20 days of acclimatisation.

6.3.2 Suitable potting media

In *C. africanus,* treatments significantly ($P \leq 0.05$) affected stem diameter, number of shoots, dry shoot mass and dry root mass (Appendix 6.1-6.4), contributing 34%, 71%, 52% and 45% of TTV, respectively (Table 6.1). However, treatments had no effects ($P \leq 0.05$) on vine length and chlorophyll content (Appendix 6.5-6.6). Similarly, in *C. myriocarpus,* treatments significantly ($P \leq 0.05$) affected stem diameter, number of shoots, dry shoot mass, dry root mass and vine length, contributing 44%, 41%, 44%, 28% and 32% of TTV, respectively, without any effect on chlorophyll content.

In *C. africanus*, relative to the reference medium (sand + loam), sand + Hygromix increased stem diameter by 40%, followed by 37% in sand + compost and 20% in sand + vermiculite (Table 6.2). Sand + compost increased the number of shoots by 575%, whereas sand + Hygromix reduced the number of shoots by 75%. In contrast, in *C. africanus*, sand + vermiculite reduced dry shoot mass and dry root mass by 38% and 44%, respectively (Table 6.2), whereas sand + Hygromix increased dry shoot mass and

dry root mass by 8% and 34%, respectively, followed by 3% and 4% increases of the two variables in sand + compost, respectively (Table 6.2).

Table 6.1 Mean sum of squares for stem diameter (SD), number of shoots (NS), dry shoot mass (DSM), dry root mass (DRM), vine length (VL) and chlorophyll content (CC) in *Cucumis africanus* and *Cucumis myriocarpus* plantlets at 56 days after transplanting *ex vitro.*

Source	**DF**	**SD (mm)**		**NS**		**DSM (g)**		**DRM (g)**		**VL (m)**		**CC**	
		SS	**%**	**SS**	**%**	**SS**	**%**	**SS**	**%**	**SS**	**%**	**SS**	**%**
Cucumis africanus													
Replication	9	0.81	10*	8.60	14*	93.10	12*	0.25	16*	25.72	30*	403.27	31*
Treatment	3	2.9	34**	43.80	71**	394.92	52**	0.71	45**	8.22	10*	93.48	7*
Error	27	4.8	56	9.20	15	274.01	36	0.62	39	52.01	60	819.87	62
Total	39	8.5	100	61.60	100	762.03	100	1.57	100	85.94	100	1316.63	100
Cucumis myriocarpus													
Replication	9	1.27	17*	227.53	26*	15.51	15*	20.11	27*	0.48	6*	141.61	14*
Treatment	3	3.23	44**	360.68	41**	45.84	44**	20.77	28**	2.73	32**	35.08	3*
Error	27	2.91	39	299.58	33	41.76	41	33.63	45	5.26	62	858.16	83
Total	39	7.41	100	887.78	100	103.10	100	74.51	100	8.47	100	1034.85	100

* Significant at $P \leq 0.10$, ** significant at $P \leq 0.05$.

Table 6.2 Effects of potting media on stem diameter (mm), number of shoots, dry shoot mass (g) and dry root mass (g) of *Cucumis africanus* plantlets at 56 days after transplanting *ex vitro.*

Treatment	**Stem diameter (mm)**		**No. of shoots**		**Dry shoot mass (g)**		**Dry root mass (g)**	
	Variable[y]	**%[z]**	**Variable**	**%**	**Variable**	**%**	**Variable**	**%**
Sand + loam	1.69[b]	–	0.40[b]	–	0.72[a]	–	11.21[a]	–
Sand + vermiculite	2.02[ab]	20	0.40[b]	0	0.45[b]	–38	6.23[b]	–44
Sand + Hygromix	2.36[a]	40	0.10[b]	–75	0.79[a]	8	15.03[a]	34
Sand + compost	2.32[a]	37	2.70[a]	575	0.74[a]	3	11.65[a]	4

[y]Column means with the same letter were not different (P ≤ 0.05) according to Fisher's least significant difference test.

[z]Impact (%) = [(sand + loam/treatment – 1) x 100].

In *C. africanus*, sand + Hygromix increased vine length by 16%, followed by 15% in sand + compost, whereas in sand + vermiculite the variable was reduced by 7% (Table 6.3). Sand + Hygromix increased chlorophyll content by 2%, but the variable was reduced by 13% and 3% in sand + vermiculite and sand + compost, respectively (Table 6.3).

Table 6.3 Effects of potting media on vine length (m) and chlorophyll content of *Cucumis africanus* plantlets at 56 days after transplanting *ex vitro.*

Treatment	**Vine length (m)**		**Chlorophyll content**	
	Variable[y]	**%[z]**	**Variable**	**%**
Sand + loam	4.62[a]	–	25.57[a]	–
Sand + vermiculite	4.31[a]	–7	22.07[a]	–13
Sand + Hygromix	5.38[a]	16	25.98[a]	2
Sand + compost	5.31[a]	15	24.92[a]	–3

[y]Column means with the same letter were not different (P ≤ 0.05) according to Fisher's least significant difference test.

[z]Impact (%) = [(sand + loam/treatment – 1) x 100].

In *C. myriocarpus*, sand + Hygromix increased stem diameter by 30%, followed by 25% in sand + compost media (Table 6.4). However, in sand + vermiculite the variable was reduced by 9%. Sand + compost increased the number of shoots by 59%, whereas sand + vermiculite and sand + Hygromix reduced the variable by 49% and 20%, respectively (Table 6.4).

Table 6.4 Effects of potting media on stem diameter (mm), number of shoots, dry shoot mass (g) and dry root mass (g) of *Cucumis myriocarpus* plantlets at 56 days after transplanting *ex vitro.*

Treatment	**Stem diameter**		**No. of shoots**		**Dry shoot mass**		**Dry root mass**	
	Variable[y]	**%**[z]	**Variable**	**%**	**Variable**	**%**	**Variable**	**%**
Sand + loam	1.74[b]	–	7.60[b]	–	1.45[b]	–	1.40[b]	–
Sand + vermiculite	1.59[b]	–9	3.90[c]	–49	1.62[b]	12	0.82[b]	–41
Sand + Hygromix	2.26[a]	30	6.10[bc]	–20	3.14[a]	117	2.79[a]	99
Sand + compost	2.18[a]	25	12.10[a]	59	4.02[a]	177	1.48[b]	6

[y]Column means with the same letter were not different (P ≤ 0.05) according to Fisher's least significant difference test.

[z]Impact (%) = [(sand + loam/treatment – 1) x 100].

Sand + compost increased dry shoot mass by 177%, followed by 117% in sand + Hygromix and 12% in sand + vermiculite (Table 6.4). Sand + Hygromix increased dry root mass by 99%, followed by 6% in sand + compost, whereas sand + vermiculite reduced the variable by 41% (Table 6.4). Sand + compost increased vine length by 107%, followed by 39% in sand + Hygromix and 34% in sand + vermiculite (Table 6.5). All three potting media slightly reduced chlorophyll content (Table 6.5).

Table 6.5 Effects of potting media on vine length (m) and chlorophyll content of *Cucumis myriocarpus* plantlets at 56 days after transplanting *ex vitro.*

Treatment	**Vine length (m)**		**Chlorophyll content**	
	Variable[y]	**%[z]**	**Variable**	**%**
Sand + loam	0.67[b]	–	39.20[a]	–
Sand + vermiculite	0.90[b]	34	36.91[a]	–6
Sand + Hygromix	0.93[b]	39	39.14[a]	–0
Sand + compost	1.39[a]	107	38.78[a]	–1

[y]Column means with the same letter were not different (P ≤ 0.05) according to Fisher's least significant difference test.

[z]Impact (%) = [(sand + loam/treatment – 1) x 100].

6.4 Discussion

6.4.1 Acclimatisation of *in vitro* plantlets

The procedure used in this study for acclimatising *in vitro*-propagated *Cucumis* species resulted in higher survival percentages of the plantlets. Generally, soilless media is

used for transferring the plantlets from *in vitro* to *ex vitro* conditions (Ahuja, 1993). The addition of vermiculite medium to plantlets, while still rooted in agar medium was intended to gradually allow essential nutrients in the MS medium to be depleted, allowing plants to become sturdy and thereby promote acclimatisation (Purohit *et al*., 1998). Also, vermiculite medium inside the culture vessels enabled the plantlets to slowly adapt to course growing substrate (Selvaraj *et al*., 2002). Irrigation of *Cucumis* plantlets during acclimatisation stage provided the plants with an opportunity to develop an absorption-transpiration continuum, which is central in the development of the Kingdom Plantae (Salisburry and Ross, 1992), particularly in harsh conditions. The wilting of un-acclimatised explants transferred from *in vitro* to *ex vitro* conditions is probably a sign of a poorly developed absorption-transpiration continuum. Under standard tissue culture conditions, where RH is usually more than 95%, *in vitro* leaves may also not develop a waxy cuticle to the same extent as those found in *ex vitro* conditions (Gilly *et al*., 1997). Covering the culture vessels with clear plastic bags maintained high RH at acclimatisation stage, with perforation of the plastic bags 7-days later, gradually reducing RH, thereby adapting the plantlets to *ex vitro* conditions (Chabukswar and Deodhar, 2005).

6.4.2 Potting media

Generally, stem diameter, number of shoots, dry shoot mass, dry root mass, vine length and chlorophyll content in propagated plants, were usually used as good indicators for assessing growth and development (Wood and Ropper, 2000). In this study, the continuous reduction of growth indicators in *C. africanus* and *C. myriocarpus* plantlets,

transplanted in sand + vermiculite potting medium, suggested that the potting medium was unsuitable for growth and development of the two *Cucumis* species. Although the two *Cucumis* species are known to grow well in sandy loam soil (Van Wyk and Gericke, 2000), its combination with vermiculite reduced growth. Sand is not a suitable growing medium as observed in growth of *Alysicarpus* species, in which yield components were generally negatively correlated with high sand (76-94%) content (Mashela *et al*., 1991). Sand has the largest particles among different soil types and cannot retain nor hold water and nutrients required by the growing plants (Hartmann *et al.*, 2007). When sand is used as a potting medium, water drains quickly to locations where the root system of potted plants, particularly those of transplants and seedlings cannot reach (Hartmann *et al*., 2010). Sand also has a neutral pH and hardly provides fertilisers to plants (Scholes, 1990). However, certain plants like the Transvaal RedMilkwood (*Mimusops zeyheri*), which is indigenous to South Africa, excel in marginal soils like sand (Mashela *et al*., 2013).

A reduction in dry shoot mass and dry root mass due to sand + vermiculite suggested poor overall plant productivity (Wood and Ropper, 2000). Vermiculite is a completely sterile growing medium with a neutral pH and possesses CEC. It holds and makes available, ammonium, calcium, magnesium and potassium to the growing plants (Bunt, 1988). Scarratt (1986) reported low levels of all nutrients required for plant growth in standard peat-vermiculite growing medium, analysed for a variety of essential nutrient elements and other chemical properties. Generally, because of its structurally unstable particles under moist conditions, vermiculite should not be used alone or with sand

(Bunt, 1988) ─ primarily because it compresses over time (Ward *et al*., 1987). The leaf area from the developed shoots is a major source of carbohydrates for growth, whereas a good root system anchors the plant firmly in the potting media, thereby absorbing nutrients and water required by the growing plants (Hartmann *et al*., 2007). In this study, the combination of sand + vermiculite as a potting medium proved to be unsuitable for the two *Cucumis* species.

The use of sand + Hygromix as a potting medium increased most indicators used to assess growth far better than sand + vermiculite, suggesting that the combination of the two media has the potential to be used as a potting medium for the two *Cucumis* species. Sand + Hygromix potting medium have been used successfully in seedling establishment of other plant species, including the two *Cucumis* species (Fessehazion *et a*l., 2008; Nkgapele *et al*., 2012), along with transplants of various vegetables and ornamentals (Maboko *et al*., 2008; Mafeo and Mashela, 2010). Hygromix potting medium also contains some vermiculite, which ensures additional aeration, improved water holding capacity, faster root growth and quick anchorage to young developing roots (Bezuidenhout and Lamprecht, 2010). Also, it contains polystyrene beads, which improves drainage, aeration and reduces the mass of potting media (Warncke and Krauskopf, 1983). In this study, the combination of sand + Hygromix as a potting medium, improved growth and development of the two *Cucumis* species.

The potting media in which plants grow should be porous enough for root aeration, drainage, capable of retaining water and nutrients. A good growing medium should also

be reproducible in order to guarantee crop uniformity and maintain crop production schedules (Whitcomb, 1988). In this study, sand + compost were also found to be a more suitable potting medium for the two *Cucumis* species, in which all the growing indicators measured were increased and some being the highest. For instance, this medium had the highest number of shoots development per plant in the two *Cucumis* species and the highest in vine length of *C. myriocarpus*. The observation in sand + compost medium in this study, supported results in *Cucumis sativas* (L.), in which yields were increased in plants grown in composted growing medium (Al Naddaf *et al*., 2011).

Compost is organic matter that has been decomposed and recycled as a fertiliser and soil amendment (Nair *et al*., 2011). It is used as a soil conditioner, a fertiliser, addition of vital humus or humic acids and as a natural pesticide for soil. Compost is rarely used alone as a potting medium because it does not have the optimal water holding capacity and its soluble salt levels may be higher than optimal for potting mix (Smith and Doran, 1996). Organic materials are an important component of growing media, because they generate a large proportion of micropores and have a high water-holding capacity, yet are resilient enough to resist compaction (Haynes and Naidu, 1998). Compost also has a high CEC and can therefore, retain nutrient ions against leaching and provide a buffer against rapid changes in salinity (Pinamonti *et al*., 1997). The ability of compost to provide most nutrients required by the two potted *Cucumis* species for growth and development and the ability of sand to promote drainage and aeration in the medium, promoted growth and yield, respectively.

