The role of *meta*-topolins on the physiology of micropropagated „Williams' bananas (*Musa* spp. AAA)

By

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Table of Contents

College of Agriculture, Engineering and Science Declaration 1 - Plagiarism..... v
Student Declaration vi
Declaration by Supervisors.....vii
Publications from this thesis viii
Conference contributions from this thesis ix
College of Agriculture, Engineering and Science Declaration 2 - Publications x
Acknowledgements xii
List of Figures xiv
List of Tables.....xviii
List of Abbreviations xx
Abstract xxiii

Chapter 1: General introduction 1

1.1 *Musa* spp. and their importance 1

1.2 Origin, distribution and diversity of *Musa* spp. 1

1.3 Morphology, taxonomy, and genomics of *Musa* spp. 2

1.4 Propagation of *Musa* spp. 4
1.4.1 Conventional method 4
1.4.2 Micropropagation of *Musa* spp. 5
1.4.2.1 Shoot-tip culture 6
1.4.2.2 Somatic cell culture 7

1.5 Significance of the study 9

1.6 Aims and objectives: 9

1.7 General overview of the thesis 10

Chapter 2: Literature review.....12

2.1 Introduction 12

2.2 Cytokinins in plant tissue culture 12
2.2.1 Cytokinins – Their Discovery, function and diversity 14
2.2.2 Cytokinins biosynthesis and metabolism 17
2.2.3 Cytokinin structure-activity relationships.....20
2.2.4 The role of topolins on various parameters in plant tissue culture.....25
2.2.4.1 Shoot proliferation and elongation 25
2.2.4.2 Rooting and *ex vitro* acclimatization.....26

2.2.4.3 Alleviation of *in vitro*-induced physiological disorders 28

2.3 Somaclonal variation in plant tissue culture....31
2.3.1 Frequency rates, sources and causes of somaclonal variation....32
2.3.1.1 Pre-existing variation....33
2.3.1.2 Tissue culture induced variation....33
2.3.2 Methods of detecting somaclonal variants....38
2.3.2.1 Morphological detection....38
2.3.2.2 Physiological/biochemical detection....39
2.3.2.3 Molecular detection....40
2.3.2.3.1 Cytological methods....41
2.3.2.3.2 Proteins and isozymes....42
2.3.2.3.3 Restriction fragment length polymorphism....43
2.3.2.3.4 Polymerase chain reaction based techniques....44
2.3.2.3.4.1 Random amplified polymorphic DNA....45
2.3.2.3.4.2 Amplified fragment length polymorphism....46
2.3.2.3.4.3 Microsatellite markers....48

2.4 Concluding remarks....49

Chapter 3: Micropropagation and *in vitro* phenolic production in 'Williams' bananas....52

3.1 Introduction....52

3.2 Materials and methods....54
3.2.1 Chemicals....54
3.2.2 Source of initial plant material....54

3.2.3 Explant initiation, decontamination, medium composition and growth room conditions....55
3.2.4 *In vitro* shoot proliferation....56
3.2.5 *In vitro* rooting and acclimatization....56
3.2.6 *In vitro* phenolic content....57
3.2.6.1 Determination of total phenolic content....57
3.2.6.2 Determination of total flavonoid content....58
3.2.6.3 Determination of total proanthocyanidin content....58
3.2.7 Data analyses....58

3.3 Results and discussion....59
3.3.1 Explant decontamination frequency....59
3.3.2 Effect of the aromatic cytokinins on *in vitro* shoot proliferation....59
3.3.3 Effect of the aromatic cytokinins on *in vitro* rooting and acclimatization....66
3.3.4 Effect of the cytokinins on phenolic compound accumulation....70

3.4 Concluding remarks....74

Chapter 4: Influence of *meta*-topolins on photosynthetic pigment profiles and foliar structures of micropropagated 'Williams' bananas 76

4.1 Introduction 76

4.2 Materials and methods 78
4.2.1 Cytokinin and explant sources, medium composition and growth conditions 78
4.2.2 Determination of photosynthetic pigment content 78
4.2.3 Scanning electron microscopy of *in vitro* regenerated banana leaves 79
4.2.4 Data analyses 80

4.3 Results and discussion 80
4.3.1 Effect of cytokinins on the photosynthetic pigment content 80
4.3.2 Effect of cytokinins on the morphometric features of the foliar surfaces of the regenerated banana plantlets 90

4.4 Concluding remarks 94

Chapter 5: Application of smoke-water and karrikinolide in *in vitro* propagation compared to *meta*-topolin 95

5.1 Introduction 95

5.2 Materials and methods 96
5.2.1 Sources of materials used for *in vitro* propagation and growth conditions 96
5.2.2 Growth, *in vitro* phenolic and photosynthetic pigment quantification 97
5.2.3 Synergistic effect of *meta*-topolin with either smoke-water or karrikinolide 97
5.2.4 Data analyses 98

5.3 Results and discussion 98

5.4 Concluding remarks 106

Chapter 6: Enhancement of the acclimatization potential of *in vitro*-cultured 'Williams' bananas in the greenhouse 107

6.1 Introduction 107

6.2 Materials and methods 109
6.2.1 Source of „Williams' banana plantlets, smoke-water and vermicompost leachate solutions 109
6.2.2 Experimental design and growth conditions 109
6.2.3 Data collection and analyses 110

6.3 Results and discussion 110

6.4 Concluding remarks 119

Chapter 7: Physiological responses and endogenous cytokinin profiles in relation to roscovitine and an inhibitor of cytokinin oxidase/dehydrogenase (INCYDE) treatments 121

7.1 Introduction 121

7.2 Materials and methods 124
7.2.1 Explant culture and growth conditions 124
7.2.2 Quantification of photosynthetic pigment and phytochemical content 125
7.2.3 Extraction, purification and quantification of endogenous cytokinins 125
7.2.4 Data analyses 126

7.3 Results and discussion 127
7.3.1 Effect of roscovitine and INCYDE on the growth of „Williams' bananas 127
7.3.2 Effect of roscovitine and INCYDE on endogenous cytokinin content 134
7.3.3 Effect of roscovitine and INCYDE on photosynthetic and phenolic contents 141

7.4 Concluding remarks 144

Chapter 8: Influence of high cytokinin concentration on the genetic stability of tissue-cultured 'Williams' bananas 146

8.1 Introduction 146

8.2 Materials and methods 147
8.2.1 Plant material 147
8.2.2 Isolation and quantification of plant genomic DNA 148
8.2.3 Amplification of isolated DNA using inter simple sequence repeat (ISSR) primers 148
8.2.4 Amplified DNA product electrophoresis 149
8.2.5 Band scoring and data analysis 149

8.3 Results and discussion 150

8.4 Concluding remarks 152

Chapter 9: General conclusions and recommendations 153

References 157

APPENDIX 1: Protocol for modified MS basal medium for *Musa* spp. shoot-tip culture .206

College of Agriculture, Engineering and Science Declaration 1 - Plagiarism

I, **Adeyemi Oladapo Aremu (207526996)**, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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Student Declaration

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I, **ADEYEMI OLADAPO AREMU**

Student Number **207526996**

declare that :

(i) The research reported in this dissertation, except where otherwise indicated, is the result of my own endeavours in the Research Centre for Plant Growth and Development, School of Life Sciences, University of KwaZulu-Natal Pietermaritzburg;

(ii) This dissertation has not been submitted for any degrees or examination at any other University;

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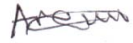
Signed at **UKZN Pietermaritzburg campus** on the **10th** day of **September, 2012**.



10th September, 2012

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Declaration by Supervisors

We hereby declare that we acted as Supervisors for this PhD student:

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

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CO-SUPERVISOR: DR M.W. BAIRU

Publications from this thesis

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College of Agriculture, Engineering and Science Declaration 2 - Publications

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this thesis (include publications in preparation, submitted, *in press* and published and give details of the contributions of each author to the experimental work and writing of each publication)

Publication 1

Contributions: Tissue culture, photosynthetic and phytochemical experiments were done by AOA. Endogenous cytokinin isolation, quantification and analysis were performed by LP, ON, KD and MS. Roscovitine and INCYDE was synthesized and provided by MZ, KD, MS. Manuscript was drafted by AOA under the guidance and supervision of JVS, JFF, MWB and KD.

Publication 2

Contributions: All experimental and microscopy work were done by AOA while the topolins were synthesized and provided by LS. Manuscript was drafted by AOA under the guidance and supervision of JVS, JFF and MWB.