6.5 Conclusion

In conclusion, the protocols developed in this study successfully acclimatised *in vitro C. africanus* and *C. myriocarpus* plantlets to *ex vitro* conditions. Sand + compost growing medium were the most suitable potting media for *C. africanus* and *C. myriocarpus* plantlets during weaning from *in vitro* to *ex vitro* conditions. Along with the *in vitro* protocols developed previously (Chapter 4), the current protocols improved the probability of developing uniform, true-to-type and disease-free plants of the two *Cucumis* species as future crops for various industries in the context of climate-smart agriculture.

CHAPTER 7
DEGREE OF NEMATODE RESISTANCE ON *IN VITRO*-PRODUCED PLANTLETS OF NEMATODE-RESISTANT INDIGENOUS *CUCUMIS* SPECIES

7.1 Introduction

Wild watermelon (*Cucumis africanus* LF.) and wild cucumber (*Cucumis myriocarpus* Naude.), indigenous to Limpopo Province, South Africa (Kristkova *et al*., 2003), are highly resistant to the root-knot (*Meloidogyne* species) nematodes (Pofu and Mashela, 2012; Pofu and Mashela, 2011; Pofu *et al*., 2012a,b; Pofu *et al*., 2010a,b; Pofu *et al*., 2009). Generally, nematode resistance in plants is genetically expressed (Thurau *et al*., 2010), with various environmental factors playing a role in the degree of resistance. Nematode resistance in tomato varieties was lost when soil temperatures were above 28°C (Dropkin, 1969). In citrus, cyclic salinity, which is common in the rhizosphere, resulted in the loss of nematode resistance to the citrus nematode (*Tylenchulus semipenetrans* Cobb) (Mashela *et al*., 1992). Also, honeydew-inducing insects such as whiteflies (*Trialeurode vaporariorum* Westwood), resulted in loss of resistance to *M. incognita* in *C. africanus* seedlings under greenhouse conditions (Pofu *et al*., 2011). However, Pofu and Mashela (2012) demonstrated that inter-generic grafting of highly nematode-susceptible watermelon (*Citrullus lanatus* Thunb.) cultivars on *C. africanus* and *C. myriocarpus* seedling rootstocks had no effect on the degree of nematode resistance to various *Meloidogyne* species. Mechanisms involved in the loss of nematode resistance are not yet clear, but literature is replete with evidence that energy proteins, secondary metabolites and plant growth regulators (PGRs) are, to a certain

degree, involved in plant resistance to various pathogens (Pofu *et al*., 2011; Thurau *et al*., 2010).

The two *Cucumis* species had been successfully propagated using *in vitro* techniques (Chapters 5-6). The successful use of *in vitro* techniques depends on the manipulation of the concentrations of PGRs (Murashige and Skoog, 1962; Skoog and Muller, 1957), which are known to play a role in the stability of the genes. Mutations, due to changes in genetic make-up of cells in plantlets produced through *in vitro* techniques, had been observed in various plant species (Van Harten, 1998). Generally, mutations increase genetic variability in various plant species (Lo Schiavo *et al*., 1989) and might result in the loss of certain desired traits, *vice versa*, whereas certain undesired traits could be greatly amplified (Van Harten, 1998). The influence of the PGRs on the degree of resistance to nematodes in plantlets is not documented. The objective of this study, therefore, was to determine whether *in vitro*-produced plantlets from nematode-resistant *C. africanus* and *C. myriocarpus* would retain their resistance to *M. incognita* race 2 under greenhouse conditions.

7.2 Materials and methods

7.2.1 Location and preparation of materials

Ex vitro trials were conducted at the Green Technologies Research Centre, University of Limpopo, South Africa (23°53'10" S, 29°44'15" E) during spring to summer (October-January, 2013/2014). Ambient day/night temperatures in the greenhouse averaged 25/16°C, with maximum temperatures controlled using thermostatically-activated fans, whereas minimum temperatures depended upon the greenhouse effect. Relative

humidity (RH), photosynthetically active radiation and solar radiation were not recorded. *Meloidogyne incognita* race 2 for inoculation was prepared by extracting eggs and second-stage juveniles (J2s) from roots of nematode-susceptible kenaf (*Hibiscus cannabinus* L.) plants in 1% NaOCl solution (Hussey and Barker, 1973).

7.2.2 Preparation of plantlets

Three-month-old healthy plantlets of *C. africanus* and *C. myriocarpus*, measuring 15 cm long, with at least five leaves and a well-developed root system, were transplanted in 15-cm-diameter pots, filled with 1 000 ml steam-pasteurised sand (300°C for 1 h) and compost at 3:1 ratio (v/v). Pots were placed on greenhouse benches at 0.5 m inter-row and 0.6 m intra-row spacing and allowed to establish for 14 days (Figure 7.1). Each plant was fertilised with 0.63 g Multifeed P (Plaaskem®, Witfield) to provide 0.12 g N, 0.05 g P, 0.10 g K, 0.59 mg Mg, 0.23 mg Zn, 0.65 mg B, 0.05 mg Mol, 0.05 mg Fe, 0.20 mg Mn and 0.50 mg Cu per ml water.

7.2.3 Treatments and experimental design

At 14 days, plantlets were each inoculated by dispensing 0, 200, 600, 1 000, 1 400, 1 800 and 2 200 eggs and J2s of *M. incognita* race 2 using a 20 ml plastic syringe. The inocula were placed into 2.5-cm-deep holes on the cardinal points of the stem, with holes covered with the growing media. The zero untreated control plantlets received filtrate (25 µm screen) of nematode suspension to establish any microbes associated with *Meloidogyne* species in their rhizosphere. Treatments were arranged in a randomised complete block design, with ten replicates (n = 70). Plantlets were irrigated

with 250 ml tapwater every other day. Plants were scouted for greenhouse whiteflies and sprayed with 1.33 ml Lebaycid (a.i. fenthion 50% ml) per litre water when populations increased to above 10 whiteflies per plant.

Figure 7.1 *In vitro*-produced *Cucumis africanus* and *Cucumis myriocarpus* plantlets established for *Meloidogyne incognita* race 2 nematodes inoculation.

7.2.4 Data collection

At 56 days after inoculation, vine length was measured from the tip of the plant to the basal-end of the short stem, with multiple vine lengths combined to constitute vine length/plant. The number of shoots developed from the lateral buds of the vines was counted. Mature leaves were randomly selected and measured for chlorophyll content using Chlorophyll meter (SPAD 502). Stem root-collar diameters were measured at 3 cm above the soil surface using a digital vernier caliper. Vines were then severed at the

soil surface using scalpel blade, roots removed from pots, immersed in water to remove excess soil particles and blotted dry. Fresh root mass was measured to facilitate the calculation of nematode numbers per total root system per plant. Root galls, when necessary, were assessed using the North Carolina Differential Scale of 0 = no galls, 1 = 1-2 galls, 2 = 3-11 galls, 3 = 11-30 galls, 4 = 31-100 galls and 5 > 100 galls (Taylor and Sasser, 1978). Shoots were oven-dried for 72 h at 70°C for determination of dry matter.

7.2.5 Nematode extraction and counting

Eggs and J2s were extracted from 5 g roots per plant in 1% NaOCl solution using the maceration and blending method for 30 seconds (Hussey and Barker, 1973). The aliquots were passed through 150, 45 and 25-µm-opening nested screens, with contents from 25 µm screen poured into 100 ml plastic containers and adjusted using tapwater to the mark. Soil samples/pot were collected, with J2s extracted from 250 ml subsample using the modified sugar-floatation and centrifugation method (Coolen and D'Herde, 1972). Briefly, subsamples were separately washed through a 45 µm screen into a 20 L bucket, which was quarter-filled with water and mixed in a swill. After the swill had stopped, the aliquot was decanted onto a 25 µm screen with contents washed into 100 ml plastic centrifuge tubes. Contents of each tube were mixed with 2.5 g kaolin and centrifuged at 1 750 revolutions per minute for five minutes. After decanting the kaolin solution, a 469 g sugar/L tapwater was poured into the centrifuge tubes and stirred once prior to centrifuging for 30 seconds. The aliquot was decanted onto a 25 µm screen with sugar rinsed off the J2s, which were then washed into 100 ml plastic

containers for counting under a stereomicroscope. Throughout counting, which was completed in less than 12 days, uncounted samples were stored at 5°C. Eggs and J2s from roots were converted to nematodes per total root system per plant, whereas soil J2 numbers were converted to 1 000 ml soil per pot to estimate the final nematode population density (Pf). The reproductive factor (RF = Pf/Pi), which is a proportion of Pf to initial population density (Pi), was computed for each treatment, to allow for the assessment of host-status.

7.2.6 Data analysis

Prior to analysis of variance (ANOVA), nematode data were transformed through $\log_{10}(x + 1)$ to homogenise the variances (Gomez and Gomez, 1984), but untransformed data were recorded. Data were subjected to ANOVA through the SAS software (SAS Institute, Inc. 2008) to determine the effects of Pi on RF values and the yield components. Mean separation for significant ($P \leq 0.05$) treatments was achieved through the Waller-Duncan multiple range test. Unless otherwise stated, only treatment means that were significant at the probability level of 5% were discussed.

7.3 Results

7.3.1 Host-status

Generally, roots of plantlets had no galls, whereas more J2s were in roots than in soil. Overall, Pf_{total} values in both *Cucumis* species were less than those used for inoculation (Table 7.1). In all inoculation levels, RF values for *M. incognita* were less than one in

both *C. africanus* and *C. myriocarpus* (Table 7.1). Generally, RF values were higher at low inoculation levels than at high inoculation levels.

7.3.2 Host-sensitivity

Treatments had no effect on vine length, stem diameter, number of shoots, chlorophyll content, dry shoot mass and dry root mass in *C. africanus* and *C. myriocarpus* plantlets (Figure 7.2). In plant nematology, plant variables are important for concluding whether the plant was susceptible, tolerant or resistant to the test nematode (Seinhorst, 1967) and therefore, treatment means of plant variables were reported (Table 7.2).

Figure 7.2 *Cucumis africanus* and *Cucumis myriocarpus* plants after infested with *Meloidogyne incognita* race 2 nematodes.

Table 7.1 Responses of final population densities (Pf) and the reproductive factor (RF) of *Meloidogyne incognita* race 2 in various levels of initial population densities (Pi) on *in vitro*-produced *Cucumis africanus* and *Cucumis myriocarpus* plantlets under greenhouse conditions.

	Cucumis africanus				*Cucumis myriocarpus*			
Pi	Pf_{soil}	Pf_{root}	Pf_{total}	RF^z	Pf_{soil}	Pf_{root}	Pf_{total}	RF^z
200	20	139	159	0.80^a	0	100	100	0.51^a

600	48	147	194	0.32[ab]	0	0	0	0.00[b]
1000	44	396	440	0.44[b]	50	25	75	0.08[ab]
1400	48	98	146	0.11[b]	50	0	50	0.04[b]
1800	172	868	1040	0.58[b]	0	0	0	0.00[b]
2200	32	503	535	0.24[b]	0	0	0	0.00[b]

[z]Column means with the same letter were not different ($P \leq 0.05$) according to Waller-Duncan multiple range test.

Table 7.2 Responses of vine length (VL), number of shoots (NS), stem diameter (SD), chlorophyll content (CC), dry shoot mass (DSM) and dry root mass (DRM) to various levels of initial population densities (Pi) of *Meloidogyne incognita* race 2 on *in vitro*-produced *Cucumis africanus* and *Cucumis myriocarpus* plants under greenhouse conditions.

Pi	**VL (m)**	**NS**	**SD (mm)**	**CC**	**DSM (g)**	**DRM (g)**	**VL (m)**	**NS**	**SD (mm)**	**CC**	**DSM (g)**	**DRM (g)**
	Cucumis africanus						***Cucumis myriocarpus***					
0	5.01	0.40	1.56	25.31	11.59	0.77	1.45	2.60	3.24	38.95	1.28	0.76
200	4.36	0.90	1.32	22.08	9.63	0.92	1.46	1.70	2.39	38.86	0.86	0.34
600	4.58	0.90	1.32	25.57	10.72	0.91	1.41	1.80	2.89	32.61	1.26	0.73
1000	5.20	1.20	1.13	25.98	10.82	0.93	1.35	2.20	2.07	29.25	0.81	0.33
1400	4.35	0.60	1.69	22.10	9.63	1.19	1.45	2.20	2.79	26.79	1.29	0.78
1800	4.16	0.40	1.72	24.25	10.16	1.17	1.46	1.70	2.29	32.82	1.31	0.83
2200	4.47	0.80	1.42	23.00	16.48	0.96	1.45	1.70	2.43	34.53	0.80	0.37
$LSD_{0.05}$	0.58	0.33	0.24	2.67	3.98	0.22	0.08	0.57	0.39	12.75	0.35	0.48

7.4 Discussion

The relatively higher number of juveniles in root systems of both *C. africanus* and *C. myriocarpus* than in soil agreed with observations that juveniles locate and penetrate roots of nematode-susceptible and nematode-resistant plants equally (Kaplan and Davis, 1987). Also, the high number of juveniles in roots confirmed observations by Pofu and Mashela (2011) that nematode resistance in the two *Cucumis* species was apparently post-infectional as observed in other plant species (Acedo *et al*., 1984; Huang, 1986; Pofu *et al*., 2010a; Raja and Dasgupta, 1986; Steele and Savitsky, 1981; Weischer, 1982). However, in cases where chemicals with nematicidal properties are released into the rhizosphere, juvenile penetration is restricted, with pre-infectional nematode resistance being expressed (Caswell *et al*., 1991; McSorley and Gallaher, 1991; Roberts, 1993). The degree of nematode resistance in plants had been described better using three concepts, *viz*. host-status, host-sensitivity and nematode resistance (Seinhorst, 1967).