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Publication 7

Contributions: Concept was conceived with the assistance of KD and MWB. KD supplied valuable literature and made valuable suggestions. Manuscript was drafted by AOA under the guidance and supervision of JVS, JFF and MWB.

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List of Figures

Figure 2.1: Molecular structures of benzyladenine (most widely-used cytokinin) and different topolin derivatives used for micropropagation and investigated in the current study..... 16

Figure 3.1: Initiation stages of „Williams' banana cultures (A) = one-month-old plantlets in the mist-house (B) = four-month-old greenhouse plantlets; (C) = excised explant used for decontamination and (D) = initiated explants in culture tubes. Bar = 50 mm..... 55

Figure 3.2: Effect of 0.1% mercuric chloride ($HgCl_2$) solution treatment on the decontamination rate (%) of the shoot-tip explants of „Williams' banana..... 59

Figure 3.3: Morphological appearance of shoots regenerated from different cytokinins compared to the control. Me*m*T = *meta*-Methoxy topolin and *m*TR = *meta*-Topolin riboside. 60

Figure 3.4: Effect of rooting compounds on the number of regenerated roots. A= *meta*-Topolin; B = *meta*-Topolin riboside; C = *meta*-Methoxy topolin; D = *meta*-Methoxy topolin riboside; E = Benzyladenine and F = *meta*-Methoxy topolins 9-tetrahydropyran-2-yl. IAA = Indole-3-acetic acid; IBA = Indole-3-butyric acid; NAA = Naphthalene acetic acid; SW = Smoke-water; KAR_1 = Karrikinolide. Concentrations of root inducing compounds: IAA, IBA and NAA = 1 µM; KAR_1 = 4.8 X 10^{-22} M and SW = 1:1000 dilution. In each graph, bars with different letter(s) are significantly different (*P* = 0.05) according to Duncan's multiple range test. 67

Figure 3.5: Effect of rooting compounds on the length of regenerated roots. A= *meta*-Topolin; B = *meta*-Topolin riboside; C = *meta*-Methoxy topolin; D = *meta*-Methoxy topolin riboside; E = Benzyladenine and F = *meta*-Methoxy topolin 9-tetrahydropyran-2-yl. IAA = Indole-3-acetic acid; IBA = Indole-3-butyric acid; NAA = Naphthalene acetic acid; SW = Smoke-water; KAR_1 = Karrikinolide. Concentrations of root inducing compounds: IAA, IBA and NAA = 1 µM; KAR_1 = 4.8 X 10^{-22} M and SW = 1:1000 dilution. In each graph, bars with different letter(s) are significantly different (*P* = 0.05) according to Duncan's multiple range test. 68

Figure 4.1: Effect of different cytokinins on the chlorophyll a content (µg/g FW) of micropropagated „Williams' bananas. A = 10 µM; B = 20 µM and C = 30 µM cytokinin treatments with each bar representing mean value (± standard error, n = 6) *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Me*m*T =

meta-Methoxy topolin; Me*m*TR = *meta*-Methoxy topolin riboside; BA = Benzyladenine; Me*m*TTHP = *meta*-Methoxy topolin 9-tetrahydropyran-2-yl. 82

Figure 4.2: Effect of different cytokinins on the chlorophyll b content (µg/g FW) of micropropagated „Williams' bananas. A = 10 µM; B = 20 µM and C = 30 µM cytokinin treatments with each bar representing mean value (± standard error, n = 6). *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Me*m*T = *meta*-Methoxy topolin; Me*m*TR = *meta*-Methoxy topolin riboside; BA = Benzyladenine; Me*m*TTHP = *meta*-Methoxy topolin 9-tetrahydropyran-2-yl. 83

Figure 4.3: Effect of different cytokinins on the total chlorophyll content (µg/g FW) of micropropagated „Williams' bananas. A = 10 µM; B = 20 µM and C = 30 µM cytokinin treatments with each bar representing mean value (± standard error, n = 6). *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Me*m*T = *meta*-Methoxy topolin; Me*m*TR = *meta*-Methoxy topolin riboside; BA = Benzyladenine; Me*m*TTHP = *meta*-Methoxy topolin 9-tetrahydropyran-2-yl. 84