7.4.1 Host-status

Mean RF values provide an indication of whether a plant is a host or a non-host to the test nematode, with less than one values suggesting the non-host status, whereas the greater than one values imply the host-status (Seinhorst, 1967; Windham and Williams, 1988). Mean RF values of less than one in the current trial suggested that *M. incognita* race 2 failed to feed and reproduce on *in vitro*-produced *C. africanus* and *C. myriocarpus* plantlets. Generally, in the absence of feeding in *Meloidogyne* species, J2s are converted to non-feeding males (Windham and Williams, 1988), which were occasionally observed in the two *Cucumis* species in other studies (Pofu and Mashela, 2011). Since J2s of *M. incognita* race 2 failed to

develop and reproduce on *in vitro*-produced *C. africanus* and *C. myriocarpus* plantlets, the two *Cucumis* species retained their non-host status to this nematode species, which confirmed observations on ungrafted *Cucumis* species (Pofu *et al*., 2009; Pofu *et al*., 2010a,b) and inter-generic grafting on *Citrullus lanatus* cultivars (Pofu and Mashela, 2012). Apparently, the formation of callus as propagules when transferred from medium to medium had negligible effects on properties responsible for nematode resistance in the two *Cucumis* species.

7.4.2 Host-sensitivity

Generally, plants respond to nematodes in one of three ways, namely, stimulated, no response or inhibited growth. At low infection levels prior to the damage threshold level, plants may respond to nematode infection in one of the two ways: (a) no response (Sikora and Fernàndez, 2005) or (b) plant growth may be stimulated (Wallace, 1973). Stimulation of tomato growth had been confirmed when *Meloidogyne* species were below the damage threshold level in various tomato-nematode trials (Mashela and Nthangeni, 2002). Generally, the three nematode-plant relations (stimulated, neutral and inhibited growth) are density-dependent growth patterns (Liu *et al*., 2003), an important concept in biological systems (Salisbury and Ross, 1992). In the two *Cucumis* plantlets, growth of variables was not affected, therefore, both *C. africanus* and *C. myriocarpus* were not susceptible to *M. incognita* race 2. The observed non-susceptibility confirmed results of studies under greenhouse (Pofu *et al*., 2010a), microplot (Pofu *et al*., 2010b) and field (Pofu *et al*., 2009) conditions.

7.4.3 Nematode resistance

According to the Seinhorst (1967) model, when mean RF values were greater than one and nematode infection reduced plant growth, the host was viewed as being susceptible to the target nematode. However, when mean RF values were greater than one and nematode infection had no effect on plant growth, the plant was said to be tolerant. In contrast, when mean RF values were less than one and infection did not result in reduced plant growth, the plant was said to be resistant to the test nematode species. Using the described Seinhorst (1967) model, *in vitro*-produced *C. africanus* and *C. myriocarpus* plantlets retained their resistance to *M. incognita* race 2. The retaining of resistance to *Meloidogyne* species in the two *Cucumis* species after exposure to PGRs is beneficial since both species could be mass produced *in vitro* for use as nematode-resistant rootstocks in inter-generic grafting technologies, especially with *Citrullus lanatus* cultivars, where genotypes that are resistant to *Meloidogyne* species do not exist (Montalvo and Esnard, 1994; Pitrat *et al.,* 1999; Thomason and McKinney, 1959; Winstead and Riggs, 1959). The two *Cucumis* species were shown to be highly resistant to *M. incognita* races 2 and 4 (Pofu and Mashela, 2012) and *M. javanica* (Pofu *et al*., 2012a,b; Pofu *et al*., 2011; Pofu *et al*., 2010a,b; Pofu *et al*., 2009), which are widely distributed in South Africa (Kleynhans *et al*., 1996).

7.5 Conclusion

In conclusion, results of this study suggested that *in vitro*-propagated *C. africanus* and *C. myriocarpus* plantlets retained their resistance to *M. incognita* race 2 after exposure to various PGRs. Therefore, the two *Cucumis* species have the potential to

be mass produced under *in vitro* conditions and serve as future crops in various industries, especially in marginal communities where high population densities of *Meloidogyne* species inhibit economic production of various exotic crops.

CHAPTER 8
SUMMARY, SIGNIFICANCE OF FINDINGS, FUTURE RESEARCH AND CONCLUSIONS

8.1 Summary

Leaching alone eliminated chemical dormancy in wild watermelon (*Cucumis africanus* LF.) and seeds germinated *in vitro*, whereas in wild cucumber (*Cucumis myriocarpus* Naude.) chemical dormancy was not eliminated and seeds did not germinate. In *C. africanus* seeds, cucurbitacin B was deposited exogenously to the testa, whereas in *C. myriocarpus* cucurbitacin A was deposited endogenously to the testa. In the two *Cucumis* species, combining leaching and scarification eliminated both chemical and physical dormancies. Seeds of each *Cucumis* species have two "water gaps", which acted as "water canals" for imbibition and the subsequent exit of the radicle. Generally, seeds of *C. myriocarpus* had five distinct layers, whereas those in seeds of *C. africanus* had four layers, like in most cucurbits (Singh and Dathan, 1972). In both *Cucumis* species, nodal and apical buds were the most suitable propagules for *in vitro* mass propagation, dispelling the notion that cucurbitacin B, which is uniformly distributed in all organs of *C. africanus* could be auto-allelopathic. After acclimatisation of *in vitro*-produced *C. africanus* and *C. myriocarpus* plantlets to greenhouse conditions, sand + compost mixture at 3:1 v/v was the most suitable media for hardening the two *Cucumis* species. Also, *in vitro* propagated plants retained their nematode resistance to *Meloidogyne incognita* race 2.

8.2 Significance of findings

Seed dormancy is widespread in plant species within the Cucurbitaceae Family, which had since induced interest in various industries. The protocols developed to eliminate the two forms of dormancies in this study will help nursery people and researchers to manage dormancies during seed germination within the family. The findings have added new knowledge on chemical and physical dormancies of *C. africanus* and *C. myriocarpus*, which explained variation in germination of seed from the two *Cucumis* species. Cucurbitacin B and cucurbitacin A in *C. africanus* and *C. myriocarpus* seeds, respectively, are respectively accumulated outside and inside the testa. Additionally, the number of layers in the testa differed with *C. africanus* testas having four layers as in other cucurbits (Singh and Dathan, 1972), whereas those of *C. myriocarpus* seeds had five layers. Also, the two “water gaps” at the micropylar and chalaza region, suggested that for sexual propagation, scarification on either region could improve imbibition and thereby, germination after leaching the auto-allelopathic cucurbitacins.

8.3 Future research

Although wild *Cucumis* species had been widely used in various industries, propagation had been a major limitation on consideration to cultivate the species. In addition to pursuing the use of *in vitro*-propagated plantlets in various industries, the use of plantlets in inter-generic grafting technologies for inter-generic grafting with other nematode susceptible cultivars within the Cucurbitaceae Family should be escalated. The plantlets were resistant to *M. incognita* race 2 and should also be tested against *M. incognita* race 4 and *M. javanica*, which are common in South Africa (Kleynhans *et al*., 1996). The highly nutritious *C. myriocarpus* leaves should be explored as leafy vegetable and for generating health soups using the developed

in vitro protocols. Also, the production cost for plantlets and seedlings should be compared in order to enhance decision-making in the choice of the propagation protocol. The protocols developed in this study could also be used in other genera within the Cucurbitaceae Family.

8.4 Conclusions

In conclusion, seeds of the two *Cucumis* species were both chemically and structurally different, but both limitations were ameliorated to enhance seed germination. *In vitro* mass propagation protocols of the two wild *Cucumis* species produced in this study allowed for the propagation of uniform, true-to-type and disease-free plantlets for various industries.

REFERENCES

Abu-Romman, S., Suwwan, M. and E.A.D. Al-Ramamneh. 2013. The influence of plant growth regulators on callus induction from hypocotyls of cucumber (*Cucumis sativas* L.). *Advances in Environmental Biology* 7.

Acedo, J.R., Dropkin, V.H. and V.D. Luedders. 1984. Nematode population attrition and histopathology of *Heterodera glycines*–soybean associations*. Journal of Nematology* 16:48-57.

Adelberg, J.W., Rhodes, B.B. and H.T. Skorupska. 1993. Generating tetraploid melons in tissue culture. *Acta Horticulturae* 336:370-380.

Agarwal, M. and R. Kamal. 2004. *In vitro* clonal propagation of *Momordica charantia* L. *Indian Journal of Biotechnology* 3*:*426-430.

Agricultural Research Council ─ Plant Protection Research Institude (ARC-PPRI)., 2011. Virology sample report letter: Ms Marika van der Merwe. Pretoria, South Africa.

Ahmad, N. and M. Anis. 2005. *In vitro* mass propagation of *Cucumis sativas* L. from nodal segments. *Turkish Journal of Botany* 29:237-240.

Ahuja, M.R. 1993. Micropropagation a` la Carte. *In*: Ahuja, M.R. (ed.), Micropropagation of Woody Plants. Kluwer Academic Publishers: Dordrecht.

Akinnifesi, F.K., Sileshi, G., Mkonda, A., Ajayi, O.C., Mhango, J. and T. Chilanga. 2007. Germplasm Supply, Propagation and Nursery Management of Miombo Fruit Trees. *In*: Akinnifesi, F.K., Sileshi, G., Ajaji, O., Tchoundjeu, Z. and P.

Matakala (eds.), Indigenous Fruit Trees in the Tropics: Domestication, Utilization and Commercialization. CABI publishing: UK.

Alper, Y., Adelberg, J.W., Young, R.E. and B.B. Rhodes. 1994. Unitized, non-selective cutting of *in vitro* watermelon. *Transactions of the American Society of Agricultural Engineers* 37:1331-1336.

Al Naddaf, O., Livieratos, I., Stamatakis, A., Tsirogiannis, I., Gizas, G. and D. Savvas. 2011. Hydraulic characteristics of composted pig manure, perlite and mixtures of them and their impact on cucumber grown on bags. *Scientia Horticulturae* 129:135-141.

Amin, M.N., Azad, M.A.K. and S.M.M. Alam. 1997. Regeneration of plantlets *in vitro* from a medicinal shrub-Basak. 9th Biennial Botanical Conference, University of Dhaka.

Ananthakrishnan, G., Xia, X., Elman, C., Singer, S., Paris, H.S., Galon, A. and V. Gaba. 2003. Shoot production in squash (*Cucurbita pepo* L.) by *in vitro* organogenesis. *Plant Cell Reports* 21:739-746.

Andersson, L., Milberg, P. and A. Noronha. 1997. Germination response of weed seeds to light of short duration and darkness after stratification in soil. *Swedish Journal of Agricultural Research* 27:113-120.

Anon., 2011. Abstract of Agricultural Statistics, Department of Agriculture Forestry and Fisheries. Government Printers: Pretoria.

Arulpragasem. and R. Latiff. 1986. Studies on the tissue culture of tea (*Camellia sinensis* (L.) O. Kuntze): A development of a culture method for the multiplication of shoots. *Sri Lanka Journal of Tea Science* 55:44-47.

Association of Official Seed Analysts (AOSA)., 1990. Rules for testing seeds. *Journal of Seed Technology* 12:1-112.

Attard, E. and A. Scicluna-Spiteri. 2003. The cultivation of cucurbitacin content of *Ecballium elaterium* (L.) A. Rich. *Cucurbit Genetics Cooperative Report* 26:66-69.

Barnea, A., Harborne, J.B. and C. Pannell. 1993. What parts of fleshy fruits contain secondary compounds toxic to birds and why? *Biochemical Systematics and Ecology* 21:421-429.

Barua, P.K. and A.C. Dutta. 1959. Leaf sclereids in the taxonomy of the camellias. *Phytomorphology* 9:372-382.

Basak, O., Demir, I., Mavi, K. and S. Matthews. 2006. Controlled deterioration test for predicting seedling emergence and longevity of pepper (*Capsicum annuum* L.) seed lots. *Seed Science and Technology* 34:723-734.

Baskin, C.C. 2003. Breaking physical dormancy in seeds – focussing on the lens. *New Phytologist* 158:229-232.

Baskin, C.C. and J.M. Baskin. 2005. Seed dormancy in trees of climax tropical vegetation types. *Tropical Ecology* 46:17-28.

Baskin, J.J. and C.C. Baskin. 2004. A classification system for seed dormancy. *Seed Science Research* 4:1-16.

Baskin, C.C. and J.M. Baskin. 2001. Seeds: Ecology, Biogeography and Evolution of Dormancy and Germination. Elsevier.

Baskin, C.C. and J.M. Baskin. 1998. Seeds: Ecology, Biogeography and Evolution of Dormancy and Germination. Academic Press: San Diego, USA.

Baskin, J.M., Baskin, C.C. and X. Li. 2000. Taxonomy, ecology and evolution of physical dormancy in seeds. *Plant Species Biology* 15:139-152.

Baskin, C.C., Baskin, J.M., Yoshinaga, A. and K. Thompson. 2005. Germination of drupelets in multi-seeded drupes of the shrub *Leptecophylla tameiameiae*

(Ericaceae) from Hawaii: A case for deep physiological dormancy broken by high temperatures. *Seed Science Research* 15:349-356.

Bass, L. 1999. Container Vegetable Gardening. North Carolina Cooperative Extension Service.

Bell, E.A. 1980. Secondary Plant Products. *In*: Bell, E.A. and B.W. Charlwood (eds.), Encyclopaedia of Plant Physiology. Springer-Verlag: Berlin.