Figure 4.4: Effect of different cytokinins on the total carotenoid content (µg/g FW) of micropropagated „Williams' bananas. A = 10 µM; B = 20 µM and C = 30 µM cytokinin treatments with each bar representing mean value (± standard error, n = 6). *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Me*m*T = *meta*-Methoxy topolin; Me*m*TR = *meta*-Methoxy topolin riboside; BA = Benzyladenine; Me*m*TTHP = *meta*-Methoxy topolin 9-tetrahydropyran-2-yl. 85

Figure 4.5: Effect of different cytokinins on the chlorophyll a/b ratio of micropropagated „Williams' bananas. A = 10 µM; B = 20 µM and C = 30 µM cytokinin treatments with each bar representing mean value (± standard error, n = 6). *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Me*m*T = *meta*-Methoxy topolin; Me*m*TR = *meta*-Methoxy topolin riboside; BA = Benzyladenine; Me*m*TTHP = *meta*-Methoxy topolin 9-tetrahydropyran-2-yl..... 86

Figure 4.6: Effect of different cytokinins on the total chlorophyll/carotenoid ratio of micropropagated „Williams' bananas. A = 10 µM; B = 20 µM and C = 30 µM cytokinin treatments with each bar representing mean value (± standard error, n = 6). *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Me*m*T = *meta*-Methoxy topolin; Me*m*TR = *meta*-Methoxy topolin riboside; BA = Benzyladenine; Me*m*TTHP = *meta*-Methoxy topolin 9-tetrahydropyran-2-yl. 87

Figure 4.7: Effect of 10 µM cytokinins on the foliar surface of micropropagated „Williams' bananas. A = Control; B = *meta*-Methoxy topolin 9-tetrahydropyran-2-yl; C = Benzyladenine and D = *meta*-Topolin. Magnification = 500x, Bar = 100 µm..... 92

Figure 4.8: Effect of 10 µM cytokinins on the stomata structure of micropropagated „Williams' bananas. A = Benzyladenine; B = *meta*-Methoxy topolin; C = *meta*-Methoxy topolin 9-tetrahydropyran-2-yl and D = Control. Magnification = 5000x, Bar = 10 µm. 93

Figure 5.1: Effect of different concentrations of karrikinolide and smoke-water on micropropagated „Williams' banana photosynthetic pigment contents (A) Chlorophyll a (B) Chlorophyll b (C) Carotenoid and (D) Chlorophyll a/b ratio. In each graph, bars with different letter(s) are significantly different (*P* = 0.05) according to Duncan's multiple range test. n/s = Not significant. 101

Figure 6.1: Effect of smoke-water and vermicompost leachate on growth of „Williams' bananas (A) Foliar application using smoke-water and (B) Soil drenching using smoke-water and vermicompost leachate. SW = Smoke-water; VCL = vermicompost leachate; scale bars = 5 cm. 111

Figure 6.2: Effect of different concentrations of smoke-water and vermicompost leachate on the vegetative growth of „Williams' bananas (A) Mean shoot:root ratio of regenerated off-shoot plants; (B) Mean shoot:root ratio of parent plants; and (C) Mean number of off-shoots regenerated from the parent plant. In each graph, bars with different letter(s) are significantly different (*P* = 0.05) according to Duncan's multiple range test. 115

Figure 7.1: Effect of roscovitine and INCYDE on growth of the micropropagated „Williams' bananas. A = regeneration rate; B = shoot number; C = shoot length; D = fresh weight and E = dry weight. 1 = *meta*-topolin; 2 = benzyladenine; 3 = benzyladenine + INCYDE; 4 = benzyladenine + roscovitine; 5 = benzyladenine + INCYDE + roscovitine; 6 = *meta*-topolin + INCYDE; 7 = *meta*-topolin + roscovitine; 8 = *meta*-topolin + INCYDE + roscovitine. In each graph, bars with different letter(s) are significantly different (*P* = 0.05) based on Duncan's multiple range test. 128

Figure 7.2: Total isoprenoid and aromatic cytokinin content (%) in aerial (A) and underground (B) parts of the micropropagated „Williams' bananas. 1 = *meta*-topolin; 2 = benzyladenine; 3 = benzyladenine + INCYDE; 4 = benzyladenine + roscovitine; 5 = benzyladenine + INCYDE + roscovitine; 6 = *meta*-topolin + INCYDE; 7 = *meta*-topolin + roscovitine; 8 = *meta*-topolin + INCYDE + roscovitine. 135

Figure 7.3: Relative abundance (%) and distribution pattern of the isoprenoid and aromatic cytokinins in the micropropagated „Williams' bananas. A = aerial and B = underground regions. 1 = *meta*-topolin; 2 = benzyladenine; 3 =

benzyladenine + INCYDE; 4 = benzyladenine + roscovitine; 5 = benzyladenine + INCYDE + roscovitine; 6 = *meta*-topolin + INCYDE; 7 = *meta*-topolin + roscovitine; 8 = *meta*-topolin + INCYDE + roscovitine. 136

Figure 7.4: Total cytokinin pool (%) of the different cytokinin forms in aerial (A) and underground (B) parts of the micropropagated „Williams' bananas. 1 = *meta*-topolin; 2 = benzyladenine; 3 = benzyladenine + INCYDE; 4 = benzyladenine + roscovitine; 5 = benzyladenine + INCYDE + roscovitine; 6 = *meta*-topolin + INCYDE; 7 = *meta*-topolin + roscovitine; 8 = *meta*-topolin + INCYDE + roscovitine. 139

Figure 7.5: Relative abundance (%) and distribution pattern of the different cytokinin forms in the micropropagated „Williams' bananas. A = aerial and B = underground regions. 1 = *meta*-topolin; 2 = benzyladenine; 3 = benzyladenine + INCYDE; 4 = benzyladenine + roscovitine; 5 = benzyladenine + INCYDE + roscovitine; 6 = *meta*-topolin + INCYDE; 7 = *meta*-topolin + roscovitine; 8 = *meta*-topolin + INCYDE + roscovitine. 140

List of Tables

Table 2.1: The influence of topolins in comparison to other cytokinins on various growth parameters and physiological disorders 21

Table 2.2: Examples of *Musa* spp. with incidence of somaclonal variation, possible causes, detection methods and their desirability 36

Table 3.1: Effect of six aromatic cytokinins at different concentrations on the growth of micropropagated „Williams' bananas 61

Table 3.2: Effect of six aromatic cytokinins at different concentrations on the rooting parameters of micropropagated „Williams' bananas during the shoot proliferation phase 64

Table 3.3: Effect of five rooting compounds on the rooting and production of new off-shoots frequencies (%) of different cytokinin-derived shoots of „Williams' bananas 65

Table 3.4: Effect of aromatic cytokinins on the accumulation of phenolic compounds in micropropagated „Williams' bananas 71

Table 4.1: Effect of type and concentration of cytokinins on morphometric parameters of foliar surfaces of regenerated banana plantlets 91

Table 5.1: Effect of different concentrations of smoke-water (SW) and karrikinolide (KAR_1) on the growth of micropropagated „Williams' bananas 99

Table 5.2: Synergistic effect of *meta*-topolin with either smoke-water (SW) or karrikinolide (KAR_1) on the growth of micropropagated „Williams' bananas 100

Table 5.3: Synergistic effect of *meta*-topolin (*m*T) with either smoke-water (SW) or karrikinolide (KAR_1) on photosynthetic pigment content of micropropagated „Williams' bananas 101

Table 5.4: Effect of *meta*-topolin (*m*T), smoke-water (SW) and karrikinolide (KAR_1) on secondary metabolite accumulation in micropropagated „Williams' bananas 104

Table 5.5: Synergistic effect of *meta*-topolin (*m*T) with either smoke-water (SW) or karrikinolide (KAR_1) on secondary metabolite accumulation in micropropagated „Williams' bananas 105

Table 6.1: Effect of soil drenching of different concentrations of vermicompost leachate (VCL) and smoke-water (SW) on the growth parameters of „Williams' bananas..... 113

Table 6.2: Effect of soil drenching of different concentrations of vermicompost leachate (VCL) and smoke-water (SW) on the growth parameters of produced off-shoots in „Williams' bananas 114

Table 7.1: Effect of roscovitine and INCYDE with aromatic cytokinins on endogenous cytokinins content (pmol/g FW) in „Williams' banana aerial parts 130