Benjamin, B.D., Roja, P.C., Heble, M.R. and M.S. Chandha. 1987. Multiple shoot cultures of *Atropa belladona*: Effect of physico-chemical factors on growth and alkaloid formation. *Journal of Plant Physiology* 192:129.

Berenson, M.L. and D.M. Levine. 1996. Basic Business Statistics: Concepts and Applications. Prentice-Hall: Englewood Cliffs, New Jersey.

Bewley, J.D. 1997. Seed germination and dormancy. *Plant Cell* 96:1055-1066.

Bewley, J.D., Black, M. and P. Halmer. 2006. The Encyclopedia of Seeds: Science, Technology and Uses. CAB International: Wallingford, UK.

Bezuidenhout, C.M. and S.C. Lamprecht. 2010. Pathogenicity and cross-pathogenicity of *Fusarium oxysporum* on Proteaceae. *In XXVIII International Horticultural Congress on Science and Horticulture for People (IHC2010): International Symposium* 937:757-762.

Blum, U. 1999. Designing Laboratory Plant Debris Soil Bioassays: Some Reflections. *In*: Inderjit, D.K.M.M. and C.L. Foy (eds.), Principles and Practices of Plant Ecology: Allelochemical Interactions. CRC Press: Florida.

Boesewinkel, F.D. and W. Been. 1979. Development of ovule and testa of *Geranium pratense* L. and some other representatives of the Geraniaceae. *Acta Botanica Neerlandica* 28:335-348.

Bolar, J.P., Norelli, J.L., Aldwinckle, H.S. and V. Hanke. 1998. An efficient method for rooting and acclimation of micropropagated apple cultivars. *HortScience* 37:1257-1252.

Bretzloff, L.V. and N.E. Pellett. 1979. Effect of stratification and gibberellic acid on the germination of *Carpinus caroliniana* Walt. *HortScience* 14:621-622.

Broome, O.C. and R.H. Zimmerman. 1978. *In vitro* propagation of blackberry. *HortScience* 13:151-153.

Brown, N.A.C. and J. Van Staden. 1973. The effect of stratification on the endogenous cytokinin levels of seed at *Protea compacta* and *Leucadendron daphnoides. Physiologia Plantarum* 28:388-92.

Browning, S. and L. Hodges. 2004. Cucumber Crop Information: Bitterness in Zucchini Squash and Cucumber. University of Nebraska Lincoln, NE 68583. August Newsletter.

Bunt, A.C. 1988. Media and Mixes for Container-grown Plants. Unwin Hyman: London.

Campbell, N.A. 1990. Biology. Benjamin/Cummings Publisher: Redwood City.

Caswell, E.P., Defrank, J., Apt, W.J. and C.S. Tang. 1991. Influence of non-host plants on population decline of *Rotylenchulus reniformis*. *Journal of Nematology* 23:91-98.

Chabukswar, M.M. and M.A. Deodhar. 2005. Rooting and hardening of *in vitro* plantlets of *Garcinia indica* Chois. *Indian Journal of Biotechnology* 4:409-413.

Chambliss, O.L. and C.M. Jones. 1966. Cucurbitacins: Specific insect attractants in Cucurbitaceae. *Science* 153:1392-1393.

Chen, S.Y., Kuo, S.R., Baskin, J.M., Baskin, C.C. and C.T. Chien. 2006. Seed dormancy and germination in *Neolitsea acuminatissima* (Lauraceae). *Taiwan Journal of Forest Science* 21:125-129.

Chen, J.C., Chiu, M.H., Nie, R.L., Cordell, G.A. and S.X. Qiu. 2005. Cucurbitacins and cucurbitane glycocides: Structures and biological activities. *Natural Products Report* 22:386-399.

Chen, W.S., Chiu, C.C., Liu, H.Y., Lee, T.L., Cheng, J.T., Lin, C.C., Wu, Y.J. and H.Y. Chang. 1998. Gene transfer via pollen-tube pathway for anti-*Fusarium wilt* in watermelon. *Biochemistry and Molecular Biology International* 46:1201-1209.

Coley, P.D., Bryant, J.P. and F. Stuart Chapin III. 1985. Resource availability and plant anti-herbivore defence. *Science* 230:895-899.

Compton, M.E. 2000. Interaction between explant size and cultivar affects shoot organogenic competence of watermelon cotyledons. *HortScience* 35:749-750.

Compton, M.E. 1999. Dark pre-treatment improves adventitious shoot organogenesis from cotyledons of diploid watermelon. *Plant Cell, Tissue and Organ Culture* 58:185-188.

Compton, M.E. 1997. Influence of seedling pre-treatment and explant type on watermelon shoot organogenesis. *Horticultural Science* 32:514-515.

Compton, M.E. and D.J. Gray. 1993. Shoot organogenesis and plant regeneration from cotyledons of diploid, triploid and tetraploid watermelon. *Journal of the American Society for Horticultural Science* 118:151-157.

Compton, M.E., Gray, D.J. and G.W. Elmstrom. 1993. A simple protocol for micropropagating diploid and tetraploid watermelon using shoot-tip explants. *Plant Cell, Tissue and Organ Culture* 33:211-217.

Conner, A.J. and M.B. Thomas. 1981. Re-establishing plantlets from tissue culture: A review. Proceedings of the International Plant Propagators Society 31:342-351.

Coolen, W.A. and C.J. D'Herde. 1972. A Rapid Method for the Quantitative Extraction of Nematodes From Plant Tissue. State Agricultural Research Center, Ghent: Belgium.

Costich, D.E. 1995. Gender specialization across a climatic gradient: Experimental comparison of monoecious and dioecious *Ecballium*. *Ecology* 76:1036-1050.

Dabauza, M., Gray, D.J. and V.P. Gaba. 1997. Plant regeneration and agrobacterium-mediated transformation of cotyledon explants of *Citrullus colocynthis* (L.) Schrad. *Plant Cell Reports* 16:888-892.

Dalzell, H.W. 1987. Soil management: Compost production and use in tropical and subtropical environments. Food and Agriculture Organization.

Dangl, J.L. and J.D. Jones. 2001. Plant pathogens and integrated defence responses to infection. *Nature* 411:826-833.

Das, U.N. 1985. Studies on Some Growth Regulators. PhD Thesis, Kalyani University. Kolkata.

Debeaujon, I. and M. Branchard. 1992. Induction of somatic embryogenesis and calogenesis from cotyledon and leaf protoplast-derived colonies of melon (*Cucumis melo* L.). *Plant Cell Reports* 12:37-40.

Demir, I., Ermis, S., Mavi, K. and S. Matthews. 2008. Mean germination time of pepper seed lots (*Capsicum annuum* L.) predicts size and uniformity of seedlings and germination tests and transplant modules. *Seed Science and Technology* 36:21-30.

Dennis Thomas, T. and K.R. Sreejesh. 2004. Callus induction and plant regeneration from cotyledon explant of ash gourd (*Benincasa hispida* L.). *Scientia Horticulturae* 100:359-367.

Desjardins, Y. 1995. Photosynthesis *in vitro* on the factors regulating CO_2 assimilation in micropropagation systems. *Acta Horticulturae* 393:45-61.

Devendra, N.K., Attard, E.G., Raghunandan, D. and Y.N. Seetharam. 2011. Study on seasonal variation on the content of cucurbitacin of various vegetative parts of *Trichosanthes cucumerina* L. var. Cucumerina. *International Journal of Plant Research* 1:25-28.

Devran, Z. and M.A. Sogut. 2011. Characterising races of *Meloidogyne incognita*, *Meloidogyne javanica* and *Meloidogyne arenaria* in the West Mediterranean region of Turkey. *Crop Protection* 30:451455.

Dietrich, R., Ploss, K. and M. Heil. 2005. Growth responses and fitness costs after induction of pathogen resistance depend on environmental conditions. *Plant, Cell and Environment* 28:211-222.

Dirks, R. and M. Van Buggenum. 1989. *In vitro* plant regeneration from leaf and cotyledon explants of *Cucumis melo* L. *Plant Cell Reports* 7:626-627.

Dong, J.Z. and S.R. Jia. 1991. High efficiency plant regeneration from cotyledons of watermelon (*Citrullus vulgaris* Schrad*.). Plant Cell Reports* 9:559-562.

Dropkin, V.H. 1969. Cellular responses of plants to nematode infections. *Annual Review Phytopathology* 7:101-122.

Earle, E.D. and R.W. Langhans. 1975. Carnation propagation from shoot-tips cultured in liquid medium. *HortScience* 10:608-610.

Egley, G.H. 1989. Water-impermeable Seed Coverings as Barriers to Germination. *In*: Taylorson, R.B. (ed.), Recent Advances in the Development and Germination of Seeds. Plenum Press: New York.

Eira, M.T.S. and L.S. Caldas. 2000. Seed dormancy and germination as concurrent processes. *Revista Brasileira de Fisiologia Vegetal* 85:85-104.

Ellis, R.A. and E.H. Roberts. 1981. The quantification of ageing and survival in orthox seeds. *Seed Science Technology* 9:373-409.

Ellis, R.H. and E.H. Roberts. 1980. Towards a Rational Basis for Testing Seed Quality. *In*: Hebblethwaite, P.D. (ed.). Butterworths: London.

Emery, D.E. 1987. Seed Propagation of Native California Plants. Santa Barbara Botanic Garden, Santa Barbara, CA.

Érzek, T. and Z. Kiraly. 1986. Phytoalexins and questions that remain unresolved forty-five years after their discovery. *Acta Phytopathologica et Entomolologica Hungarica* 21:5-14.

Esau, K. 1977. Anatomy of Seed Plants. John Wiley and Sons: New York.

Esau, K. 1965. Plant Anatomy. John Wiley and Sons: New York.

Esau, K. 1960. Anatomy of Seed Plants. John Wiley and Sons: New York.

Ferguson, J.E., Metcalf, E.R., Metcalf, R.L. and A.M. Rhodes. 1983. Influence of cucurbitacin content in cotyledons of Cucurbitaceae cultivars upon feeding behaviour of *Diabroticina* beetles (Coleoptera: *Chrysomelidae*). *Journal of Economic Entomology* 76:47-51.

Fessehazion, M.K., Marais, D. and P.J. Robbertse. 2008. Predicting seedling emergence of cabbage and onion using vigour tests. *South African Journal of Plant and Soil* 25:1-7.

Finch-Savage, W.E. and G. Leubner-Metzger. 2006. Seed dormancy and the control of germination. *New Phytologist* 171:501-523.

Finch-Savage, W.E., Bergervoet, J.H.W., Bino, R.J., Clay, H.A. and S.P.C. Groot. 1998. Nuclear replication activity during seed-dormancy breakage and germination in the three tree species: Norway maple (*Acer platanoides* L.), sycamore (*Acer pseudoplatanus* L.) and cherry (*Prunus avium* L.). *Annals of Botany* 81:519-526.

Gama-Arachchige, N.S., Baskin, J.M., Geneve, R.L. and C.C. Baskin. 2011. Acquisition of physical dormancy and ontogeny of the micropyle water gap complex in developing seeds of *Geranium carolinianum* (Geraniaceae). *Annals of Botany* 108: 51-64.

Ganasan, K. and F. Huyop. 2010. *In vitro* regeneration of *Citrullus lanatus* cv. Round dragon. *Journal of Biological Science* 10:131-137.

George, E.F. 1993. Plant Propagation by Tissue Culture in Practice. Exegetics Ltd: Edington.

George, E.F., Hall, M.A. and G.J. De Klerk. 2008. Micropropagation: Uses and Methods. Springer: Netherlands.

Gilly, C., Rohr, R. and A. Chamel. 1997. Ultrastructure and radio labelling of leaf cuticles from ivy (*Hedera helix* L.) plants *in vitro* and during *ex vitro* acclimatization. *Annals of Botany* 80:139-145.

Giraudat, J., Hauge, B.M., Valon, C., Smalle, J., Parcy, F. and H.M. Goodman. 1992. Isolation of the Arabidopsis *AB13* gene by positional cloning. *Plant cell* 4:1251-1261.

Gomez, K.A. and A.A. Gomez. 1984. Statistical Procedures for Agricultural Research. Wiley: New York.

Groot, S.P.C. and C.M. Karssen. 1987. Gibberellins regulate seed germination in tomato by endosperm weakening: A study with gibberellin-deficient mutants. *Planta* 171:525-531.

Grunert, O., Perneel, M. and S. Vandaele. 2008. Peat-based organic grow-bags as a solution to the mineral wool waste problem. *Mires and Peat* 3:1-5.

Guha, J. and S.P. Sen. 1973. Anti-gibberellins of the Cucurbitaceae. *Nature New Biology* 244:223-224.

Gunawardena, A.H., Pearce, D.M., Jackson, M.B., Hawes, C.R. and D.E. Evans. 2001. Characterisation of programmed cell death during aerenchyma formation induced by ethylene or hypoxia in roots of maize (*Zea mays* L.). *Planta* 212:205-214.

Hartmann, H.T., Kester, D.E., Davies, F.T. and R.I. Geneve. 2010. Plant Propagation: Principles and Practices. Prentice Hall: New Jersey.

Hartmann H.T., Kester, D.E., Davies, F.T. and R.I. Geneve. 2007. Hartmann and Kester's Plant Propagation. Principles and Practices. Prentice-Hall of India Private limited.

Hather, J.G. 2000. Archaeological Parenchyma. Archetype Publications: London.

Haque, M.I., Hoque, M.M., Begum, A. and A.S. Islam. 1984. *In vitro* regeneration of plantlets from different explants of *Vigna mungo* L. Hepper. Bangladesh. *Journal of Botany* 13:45-56.