Table 7.2: Effect of roscovitine and INCYDE with aromatic cytokinins on endogenous cytokinins content (pmol/g FW) in „Williams' bananas underground parts.. 132

Table 7.3: Effect of roscovitine and INCYDE on the phenolic content of micropropagated „Williams' bananas 141

Table 7.4: Effect of roscovitine and INCYDE on the photosynthetic pigment contents of micropropagated „Williams' bananas 143

Table 8.1: Characteristics of the ISSR primers used for the current study 149

Table 8.2: ISSR analysis of the genetic stability of „Williams' banana plantlets using different cytokinins..... 151

List of Abbreviations

Ad	Adenine
AdS	Adenine sulphate
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
BA	6-Benzyladenine
BA9G	6-Benzyladenine-9-glucoside
BA9R	6-Benzyladenine-9-riboside
BAR	6-Benzyladenosine riboside
BAR5′MP	6-Benzyladenosine-5′-monophosphate
BPA	6-Benzyl-9-(2-tetrahydropyranylamino)purine
CBPs	Cytokinin binding proteins
CCE	Cyanidin chloride equivalents
CDK	Cyclin-dependent kinase
CE	Catechin equivalents
CK	Cytokinin
CKX	Cytokinin oxidase/dehydrogenase
CPA	4-Chlorophenoxyacetic acid
CPPU	6-(2-chloro-4-pyridyl)-N'-phenylurea
CYCB1	Cyclin B1
*c*Z	*cis*-Zeatin
*c*Z9G	*cis*-Zeatin-9-glucoside
*c*ZOG	*cis*-Zeatin-*O*-glucoside
*c*ZR	*cis*-Zeatin riboside
*c*ZR5′MP	*cis*-Zeatin riboside-5′-monophosphate
*c*ZROG	*cis*-Zeatin-*O*-glucoside riboside
DHZ	Dihydrozeatin
DHZ9G	Dihydrozeatin-9-glucoside
DHZOG	Dihydrozeatin-*O*-glucoside
DHZR	Dihydrozeatin riboside
DHZR5′MP	Dihydrozeatin riboside-5′-monophosphate
DHZROG	Dihydrozeatin-*O*-glucoside riboside
DW	Dry weight
F*m*T	*meta*-Fluoro-topolin
F*m*TR	*meta*-Fluoro-topolin riboside

Folin-C	Folin-Ciocalteu
FW	Fresh weight
GAE	Gallic acid equivalents
IAA	Indole-3-acetic acid
IAC	Immunoaffinity chromatography
IBA	Indole-3-butyric acid
INCYDE	2-Chloro-6-(3-methoxyphenyl)aminopurine
iP	6-Isopentenyladenine
iP9G	6-Isopentenyladenine-9-glucoside
iPR	6-Isopentenyladenosine
iPR5′MP	6-Isopentenyladenosine-5′-monophosphate
IPT	Isopentenyltransferase
IRAP	Inter-retrotransposon amplified polymorphism
KAR_1	Karrikinolide
KIN	Kinetin
KIN9G	Kinetin-9-glucoside
KINR	Kinetin riboside
KINR5′MP	Kinetin riboside-5′-monophosphate
Me*m*T	*meta*-Methoxy topolin
Me*m*TR	*meta*-Methoxy topolin riboside
Me*m*TTHP	*meta*-Methoxy topolin 9-tetrahydropyran-2-yl
Me*o*T	*ortho*-Methoxy topolin
Me*o*TR	*ortho*-Methoxy topolin riboside
MRM	Multiple reaction monitoring
MS	Murashige and Skoog medium
MSAP	Methylation-sensitive amplification polymorphism
*m*T	*meta*-Topolin
*m*T9G	*meta*-Topolin-9-glucoside
*m*TOG	*meta*-Topolin-*O*-glucoside
*m*TR	*meta*-Topolin riboside
*m*TR5′MP	*meta*-Topolin riboside-5′-monophosphate
*m*TROG	*meta*-Topolin riboside-*O*-glucoside
NAA	α-Naphthalene acetic acid
*o*T	*ortho*-Topolin
*o*T9G	*ortho*-Topolin-9-glucoside
*o*TOG	*ortho*-Topolin-*O*-glucoside