Haque, M.E., Sarkar, M.A., Mahmud, M.A., Rezwana, D. and B. Sikdar. 2008. *In vitro* propagation of pumpkin and ash gourd through nodal segments. *Journal of Biological Sciences* 16:67-71.

Haynes, R.J. and R. Naidu. 1998. Influence of lime, fertilizer and manure applications on soil organic matter content and soil physical conditions: A review. *Nutrient Cycling in Agroecosystems* 51:123-137.

Hazarika, B.N. 2003. Acclimatisation of tissue-cultured plants. *Current Science* 85:1704-1712.

Ho, C.H., Ho, M.G., Ho, S.P. and H.H. Ho. 2014. Bitter bottle gourd *Lagenaria siceraria* toxicity. *The Journal of Emergency Medicine* 46:772-775.

Hoque, A., Islam, R. and O.I. Joarder. 1995. *In vitro* plantlets differentiation in kakrol (*Momordica dioica* Roxb.). *Plant Tissue Culture* 5:119-124.

Howe, W.L., Sanborn, J.R. and A.M. Rhodes. 1976. Western corn rootworms and spotted cucumber beetle associations with *Cucurbita* and cucurbitacin. *Environmental Entomology* 5:1043-1048.

Huang, S.P. 1986. Penetration, development, reproduction and sex ratio of *Meloidogyne javanica* in three carrot cultivars. *Journal of Nematology* 18:408-412.

Hussey, R.S. and K.R. Barker. 1973. A comparison of methods of collecting inocula of *Meloidogyne* species, including a new technique. *Plant Disease Reporter* 42:865-872.

Ikuma, H. and K.V. Thimann. 1963. The role of the seed coat in germination of photosensitive lettuce seeds. *Plant Cell Physiology* 4:169-185.

Inderjit, K. and A.U. Malik. 2002. Chemical Ecology of Plants: Allelopathy in Aquatic and Terrestrial Ecosystems. Birkhauser-Verlag: Berlin.

Inderjit, D.K.M.M., Cheng, H.H. and H. Nishimura. 1999. Plants Phenolics and Terpenoids Transformation, Degradation and Potential for Allelopathic

Interactions. *In*: Inderjit, D.K.M.M. and C.L. Foy (eds.), Principles and Practices in Plant Ecology. Boca Raton, CRC Press: Florida.

Islam, M.R., Khan, M.H., Zohra, F.T., Hossain, M.B. and Z.I. Seraj. 1999. Stable transformation of jute (*Corchorus capsularis* L. var. CVL-1) calli and high frequency marker gene insertion in explants. *Plant Tissue Culture* 9:35-43.

Janzen, D.H. 1979. New Horizons in the Biology of Plant Defences. *In*: Rosenthal G.A. and D.H. Janzen (eds.), Herbivores, Their Interactions With Secondary Plant Metabolites. Academic Press: New York.

Janzen, D.H. 1974. Tropical blackwater rivers, animals and mast fruiting by the Dipterocarpaceae. *Biotropica* 6:69-103.

Jaworski, A., Gorski, P.M., Shannon, S. and R.W. Robinson. 1985. Cucurbitacin concentrations in different plant parts of *Cucurbita* species as a function of age. *Cucurbit Genetics Cooperative Report* 8:71-73.

Jayasuriya, K.M.G.G., Baskin, J.M., Geneve, R.L. and C.C. Baskin. 2007. Morphology and anatomy of physical dormancy in *Ipomoea lacunosa*: Identification of the water gap in seeds of Convolvulaceae (Solanales). *Annals of Botany* 100:13-22.

Jayasuriya, K.M.G.G., Baskin, J.M., Geneve, R.L., Baskin C.C. and C.T. Chien. 2008. Physical dormancy in seeds of the holoparasitic angiosperm *Cuscuta australis* (Convolvulaceae, Cuscuteae): Dormancy-breaking requirements, anatomy of the water gap and sensitivity cycling. *Annals of Botany* 102:39-48.

Jeffrey, C. 1980. A review of the Cucurbitaceae. *Botanical Journal of the Linnean Society* 81:233-247.

Jeffery, C. 1978. Cucurbitaceae. *In*: Launert, E. (ed.), Flora Zambesiaca Managing Committee: London, UK.

Kadleček, P. 1997. Effect of Pre-treatment by Irradiance and Exogenous Saccharose Under *In Vitro* Conditions on Photosynthesis and Growth of Tobacco (*Nicotiana tabacum* L.) Plants During Acclimatization After Transfer to Soil. Diploma Thesis, Charles University. Department of Plant Physiology, Praha.

Kaplan, D.T. and E.L. Davis. 1987. Mechanism of Plant Incompatibility with Nematodes. *In*: Veech, J.A. and D.W. Dickson (eds.), Vistas on Nematology. Society of Nematologists. Hyattsville: Maryland, USA.

Kathal, R., Bhatnagar, S.P. and S.S. Bhojwani. 1994. Plant regeneration from the callus derived from root explants of *Cucumis melo* L. cv. Pusa sharbati. *Plant Science* 96:137-142.

Kathal, R., Bhatnagar, S.P. and S.S. Bhojwani. 1988. Regeneration of plant from leaf explants of *Cucumis melo* L. cv. Pusa sharbati. *Plant Cell Report* 7:449-451.

Kaur, H., Anand, M. and D. Goyal. 2011. Optimization of potting mixture for hardening of *in vitro* raised plants of *Tylophora indica* to ensure high survival percentage. *International Journal of Medicinal and Aromatic Plants* 1:83-88.

Khalekuzzaman, M., Alam, M.F., Nuruzzman, M., Begum, N., Ahmed, M.G., Islam, M.A. and O.I. Joarder. 2003. Clonal propagation of *Averrhoa carambola* Linn. through nodal culture of mature tree. *Journal of Biological Sciences* 3:1153-1157.

Kirschman, J.C. and R.L. Suber. 1989. Recent food poisonings from cucurbitacin in traditionally bred squash. *Food Chemistry and Toxicology* 27:555-556.

Kleynhans, K.P.N., van der Berg, E., Swart, A., Marais, M. and N.H. Buckley.1996. Plant Nematodes in South Africa. Handbook No. 8. Plant Protection Research Institute: Pretoria.

Kobayashi, K. 2004. Factors affecting phytotoxic activity of allelochemicals in soil. *Weed Biology Management* 4:1-7.

Koller, D., Poljakoff-Mayber, A., Berg, A. and T. Diskin. 1963. Germination regulating mechanisms in *Citrullus colocynthis* L. *American Journal of Botany* 50:597-603.

Koricheva, J., Nykänen, H. and E. Gianoli. 2004. Meta-analysis of trade-offs among plant anti-herbivore defences: Are plants jacks-of-all-trades, masters of all? *The American Naturalist* 163:E64-E75.

Kozai, T. and M.A.L. Smith. 1995. Environmental Control in Plant Tissue Culture: General Introduction and Overview. *In*: Aitken-Christie, J., Kozai, T. and M.L. Smith (eds.), Automation and Environmental Control in Plant Tissue Culture. Kluwer Academic Publishers: Dordrecht.

Kristkova, E., Lebeda, A., Vinter, V. and O. Blahousek. 2003. Genetics resources of genus *Cucumis* and their morphological description. *HortScience* 30:14-42.

Krug, M.G.Z., Stipp, L.C.L., Rodriguez, A.P.M. and B.M.J. Mendes. 2005. *In vitro* organogenesis in watermelon cotyledons. *Pesquisa Agropecuária Brasileira* 40:861-865.

Krugman, S.L., Stein, W.I. and D.W. Schmitt. 1974. Seed Biology. *In*: Schopmeyer, C.S. (ed.), Seeds of Woody Plants in the United States Agriculture Handbook 450. USDA Forest Service, Washington, DC.

Kucera, B., Cohn, M.A. and G. Leubner-Metzger. 2005. Plant hormone interactions during seed dormancy release and germination. *Seed Science Research* 15:281-307.

Kung'u, B.J., Kihara, J., Mugendi, D.N. and H. Jaenicke. 2008. Effect of small-scale farmers tree nursery growing medium on agroforestry tree seedlings quality in Mt. Kenya region. *Scientific Research and Essays* 3:359-364.

Kurtar, E.S., Balkaya, A. and N.O. Okumus. 2010. Effects of polymers and growth mediums on *in vitro* plantlets of winter squash (*Cucurbita maxima* Duch. ex Lam.) and pumpkin (*Cucurbita moschata* Duch. ex Poir.) in acclimatization. *Annals of Biological Research* 1:148-154.

Lamberti, F. 1979. Economic Importance of *Meloidogyne* spp. in Subtropical and Mediterranean Climates. *In*: Lamberti, F. and C.E. Taylor (eds.), Root-knot Nematodes (*Meloidogyne* species): Systematics, Biology and Control. Academic press: London, New York, San Francisco.

Lattanzio, V., Lattanzio, V.M.T. and A. Cardinali. 2006. Role of phenolics in the resistance mechanisms of plant against fungal pathogens and insects. *Phytochemistry*: *Advances in Research*. Kerala, India, Research Signpost: 23-67.

Lee, D.H., Iwanski, G.B. and N.H. Thoennissen. 2010. Cucurbitacin: Ancient compound shedding new light on cancer treatment. Mini-Review. *The Scientific World Journal* 10:413-418.

Leedy, P.D. and J.E. Ormrod. 2005. Practical Research: Planning and Design. Pearson Education: New Jersey.

Lema, V., Capparelli, A. and M.L. Pochettino. 2008. Taxonomic identification of dry and carbonized archaeobotanical remains of *Cucurbita* species through seed coat micromorphology. *Vegetation History and Archaeobotany* 17:277-286.

Leroux, O. 2012. Collenchyma: A versatile mechanical tissue with dynamic cell walls. *Annals of Botany* 110:1083-1098.

Leubner-Metzger, G., Frundt, C. and F. Jr. Meins. 1996. Effect of gibberellins, darkness and osmotica on endosperm rupture and class I β-1, 3-glucanase induction in tobacco seed germination. *Planta* 199:282-288.

Li, J., Li, X.M., Qin, Y.G., Tang, Y., Wang, L., Ma, C. and H.X. Li. 2011. Optimized system for plant regeneration of watermelon (*Citrullus lanatus* Thumb.). *African Journal of Biotechnology* 10: 9760-9765.

Lindsay, A.M. 1995. Effects of Soil Compaction on Early Growth of Forest Species in South Eastern Australia. PhD Thesis, La Trobe University. Melbourne.

Little, T.M. and F.J. Hills. 1981. Statistical Methods in Agricultural Research. University of California: Davis, California.

Liu, D.L., An, M., Johnson, I.R. and J.V. Lovett. 2003. Mathematical modelling of allelopathy. III. A model for curve-fitting allelochemical dose responses. *Nonlinearity in Biology Toxicology Medicinal* 1:37-50.

Lott, J.N. 1973. A scanning electron microscope study of *Cucurbita maxima* seed coat structure. *Canadian Journal of Botany* 51:1711-1714.

Lo Schiavo, F., Pitto, L., Giuliano, G., Torti, G., Nuti-Ronchi, V., Marazitti, D., Vergara, R., Orselli, S. and M. Terrzi. 1989. DNA methylation in embryogenic carrot cell cultures and its variations as caused by mutation, differentiation, hormones and hypomethylating drugs. *Theoretical and Applied Genetics* 77:325-331.

Maboko, M.M., Du Plooy, C.P. and I. Bertling. 2008. Comparative performance of tomato cultivars in soilless vs in-soil production systems. *International Symposium on Soilless Culture and Hydroponics* 843:319-326.

Mafeo, T.P. 2014. Pre-sowing temperature treatment effect on emergence of *Cucumis myriocarpus* seedlings. *African Journal of Agricultural Research* 9:2028-2030.

Mafeo, T.P. 2012. Responses of Economically Important Crops to Crude Extracts of *Cucumis* Fruit when Used as Pre-emergent Bio-nematicide. PhD Thesis, University of Limpopo. Sovenga, South Africa.

Mafeo, T.P. 2006. Propagation, Fertilisation and Irrigation of *Cucumis myriocarpus* Masters Dissertation Submitted to the University of Limpopo. Sovenga, South Africa.

Mafeo, T.P. and M.S. Mphosi. 2012. Density-dependent growth responses of seedling emergence in monocotyledonous crops to a *Cucumis* bio-nematicide. *World Journal of Agricultural Science* 8:198-202.

Mafeo, T.P. and P.W. Mashela. 2010. Allelopathic inhibition of seedling emergence in dicotyledonous crops by *Cucumis* bio-nematicide. *African Journal of Biotechnology* 9:8349-835.

Mafeo, T.P. and P.W. Mashela. 2009. Responses of germination in tomato, watermelon and butternut squash to a *Cucumis* bio-nematicide. *American-Eurasian Journal of Agricultural and Environmental Science* 6:215-219.

Maghembe, J.A. 1995. Achievements in the Establishment of Indigenous Fruit Trees of the Miombo Woodlands of Southern Africa. *In*: Maghembe, J.A., Ntupanyama, Y. and P.W. Chirwa (eds.), Improvement of Indigenous Fruit Trees of the Miombo Woodlands of Southern Africa. Proceedings of a Conference Held on 23-27 January 1994. Mangochi, Malawi.

Mahzabin, S., Parvez, M. and M.F. Alam. 2008. Micropropagation of *Cucurbita maxima* Duch. through shoot tip culture. *Journal of Biological Sciences* 16:59-65.

Maila, K.D. and P.W. Mashela. 2013. Responses of the citrus nematode to nemarioc-A phytonematicide with and without effective micro-organisms in citrus production. 11th African Crop Science Proceedings.

Majumdar, G.P. and R.D. Preston. 1941. The fine structure of collenchyma cells in *Heracleum sphondylium* L. Proceedings of the Royal Society of London. Series B, Biological Sciences 130:201-217.

Mamphiswana, N.D., Mashela, P.W. and L.K. Mdee. 2010. Distribution of total phenolics and antioxidant activity in fruit, leaf, stem and root of *Monsonia burkeana*. *African Journal of Agricultural Research* 5:2570-2575.

Martin, P.A. and M. Blackburn. 2003. Inhibition of seed germination by extracts of bitter hawkesbury watermelon containing cucurbitacin, a feeding stimulant for corn rootworm (Coleoptera: *Chrysomelidae*). *Journal of Economic Entomology* 96:441-445.

Mashela, P.W. and M.E. Nthangeni. 2002. Efficacy of *Ricinus communis* fruit meal with and without *Bacillus* species on suppression of *Meloidogyne incognita* and growth of tomato. *Journal of Phytopathology* 150:399-402.

Mashela, P.W., Pofu, K.M. and B. Nzanza. 2013. Responses of Mmupudu (*Mimusops zeyheri*) indigenous fruit tree to three soil types. *African Journal of Agricultural Research* 8:1066-1069.

Mashela, P., Maile, K., Pelinganga, O. and K. Pofu. 2013. Responses of cowpea growth and Bradyrhizobium nodulation to post-cropping soil allelochemical

residue effects of nemafric-b phytonematicide. *African Crop Science Conference Proceedings* 11:845-850.

Mashela, P.W., de Waele, D. and K.M. Pofu. 2011. Use of indigenous *Cucumis* technologies as alternative to synthetic nematicides in management of root-knot nematodes in low-input agricultural farming systems: A review. *Scientific Research and Essays* 33:6762-6768.

Mashela, P.W., Duncan, L.W. and R. McSorley. 1992. Salinity reduces resistance to *Tylenchulus semipenetrans*. *Nematropica* 22:7-12.

Mashela, P., McSorley, R., Duncan, L.W. and R.A. Dunn. 1991. Correlation of *Belonolaimus longicaudatus*, *Hoplolaimus galeatus* and soil texture with yield of alyceclover (*Alysicarpus* spp.). *Nematropica* 21:177-184.

Matthews, S. and A. Powell. 2012. Towards automated single counts of radicle emergence to predict seed and seedling vigour. *Seed Testing* 44.

McSorley, R. and R.N. Gallaher. 1991. Nematode population changes and forage yields of six corn and sorghum cultivars. *Supplement to the Journal of Nematology* 23:673-677.

Meisert, A., Schulz, D. and H. Lehmann. 2001. The ultrastructure and development of the light line in the Geraniaceae seed coat. *Plant Biology* 3:351-356.

Meisert, A., Schulz, D. and H. Lehmann. 1999. Structural features underlying hardseededness in Geraniaceae. *Plant Biology* 1:311-314.

Metcalf, R.L. 1985. Plant kairomones and insect pest control. Ill. *Natural History Survey Bulletin* 33:175-198

Metcalf, R.L., Metcalf, R.A. and A.M. Rhodes. 1980. Cucurbitacins as kairomones for *Diabroticite* beetles. Proceedings of the National Academy of Sciences 77:3769-3772.

Metcalf, R.L., Rhodes, A.M., Metcalf, R.A., Ferguson, J., Metcalf, E.R. and P.Y. Lu. 1982. Cucurbitacin contents and *Diabroticite* (Coleoptera: Chrysomelidae) feeding upon *Cucurbita* spp. *Environmental Entomology* 11:931-937.

Metcalfe, C.R. and L. Chalk. 1979. Anatomy of the Dicotyledons. Clarendon Press: Oxford.

Milind, P. and S. Kaur. 2011. Is bottle gourd a natural guard. *International Research Journal of Pharmacy* 2:13-17.

Miller, J.H. and N. Jones. 1995. Organic and Compost-based Growing Media for Tree Seedling Nurseries. World Bank Forestry Series. Technical Paper Number 264. Washington (DC).

Miller, J.S. and P. Feeny. 1983. Effects of benzylisoquinoline alkaloids on the larvae of polyphagous Lepidoptera. *Oecologia* 58:332-339.

Misra, A.K. and S.P. Bhatnagar. 1995. Direct shoot regeneration from the leaf explants of cucumber (*Cucumis sativas* L.). *Phytomorphology* 45:47-55.

Mofokeng, M.A. 2005. Host-status of *Cucumis myriocarpus* to *Meloidogyne incognita*. Master's Mini-Dissertation Submitted to the University of Limpopo. Sovenga, South Africa.

Mohammadi, J. and N.S. Sivritepe. 2007. *In vitro* clonal propagation of *Cucumis sativas* L. by shoot tip culture. *Journal of Biological Sciences* 7:653-657.

Monohiuddin, A.K.M., Chowdhury, M.K.U., Abdullah, Z.C. and S. Napis. 1997. Influence of silver nitrate (ethylene inhibitor) on cucumber *in vitro* shoot regeneration. *Plant Cell, Tissue and Organ Culture* 51:75-78.

Montalvo, A.E. and J. Esnard. 1994. Reaction of ten cultivars of watermelon (*Citrullus lanatus* Thunb.) to a Puerto Rican population of *Meloidogyne incognita*. *Journal of Nematology* 26:640-643.

Moreno, V. and L.A. Riog. 1990. Somaclonal Variations in Cucurbits. *In*: Bajaj, Y.P.S. (ed.), Biotechnology in Agriculture and Forestry, Vol II. Somaclonal Variation in Crop Improvement I. Springer-Verlag*:* Berlin, Heidelberg, Germany.

Morrison, D.A., McClay, K., Porter, C. and S. Rish. 1998. The role of the lens in controlling heat-induced breakdown of testa-imposed dormancy in native Australian legumes. *Annals of Botany* 82:35-40.

Morrissey, J.P. and A.E. Osbourn. 1999. Fungal resistance to plant antibiotics as a mechanism of pathogenesis. *Microbiology and Molecular Biology Reviews* 63:708-724.

Murashige, T. 1974. Plant propagation through tissue culture. *Annual Review of Plant Physiology* 25:135-166.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15:473-497.

Murray, R.D.H., Mendez, J. and S.A. Brown. 1982. The Natural Coumarins. Wiley: New York.

Nair, A., Ngouajio, M. and J. Biernbaum. 2011. Alfalfa-based organic amendment in peat-compost growing medium for organic tomato transplant production. *HortScience* 46:253-259.

Nell, T.A., Marsh, P.M. and D.J. Cantliffe. 1981. Seed dormancy and germination of geranium (*Pelargonium hortorum* Bailey). *Journal of the American Society for Horticultural Science* 106:509-513*.*

Nicolson, D.H., 1960. The occurrence of trichosclereids in the Monsteroideae (Araceae). *American Journal of Botany* 47:598-602.

Nkgapele, R.J., Mphosi, M.S. and K.G. Shadung. 2012. Response of leaf, non-leaf yield characters and plant fraction interrelationship of indigenous wild cucumber (*Cucumis myriocarpus* Naude.) to varying irrigation intervals and NPK fertilizer rates. *Sustainable Irrigation and Drainage IV: Management, Technologies and Policies* 168:359.

Nocito, F.F., Pirovano, L., Cocucci, M. and G.A. Sacchi. 2002. Cadmium-induced sulfate uptake in maize roots. *Plant Physiology* 129:1872-1879.

Noble, P. 1993. Effect of potting mixture on farm tree seedlings survived in heavy soils. *Agroforestry Systems* 21:75-78.

Novak, F.J. and M. Dolezeloa. 1982. Hormone control of growth and differentiation in the *in vitro* cultured tissue of cucumber (*Cucumis sativas* L.). *Biologia* 37:283-290.

Odo, P.E. and P.E. Oleghe. 1988. The Production and Management of Gum Arabic in the Sudan and Sahelian Zones of Borno State, Nigeria.

Offord, C.A. and P.F. Meagher. 2009. Plant Germplasm Conservation in Australia: Strategies and Guidelines for Developing, Managing and Utilising *Ex Situ* Collections, Canberra. Australian Network for Plant Conservation Inc.

Osbourne, D.J. 1983. Biochemical control system operating in the early hours of germination. *Canadian Journal of Botany* 61:3568-3577.

Pal, S.P., Iftekar, A., Anisuzzamn, M., Kanak Kanti, S., Shamima Akthar, S. and M.F. Alam. 2007. Indirect organogenesis in summer squash (*Cucurbita pepo* L.). *Turkish Journal of Agriculture* 31:66-70.

Palada, M.C. and L.C. Chang. 2003. Suggested cultural practices for bitter gourd. *AVRDC International Cooperators' Guide* 03-547.

Paul, N.D., Hatcher, P.E. and J.E. Taylor. 2000. Coping with multiple enemies: An integration of molecular and ecological perspectives. *Trends in Plant Science* 5:220-225.

Pelinganga, O.M. 2013. Developing Phytonematicides Using Indigenous *Cucumis africanus* and *Cucumis myriocarpus* Fruits for Tomato Production Systems. PhD Thesis, University of Limpopo. Sovenga, South Africa.

Pelinganga, O. and P. Mashela. 2012. Mean dosage stimulation range of allelochemicals from crude extracts of *Cucumis africanus* fruit for improving growth of tomato plant and suppressing *Meloidogyne incognita* numbers. *Journal of Agricultural Science* 4:8.

Pelinganga, O., Mashela, P., Mphosi, M. and B. Nzanza. 2013. Optimizing application frequency of diluted (3%) fermented *Cucumis africanus* fruit in tomato production and nematode management. *Acta Agriculturae Scandinavica, Section B, Soil and Plant Science* 63:278-282.

Pelinganga, O.M., Mashela, P.W., Nzanza, B. and M.S. Mphosi. 2012. Baseline information on using fermented crude extracts from *Cucumis africanus* fruit for suppression of *Meloidogyne incognita* and improving growth of tomato plant. *African Journal of Biotechnology* 11:11407-11413.

Pillay, D.T.N. and L.J. Edgerton. 1965. Relationship of growth substances to rest period and germination in Mazzard cherry seeds. Proceedings, American Society for Horticultural Science 86:108-14.

Pinamonti, F., Stringari, G., and G. Zorzi. 1997. Use of compost in soilless cultivation. *Compost Science and Utilization* 5:38-46.

Pinfield, N.J. and A.K. Stobart. 1969. Hormonal regulation of germination and early seedling development in *Acer pseudoplatanus* (L.). *Planta* 104:134-45.

Pitrat, M., Chauvet, M. and C. Foury. 1999. Diversity, history and production of cultivated cucurbits. *Acta Horticulturae* 492:21-28.

Pofu, K.M. 2012. Potential Uses of Indigenous *Cucumis africanus* and *Cucumis myriocarpus* as Root-knot Nematode-resistant Rootstocks in Watermelon (*Citrullus lanatus* Thunb.) Husbandry. PhD Thesis, University of Limpopo. Sovenga, South Africa.

Pofu, K.M. and P.W. Mashela. 2014. Density-dependent growth patterns of *Meloidogyne javanica* on hemp cultivars: Establishing nematodes-sampling timeframes in host-status trials. *American Journal of Experimental Agriculture* 4:639-650.

Pofu, K.M. and P.W. Mashela. 2012. Improving survival of inter-generic grafts of nematode-susceptible watermelon cultivars and nematode-resistant *Cucumis* species. *Acta Agriculturae Scandinavica, Section B, Soil and Plant Science* 62:383-386.

Pofu, K.M. and P.W. Mashela. 2011. Using relative penetration and maleness indices in *Meloidogyne incognita* to establish resistance type in *Cucumis myriocarpus*. *African Journal of Biotechnology* 10:390-393.

Pofu, K.M., Mashela P.W. and H. Shimelis. 2012a. Inter-generic grafting in watermelon for managing *Meloidogyne* species: A review. *Scientific Research and Essays* 7:107-113.

Pofu, K.M., Mashela, P.W. and D. De Waele. 2012b. Survival, flowering and productivity of watermelon (*Citrullus lanatus*) cultivars in inter-generic grafting on nematode-resistant *Cucumis* seedling rootstocks in *Meloidogyne*-infested fields. *International Journal of Agriculture and Biology* 14:217-222.

Pofu, K.M., Mashela P.W., Mokgalong, M.N. and D. De Waele. 2011. Greenhouse whitefly infection on wild watermelon break resistance to the root-knot nematode. *African Crop Science Conference Proceedings* 10:239-244.

Pofu, K.M., Mashela, P.W. and N.M. Mokgalong. 2010a. Host-status and host sensitivity of *Cucumis africanus* and *Cucumis myriocarpus* to *Meloidogyne incognita* race 2 under greenhouse conditions. *African Journal of Agricultural Research* 5:1504-1508.

Pofu, K.M., Mashela, P.W. and M.S. Mphosi. 2010b. Responses of *Cucumis africanus* and *Cucumis myriocarpus* to *Meloidogyne incognita* race 2 under microplot conditions. *International Journal of Nematology* 20:113-118.

Pofu, K.M., Mafeo, T.P. and P.W. Mashela. 2009. The responses of *Cucumis africanus* to *Meloidogyne incognita* race 2 infections. African Crop Science Proceedings 9:659-661.

Pogson, B.J. and V. Albrecht. 2011. Genetic dissection of chloroplast biogenesis and development: An overview. *Plant Physiology* 155:1545-1551.

Pospíšilová, J., Synková, H., Haisel, D. and S. Semoradova. 2007. Acclimation of plantlets to *ex vitro* conditions: Effects of air humidity, irradiance, CO_2 concentration and abscisic acid (a review). *Acta Horticulturae* 748:29.