*o*TR	*ortho*-Topolin riboside
*o*TR5′MP	*ortho*-Topolin riboside-5′-monophosphate
*o*TROG	*ortho*-Topolin riboside-*O*-glucoside
PAS	Pasticcino
PBP	Percentage band polymorphism
PGR	Plant growth regulator
PPF	Photosynthetic photon flux density
*p*T	*para*-Topolin
PTC	Plant tissue culture
*p*TOG	*para*-Topolin-*O*-glucoside
*p*TR	*para*-Topolin riboside
*p*TR5′MP	*para*-Topolin riboside-5′-monophosphate
*p*TROG	*para*-Topolin riboside-*O*-glucoside
RAPD	Random amplified polymorphic DNA
RDA	Representational difference analysis
SEM	Scanning electron microscopy
SW	Smoke-water
*t*Z	*trans*-Zeatin
*t*Z9G	*trans*-Zeatin-9-glucoside
*t*ZOG	*trans*-Zeatin-*O*-glucoside
*t*ZR	*trans*-Zeatin riboside
*t*ZR5′MP	*trans*-Zeatin riboside-5′-monophosphate
*t*ZROG	*trans*-Zeatin riboside-*O*-glucoside
UBC	University of British Columbia
UPLC	Ultra performance liquid chromatography
Z	Zeatin
ZR	Zeatin riboside

Abstract

Banana production ranks fifth behind cereals as a food crop and has potential, along with other major crops, to feed the world's increasing population. Globally, continuous efforts and techniques including the use of plant tissue culture (PTC) have been devised for increasing the production of several *Musa* species. The choice of cytokinin (CK) is one of the most critical factors in developing a successful PTC protocol. Since the discovery of topolins as naturally occurring aromatic CKs, they have emerged as genuine alternatives to the long serving CKs (benzyladenine = BA, zeatin = Z and kinetin = KIN) in PTC. Globally, the past 15 years has witnessed a surge in the use of topolins and their derivatives in research laboratories. Topolins have demonstrated great potential during culture initiation and protocol optimization as well as for counteracting various *in vitro* induced physiological disorders in some species. In terms of general physiology (growth, phytochemical and photosynthetic pigment contents as well as genetic fidelity), the topolins were compared with BA using „Williams' bananas with minimal residual exogenous CK carry-over effects. The five topolins tested were *meta*-Topolin (*m*T); *meta*-Topolin riboside (*m*TR); *meta*-Methoxy topolin (Me*m*T); *meta*-Methoxy topolin riboside (Me*m*TR) and *meta*-Methoxy topolin 9-tetrahydropyran-2-yl (Me*m*TTHP). Based on evidence of potential CK- and auxin-like activity of smoke-water (SW) and karrikinolide (KAR_1) at low concentrations, a similar comparative study involving both compounds and *m*T was performed. For a further understanding of banana physiology *in vitro*, the effect of supplementing either *m*T- or BA-requiring cultures with roscovitine (a cyclin-dependent kinase and *N*-glucosylation inhibitor) and INCYDE (an inhibitor of CK degradation) on the endogenous CK profiles was investigated. In addition, greenhouse experiments geared towards improving the acclimatization competence of tissue-cultured banana plantlets via application of different concentrations of SW and vermicompost leachate was conducted.

Sterile shoot-tip explants were cultured on modified Murashige and Skoog (MS) media supplemented with 10, 20 or 30 µM of the tested CKs for 42 days while rooting experiments involved the use of classic auxins as well as SW and KAR_1. Apart from 10

µM BA and 30 µM Me*m*TTHP treatments, the number of shoots produced with all the CK treatments were significantly higher than the control. Treatment with 30 µM *m*T resulted in the highest number of shoots (7.3±1.0) which is an indication of the requirement of exogenous CK for increased shoot proliferation in „Williams' bananas The use of 10 µM Me*m*TTHP had the least root inhibitory effect during the shoot proliferation phase. As an indication of the toxicity of applied CK, Me*m*T- and Me*m*TR-regenerants were the most deformed while *m*TR-regenerated plantlets demonstrated the best quality across all the CKs tested. In *m*T- and BA-derived shoots, SW and KAR_1 significantly increased the number and length of roots compared to the control. During the rooting phase, topolin treatments produced more off-shoots than BA-treated ones which inevitably improved the overall number of regenerated shoots.