Pospíšilová, J., Tichá, I., Kadleček, P., Haisel, D. and Š. Plzáková. 1999. Acclimatization of micropropagated plants to *ex vitro* conditions. *Biologia Plantarum* 42:481-497.

Prasanna Kumar, G.V. and H. Raheman. 2010. Volume of vermicompost-based potting mix for vegetable transplants determined using fuzzy biomass growth index. *International Journal of Vegetable Science* 16:335-350.

Preece, J.E. and E.G. Sutter. 1991. Acclimatization of Micropropagated Plants to the Greenhouse and Field. *In*: Debergh, P.C. and R.H. Zimmerman (eds.), Micropropagation. Technology and Application. Kluwer Academic Publishers: Dordrecht, Boston, London.

Purohit, S.D., Tak, K., Joshi, P. and D. Samar. 1998. Micropropagation of Some Woody Tree Species of *Aravallis* in Rajasthan. *In*: Srivastava, P.S. (ed.), Plant Tissue Culture and Molecular Biology–Application and Prospects. Narosa Publishing House: Delhi.

Purrington, C.B. 2000. Costs of resistance. *Current Opinion in Plant Biology* 3:305-308.

Radchuk, V. and L. Borisjuk. 2014. Physical, metabolic and developmental functions of the seed coat. *Frontiers in plant science 5*.

Raja, A. and D.R. Dasgupta. 1986. Enhanced synthesis of messenger RNA in relation to resistance expression in cowpea (*Vigna unguiculata*) infected with the root-knot nematode, *Meloidogyne incognita. Journal of Nematology* 9:35-49.

Ramakrishna, P. and D. Amritphale. 2005. The perisperm-endosperm envelope in *Cucumis*: Structure, proton diffusion and cell wall hydrolysing activity. *Annals of Botany* 96:769-778.

Raven, P.H., Evert, R.F. and H. Curtis. 1981. Biology of Plants. Worth Publishers: New York.

Rice, E.L. 1984. Allelopathy. Academic Press: New York.

Rimington, P. 1938. Medicinal and Poisonous Plants of Southern and Eastern Africa. University of Natal Press: Pietermaritzburg.

Roberts, P.A. 1993. The future of nematology: Integration of new and improved management strategies. *Journal of Nematology* 25:383-394.

Robertson, L. and M.A. Diez-Rojo. 2008. New host races of *Meloidogyne arenaria*, *Meloidogyne incognita* and *Meloidogyne javanica* from horticultural regions of Spain. *Plant Disease* 93:180-184.

Rolston, M.P. 1978. Water impermeable seed dormancy. *The Botanical Review* 44:365-396.

Rout, G.R. 2004. Effect of cytokinins and auxin on micropropagation of *Clitoria ternatea* L. *Biology Letters* 41:21-26.

Roy, B.A. and J.W. Kirchner. 2000. Evolutionary dynamics of pathogen resistance and tolerance. *Evolution* 54:51-63.

Saberi, M., Shahriari, A., Tarnian, F. and S. Noori. 2011. Comparison the effect of different treatments for breaking seed dormancy of *Citrullus colocynthis* L. *Journal of Agricultural Science* 3:62.

Saha, S., Mori, H. and K. Hattori. 2007. Synergistic effect of kinetin and benzyladenine plays a vital role in high frequency regeneration from cotyledon explants of bottle gourd (*Lagenaria siceraria* Mol.) in relation to ethylene production. *Breeding Science* 57:197-202.

Salanenka, Y.A., Goffinet, M.C. and A.G. Taylor. 2009. Structure and histochemistry of the micropylar and regions of the perisperm–endosperm envelope of cucumber seeds associated with solute permeability and germination. *American Society for Horticultural Science* 134:479-487.

Salisbury, F.B. and C.W. Ross. 1992. Plant Physiology. Wadsworth: Belmont, California.

Sarowar, S., Oh, H.Y., Hyung, N.I., Min, B.W., Harn, C.H., Yang, S.K., Ok, S.H. and J.S. Shin. 2003. *In vitro* micropropagation of *Cucurbita* interspecific hybrid cultivar: A rootstock plant. *Plant Cell, Tissue and Organ Culture* 75:179-182.

SAS Institute Inc. 2008. SAS/STAT® 9.2 qualification tools user's guide. SAS Institude Inc: Carry (NC).

Scarratt, J.B. 1986. An Evaluation of Some Commercial Soluble Fertilizers for Culture of Jack Pine Container Stock. Information Report, O-X-377. Sault Sainte Marie, Ontario: Canadian Forestry Service.

Schmidt, M.G. 1980. Growing California Native Plants. University of California Press: Los Angeles.

Scholes, R.J. 1990. The influence of soil fertility on the ecology of southern African dry savannahs. *Journal of Biogeography* 17:415-419.

Schulz, D., Bachthaler, E. and U. Kunz. 1991. Seed coat structure of *Pelargonium zonale*. *Gartenbauwissenschaft* 56:118-126.

Seinhorst, J.W. 1967. The relationships between population increase and population density in plant-parasitic nematodes. II. Sedentary nematodes. *Nematology* 13:157-171.

Selvaraj, N., Vasudevan, A., Manickavasagam, M. and A. Ganapathi. 2006. *In vitro* organogenesis and plant formation in cucumber. *Biologia Plantarum* 50:123-126.

Selvaraj, N., Vengadesan, G., Vasudevan, A., Prem Anand, R., Ramesh Anbazhgan, V. and A. Ganapathi. 2002. Micropropagation of *Cucumis sativas* L. from Field Grown Plants. *In*: Maynard, D.N. (ed.), Proceedings of Cucurbitaceae 2002 Conference. ISHS Publications: Naples.

Shah, B.N., Seth, A.K. and R.V. Desai. 2010. Phytopharmacological profile of *Lagenaria siceraria*: A review. *Asian Journal of Plant Science* 9:152-157.

Sharma, G.C. and C.V. Hall. 1973. Relative attractance of spotted cucumber beetle to fruits of fifteen species of Cucurbitaceae. *Environmental Entomology* 2:154-156.

Shaukat, S.S., Munir, N. and I.A. Siddiqui. 2003. Allelopathic responses of *Conyza canadensis* (L.) Cronquist: A cosmopolitan weed. *Asian Journal of Plant Sciences* 2*:*1034-1039.

Shifriss, O. and W.L. George. 1965. Delayed germination and flowering in cucumber. *Nature* 206:424-425.

Shrivastava, A. and S. Roy. 2011. Plant regeneration from the nodal part of *Citrullus colocynthis* L. schard. *International Journal of Pharmaceutical Sciences and Research* 2:2825-2827.

Sikora, R.A and E. Fernàndez. 2005. Nematode Parasites of Vegetables. *In*: Luc. M., Sikora, R.A. and J. Bridge (eds.), Plant-parasitic Nematodes in Subtropical and Tropical Agriculture. Wallingford: CABI.

Simpson, G.M. 1990. Seed Dormancy in Grasses. Cambridge University Press: Cambridge.

Singh, D. 1967. Structure and development of seedcoat in Cucurbitaceae. In Proceedings of the Indian Academy of Sciences-Section B 65:267-274.

Singh, D. and A.S.R. Dathan. 2001. Development and Structure of Seed Coat in the Cucurbitaceae and its Implications in Systematics. *In:* Chauhan, S.V.S. and S.N. Chaturvedi (eds.), Botanical Essays: Tribute to Professor Bahadur Singh. Printwell Publishers Distributors: Jaipur, India.

Singh, D. and A.S.R. Dathan. 1998. Morphology and Embryology. *In*: Nayar N.M. and T.A. More (eds.), Cucurbits. Science Publishers: Enfield, U.S.A.

Singh, D. and A.S.R. Dathan. 1972. Structure and development of seed coat in Cucurbitaceae. IV. Seeds of *Cucurbita*. *Phytomorphology* 22:29-45.

Singh, M.N., Kathal, R. and S.P. Bhatnagar. 1990. Regeneration of plants from hypocotyls and cotyledons cultures of *Cucumis melo* L. cv. Pusa maduras. *Phytomorphology* 40:401-405.

Skoog, F. and C.O. Miller. 1957. Chemical regulation of growth and organ formation in plant tissue cultures *in vitro*. *Sympsia of the Society for Experimental Biology* 11:118-131.

Smith, J. and J.W. Doran. 1996. Measurement and use of pH and Electrical Conductivity for Soil Quality Analysis. *In*: Doran, J.W. and A.J. Jones (eds.), Methods for Assessing Soil Quality. Soil Science Society of America Special Publication. Madison, Wisconsin.

Srivastava, D.R., Andrianov, V.M. and E.S. Piruzian. 1989. Tissue culture and plant regeneration of watermelon (*Citrullus vulgaris* Schrad. cv. Melitopolski). *Plant Cell Reports* 8:300-302.

Steele, A.E. and H. Savitsky. 1981. Resistance of trisomic and diploid hybrids of *Beta vulgaris* B. procumbent to the sugarbeet nematode, *Heterodera schatii*. *Journal of Nematology* 13:352-357.

Stuart, S.G. and J.B. Loy. 1983. Comparison of testa development in normal and hull-less seeded strains of *Cucurbita pepo* L. *Botanical Gazette* 491-500.

Sultana, R.S., Bari, M.A., Rahman, M.H., Rahman, M.M., Siddique, N.A. and N. Khatun. 2004. *In vitro* rapid regeneration of plantlets from leaf explant of watermelon (*Citrullus lanatus* Thunb.). *Biotechnology* 3:131-135.

Tallamy, D.W. and V.A. Krishik. 1989. Variation and function of cucurbitacins in *Cucurbita*: An examination of current hypotheses. *American Naturalist* 133:766-786.

Tanveer, A., Tasneem, M., Khaliq, A., Javaid, M.M. and M.N. Chaudhry. 2013. Influence of seed size and ecological factors on the germination and emergence of field bindweed (*Convolvulus arvensis*). *Planta Daninha* 31:39-51.

Tao, K. and A.A. Khan. 1974. Penetration of dry seeds with chemicals applied in acetone. *Plant Physiology* 54:956-958.

Taylor, A.L. and J.N. Sasser. 1978. Biology, Identification and Control of Root-knot Nematodes (*Meloidogyne* species). North Carolina State University Press: Raleigh.

Taylor, J.E., Hatcher, P.E. and N.D. Paul. 2004. Crosstalk between plant responses to pathogens and herbivores: A view from the outside-in. *Journal of Experimental Botany* 55:159-168.

Teppner, H. 2004. Notes on *Lagenaria* and *Cucurbita* (Cucurbitaceae): Review and new contributions. *Phyton* 44:245-308.

Teppner, H. 2000. *Cucurbita pepo* L. (Cucurbitaceae): History, seed coat types, thin coated seeds and their genetics. *Phyton* 40:1-42.

Thies, J.A., Ariss, J.J., Hassell, R.L., Olson, S., Kousik, C.S. and A. Levi. 2010. Grafting for management of southern root-knot nematode, *Meloidogyne incognita*, in watermelon. *Plant Disease* 94:1195-1199.

Thomason, I.J. and H.E. McKinney. 1959. Reaction of some Cucurbitaceae to root-knot nematodes (*Meloidogyne* spp.). *Plant Disease Reporter* 43:448-450.

Thornton, M.L. 1968. Seed dormancy in watermelon *Citrullus vulgaris* Shrad. In Proceedings of the Association of Official Seed Analysts. The Association of Official Seed Analysts.

Thurau, T., Ye, W. and D. Cai. 2010. Insect and Nematode Resistance. *In:* Widholm, J.M. and T. Nagata (eds.), Biotechnique in Agriculture and Forestry. Springer-Verlag: Heidelberg, Berlin.

Tillman, O.I. 1906. The embryo sac and embryo of *Cucumis sativas* L. *Ohio Naturalist* 7:423-430.

Ting, I.P. 1982. Plant Physiology. Addison-Wesleyn Reading: Massachusetts.

Ugandhar, T., Venkateshwarrlu, M., Begum, G., Srilatha, T. and K. Jaganmohanreddy. 2011. *In vitro* plant regeneration of cucumber (*Cucumis sativum* L.) from cotyledon and hypocotyl explants. *Science Research Reporter* 1.

Van Harten, A.M. 1998. Mutation Breeding. Theory and Practical Application. Press Syndicate: University of Cambridge, UK.

Van Rooyen, N. 2001. Flowering Plants of the Kalahari Dunes. Lynnwood: Pretoria.

Van Wyk, B.E. and M. Wink. 2012. Medicinal Plants of the World: An Illustrated Scientific Guide to Important Medicinal Plants and Their Uses. Briza, Publications: Pretoria.

Van Wyk, B.E. and N. Gericke. 2000. People's Plants. A Guide to Useful Plants of Southern Africa. Briza Publications: Pretoria.

Van Wyk, B., Heerden, F. and B. Outshoorn. 2002. Poisonous Plants of South Africa. Briza Publications: Pretoria.

Vasudevan, A., Selvaraj, N., Ganapathi, A., Kasthurirengan, S., Anbazhagan, V.R. and M. Manickavasagam. 2004. Glutamine: A suitable nitrogen source for

enhanced shoot multiplication in *Cucumis sativas* L. *Biologia Plantarum* 48:125-128.

Vaughan, J.G. 1970. The Structure and Utilization of Oilseeds. Chapman and Hall: London.

Vaughn, S.F., Deppe, N.A., Palmquist, D.E. and M.A. Berhow. 2011. Extracted sweet corn tassels as a renewable alternative to peat in greenhouse substrates. *Industrial Crops and Products* 33:514-517.

Wallace, H.R. 1973. Nematode Ecology and Plant Disease. Edward Arnold: London.

Ward, J., Bragg, N.C. and B.J. Chambers. 1987. Peat-based composts: Their properties defined and modified to your needs. Combined Proceedings of International Plant Propagators Society 36:288-292.

Wardrop, A.B. 1969. The structure of the cell wall in lignified collenchyma of *Eryngium* spp. (Umbelliferae). *Australian Journal of Botany* 17:229-240.

Warncke, D.D. and D.M. Krauskopf. 1983. Greenhouse growth media: Testing and nutrition guidelines. *Extension Bulletin* E 1736.

Watkins, J.T., Cantliffe, D.J., Huber, D.J. and T.A. Nell. 1985. Gibberellic acid stimulated degradation of endosperm in pepper. *Journal of the American Society of Horticultural Science* 110:61-65.

Webb, D.P. and E.B. Dumbroff. 1969. Factors influencing the stratification process in seeds of *Acer saccharum. Canadian Journal of Botany* 47:1533-63.

Weischer, B. 1982. Histopathological studies on grape species with different degrees of resistance to *Xiphenema index*. *Nematologica* 28:178-179.

Welbaum, G.E., Muthui, W.J., Wilson, J.H., Grayson, R.L. and R.D. Fell. 1995. Weakening of muskmelon perisperm envelope tissue during germination. *Journal of Experimental Botany* 46:391-400.

Werker, E. 1997. Seed Anatomy. Gebrüder Borntraeger: Berlin.

Whitcomb, C.E. 1988. Plant Production in Containers. Lacebark Publications: Stillwater, Oklahoma.

Windham, G.L. and W.P. Williams. 1988. Reproduction of *M. javanica* on corn hybrids and inbreeds. *Annals of Applied Nematology* 2:25-28.

Winstead, N.N. and R.D. Riggs. 1959. Reaction of watermelon varieties to root-knot nematodes. *Plant Disease Reporter* 43:909-912.

Wittstock, U. and J. Gershenzon. 2002. Constitutive plant toxins and their role in defense against herbivores and pathogens. *Current Opinion in Plant Biology* 5:300-307.

Wood, A.J. and J. Roper. 2000. A simple and non-destructive technique for measuring plant growth and development. *American Biology Teacher* 62:215.

Yeung, E.C. and M.J. Cavey. 1990. Developmental changes in the inner epidermis of the bean seed coat. *Protoplasma* 154:45-52.

Zhang, J. and M.A. Maun. 1989. Seed dormancy of *Panicum virgatum* L. on the shoreline sand dunes of Lake Erie. *American Midland Naturalist* 122:77-87.

Ziv, M. 1992. The Use of Growth Retardants for the Regulation and Acclimatization of *In Vitro* Plants. *In*: Karssen, C.M., Van Loon, L.C. and D. Vreugdenhil (eds.), Progress in Plant Growth Regulation. Current Plant Science Biotechnology in Agriculture. Kluwer Academic Publishers: Dordrecht, Netherlands.

Ziv, M. 1991. Quality of micropropagated plants: Vitrification*. In vitro Cellular and Developmental Biology Plant* 27:64-69.

Ziv, M. 1986. *In Vitro* Hardening of Tissue Cultured Plants. *In*: Withers, L.A. and P.G. Alderson (eds.), Plant Tissue Culture and its Agricultural Applications. Butterworths: London.

APPENDICES

Appendix 3.1 Analysis of variance for germination percentage (GP) in leached-control (LC) and leached-scarified (LS) seeds of *Cucumis africanus in vitro*.

SOURCE	**DF**	**SS**	**%**	**F**	**P ≤**
		LC seeds			
Treatment	6	1017.1	41	3.29	0.01
Error	28	1440.0	59		
Total	34	2457.1	100		
		LS seeds			
Treatment	6	17166.7	72	11.79	0.00
Error	28	6793.0	28		
Total	34	23960.0	100		

Appendix 3.2 Analysis of variance for mean germination time (MGT) in leached-control (LC) and leached-scarified (LS) seeds of *Cucumis africanus in vitro*.

SOURCE	**DF**	**SS**	**%**	**F**	**P ≤**
		LC seeds			
Treatment	6	6595.8	35	2.55	0.06
Error	28	12037.7	65		
Total	34	18633.5	100		
		LS seeds			
Treatment	6	197.05	68	9.96	0.01
Error	28	92.34	32		
Total	34	289.39	100		

Appendix 3.3 Analysis of variance for germination index (GI) in leached-control (LC) and leached-scarified (LS) seeds of *Cucumis africanus in vitro*.

SOURCE	DF	SS	%	F	P ≤
		LC seeds			
Treatment	6	0.0154	44	0.00	0.01
Error	28	0.0200	56		
Total	34	0.0354	100		
		LS seeds			
Treatment	6	1.3036	65	8.65	0.00
Error	28	0.7035	35		
Total	34	2.0069	100		

Appendix 3.4 Analysis of variance for germination rate (GR) in leached-control (LC) and leached-scarified (LS) seeds of *Cucumis africanus in vitro*.

SOURCE	DF	SS	%	F	P ≤
		LC seeds			
Treatment	6	0.004	40	0.00	0.01
Error	28	0.006	60		
Total	34	0.010	100		
		LS seeds			
Treatment	6	0.0646	70	10.96	0.00
Error	28	0.0274	30		
Total	34	0.0921	100		

Appendix 3.5 Analysis of variance for germination percentage (GP) in leached-scarified (LS) seeds of *Cucumis myriocarpus in vitro*.

SOURCE	DF	SS	%	F	P ≤
		LC seeds			
Treatment	6	26068.6	76	14.55	0.01
Error	28	8360.0	24		
Total	34	34428.6	100		

Appendix 3.6 Analysis of variance for mean germination time (MGT) in leached-scarified (LS) seeds of *Cucumis myriocarpus in vitro*.

SOURCE	DF	SS	%	F	P ≤
		LC seeds			
Treatment	6	91.94	5	0.24	0.96
Error	28	1800.65	95		
Total	34	1892.59	100		

Appendix 3.7 Analysis of variance for germination index (GI) in leached-scarified (LS) seeds of *Cucumis myriocarpus in vitro*.

SOURCE	Df	SS	%	F	P ≤
		LC seeds			
Treatment	6	1.2949	54	5.53	0.01
Error	28	1.0920	45		
Total	34	2.3869	100		

Appendix 3.8 Analysis of variance for germination rate (GR) in leached-scarified (LS) seeds of *Cucumis myriocarpus in vitro*.

SOURCE	DF	SS	%	F	P ≤
		LC seeds			
Treatment	6	0.0574	61	7.16	0.01
Error	28	0.0374	39		
Total	34	0.0949	100		

Appendix 5.1 Analysis of variance for percentage tissue regeneration response in five propagules of *Cucumis africanus* and *Cucumis myriocarpus* treated with six concentrations of BAP (µM) *in vitro*.

SOURCE	DF	SS	%	F	P ≤
		Cucumis africanus			
Replication	4	217	0	0	0
Concentration	5	48134	19	67.67	0.01
Organ	4	148191	57	260.41	0.01
Concent. x organ	20	47529	18	16.70	0.01
Error	116	16503	6		
Total	149	260574	100		
		Cucumis myriocarpus			
Replication	4	463	0	0	0
Concentration	5	59384	25	76.64	0.01
Organ	4	116203	48	187.45	0.01
Concent. x organ	20	47629	20	15.37	0.01
Error	116	17977	7		
Total	149	241656	100		

Appendix 5.2 Analysis of variance for shoot regeneration in five propagules of *Cucumis africanus* and *Cucumis myriocarpus* treated with six concentrations of BAP

(µM) *in vitro*.

SOURCE	DF	SS	%	F	P ≤
		Cucumis africanus			
Replication	4	2.352	1	0	0
Concentration	5	29.792	7	22.53	0.01
Organ	4	252.072	61	238.30	0.01
Concent. x organ	20	97.585	24	18.45	0.01
Error	116	30.676	7		
Total	149	412.478	100		
		Cucumis myriocarpus			
Replication	4	3.465	1	0	0
Concentration	5	33.438	7	15.28	0.01
Organ	4	297.407	61	169.84	0.01
Concent. x organ	20	98.683	20	11.27	0.01
Error	116	50.783	11		
Total	149	483.776	100		

Appendix 5.3 Analysis of variance for number of shoots regenerated in *Cucumis*

africanus and *Cucumis myriocarpus* at shoot multiplication stage *in vitro*.

SOURCE	DF	SS	%	F	P ≤
		Cucumis africanus			
Treatment	5	11.78	43	3.61	0.01
Error	24	15.66	57		
Total	29	27.44	100		
		Cucumis myriocarpus			
Treatment	5	52.08	74	13.40	0.01
Error	24	18.66	26		
Total	29	70.71	100		

Appendix 5.4 Analysis of variance for vine length (cm) in *Cucumis africanus* and *Cucumis myriocarpus* at shoot multiplication stage *in vitro*.

SOURCE	DF	SS	%	F	P ≤
		Cucumis africanus			
Treatment	5	250.64	91	49.97	0.01
Error	24	24.08	9		
Total	29	274.72	100		
		Cucumis myriocarpus			
Treatment	5	65.95	65	8.75	0.01
Error	24	36.135	35		
Total	29	102.135	100		

Appendix 5.5 Analysis of variance for number of roots in *Cucumis africanus* and

Cucumis myriocarpus at rooting stage *in vitro*.

SOURCE	DF	SS	%	F	P ≤
		Cucumis africanus			
Treatment	5	227.02	84	49.97	0.01
Error	24	43.58	16		
Total	29	270.61	100		
		Cucumis myriocarpus			
Treatment	5	559.204	69	8.75	0.01
Error	24	247.096	31		
Total	29	806.300	100		

Appendix 5.6 Analysis of variance for vine length (cm) in *Cucumis africanus* and *Cucumis myriocarpus* at rooting stage *in vitro*.

SOURCE	DF	SS	%	F	P ≤
		Cucumis africanus			
Treatment	5	327.84	73	1.34	0.28
Error	24	121.967	27		
Total	29	449.807	100		
		Cucumis myriocarpus			
Treatment	5	25.228	36	2.66	0.04
Error	24	45.516	64		
Total	29	70.743	100		

Appendix 6.1 Analysis of variance for stem diameter (mm) of *in vitro* acclimatised

Cucumis africanus and *Cucumis myriocarpus* plantlets transplanted in four various potting media.

SOURCE	DF	SS	%	F	P ≤
		Cucumis africanus			
Replication	9	0.814	10	0	0
Treatments	3	2.877	34	5.35	0.01
Error	27	4.840	56		
Total	39	8.531	100		
		Cucumis myriocarpus			
Replication	9	1.271	17	0	0
Treatments	3	3.232	44	10.02	0.01
Error	27	2.905	39		
Total	39	7.408	100		

Appendix 6.2 Analysis of variance for the number of shoots for *in vitro* acclimatised

Cucumis africanus and *Cucumis myriocarpus* plantlets transplanted in four various potting media.

SOURCE	DF	SS	%	F	P ≤
		Cucumis africanus			
Replication	9	8.600	14	0	0
Treatments	3	43.800	71	42.85	0.01
Error	27	9.200	15		
Total	39	61.600	100		
		Cucumis myriocarpus			
Replication	9	227.525	26	0	0
Treatments	3	360.675	41	10.84	0.01
Error	27	299.575	33		
Total	39	887.775	100		

Appendix 6.3 Analysis of variance for dry shoot mass (g) for *in vitro* acclimatised

Cucumis africanus and *Cucumis myriocarpus* plantlets transplanted in four various potting media.

SOURCE	DF	SS	%	F	P ≤
		Cucumis africanus			
Replication	9	93.104	12	0	0
Treatments	3	394.915	52	12.97	0.00
Error	27	274.011	36		
Total	39	762.029	100		
		Cucumis myriocarpus			
Replication	9	15.505	15	0	0
Treatments	3	45.837	44	9.88	0.01
Error	27	41.756	41		
Total	39	103.098	100		

Appendix 6.4 Analysis of variance for dry root mass (g) for *in vitro* acclimatised

Cucumis africanus and *Cucumis myriocarpus* plantlets transplanted in four various potting media.

SOURCE	DF	SS	%	F	P ≤
		Cucumis africanus			
Replication	9	0.251	16	0	0
Treatments	3	0.705	45	10.29	0.01
Error	27	0.616	39		
Total	39	1.572	100		
		Cucumis myriocarpus			
Replication	9	20.107	27	0	0
Treatments	3	20.769	28	5.56	0.01
Error	27	33.634	45		
Total	39	74.510	100		

Appendix 6.5 Analysis of variance for vine length (m) for *in vitro* acclimatised

Cucumis africanus and *Cucumis myriocarpus* plantlets transplanted in four various potting media.

SOURCE	DF	SS	%	F	P ≤
		Cucumis africanus			
Replication	9	25.718	30	0	0
Treatments	3	8.215	10	1.42	0.258
Error	27	52.009	60		
Total	39	85.942	100		
		Cucumis myriocarpus			
Replication	9	0.484	6	0	0
Treatments	3	2.732	32	4.68	0.01
Error	27	5.255	62		
Total	39	8.471	100		

Appendix 6.6 Analysis of variance for chlorophyll content of *in vitro* acclimatised

Cucumis africanus and *Cucumis myriocarpus* plantlets transplanted in four various potting media.

SOURCE	DF	SS	%	F	P ≤
		Cucumis africanus			
Replication	9	403.27	31	0	0
Treatments	3	93.48	7	1.03	0.39
Error	27	819.87	62		
Total	39	1316.63	100		
		Cucumis myriocarpus			
Replication	9	141.61	14	0	0
Treatments	3	35.08	3	0.37	0.76
Error	27	858.16	83		
Total	39	1034.85	100		