Total phenolic levels were highest in 10 µM *m*T- and 30 µM Me*m*TTHP-treated plantlets detected in the aerial and underground parts, respectively. It is interesting that in the underground parts, 10 µM *m*T resulted in the production of the highest amount of proanthocyanidins which was approximately five-fold higher than in the control plants. On the other hand, 10 µM Me*m*TTHP-treated plantlets had significantly higher total flavonoids within the aerial parts. In view of the stimulation of secondary metabolites in the majority of the CK-treated plantlets, the current results indicate the role of the type and concentration of applied CK as potential elicitors in PTC.

Generally, the maximum photosynthetic pigment content was attained between 40-50 days. The control plantlets had the highest pigment content (1150 µg/g FW) while 10 µM Me*m*TTHP had the best pigment stimulatory effect among the tested CKs. Nevertheless, *in vitro* propagation of banana devoid of CKs is not a practical option due to low shoot proliferation rates. Scanning electron microscopy (SEM) of the foliar surface showed that the stomatal density was highest in 10 µM Me*m*TTHP-treated and lowest in 10 µM Me*m*TR-treated plantlets. Prolonging the culture duration as well as increasing CK concentrations reduced the pigment content. However, the drastic breakdown in chlorophyll pigments beyond 50 days was slightly inhibited by the presence of *m*T, *m*TR, Me*m*TTHP and BA compared to the control. Current findings

indicate the potential anti-senescence activity of the topolins such as *m*T, *m*TR and Me*m*TTHP under *in vitro* conditions. This study articulates that the right choice and concentration of CKs applied during *in vitro* propagation may alleviate photomixotrophic-induced physiological stress that usually accompanies the transfer of plantlets to *ex vitro* conditions.

Findings indicate that the effect of subculturing contributed significantly to the higher rate of variation in 'Williams' bananas *in vitro*. The presence of CK in the culture media apparently aggravated the stress on the explants as indicated in the relatively higher percentage polymorphic bands compared to the controls. Among the tested CKs, the use of *m*TR and Me*m*TTHP caused the least detrimental effect on the regenerants while *m*T-treated plantlets had the most polymorphic bands. Hence, it is recommended that subculturing cycles from the initial explant establishment should be limited to a maximum of five.

The use of SW and KAR_1 improved the level of photosynthetic pigment and phenolic compounds in the micropropagated bananas. However, they had a negative effect on shoot proliferation; hence their inclusion is more desired when used at the rooting phase of micropropagation. Perhaps, these compounds could be used in conjunction with auxin to increase the number of roots prior to the acclimatization stage. The enhanced photosynthetic pigment level resulting from addition of SW and KAR_1 would also play a vital role during acclimatization of the micropropagated plants. The present finding serves as an alternative approach, available to researchers for improving the quantity of secondary metabolites in micropropagated plants.

The highest regeneration rate (93%) was observed in BA + roscovitine treatment while *m*T + INCYDE-treated plantlets produced most shoots. Treatment with BA + roscovitine had the highest shoot length and biomass. Although not significant, there was more proanthocyanidins in BA + roscovitine treatments compared to the treatment with BA alone. On the contrary, total phenolics were significantly higher in *m*T + roscovitine treatment than in the *m*T-treated regenerants. The presence of roscovitine and/or

INCYDE had no significant effect on the photosynthetic pigments of the banana plantlets. Forty-seven aromatic and isoprenoid CKs categorized into nine CK-types were detected at varying concentrations. The presence of *m*T + roscovitine and/or INCYDE increased the levels of *O*-glucosides, while 9-glucosides remained the major derivative in the presence of BA. Generally, the underground parts had higher CK levels than the aerial parts; however the presence of INCYDE increased the level of CK quantified in the aerial parts of both CK treated plantlets. Apparently, the presence of INCYDE serves to enhance transportation of the CK towards the aerial regions. From a practical perspective, the use of roscovitine and INCYDE in PTC could be crucial in the alleviation of commonly observed *in vitro*-induced physiological abnormalities.

Soil drenching with SW significantly increased the root length (1:1000 and 1:500 dilutions) as well as fresh and dry weight (1:1000; 1:500 and 1:250 dilutions) when compared to foliar application. Vermicompost leachate (1:10 and 1:5 dilutions) significantly enhanced the shoot length, root length, leaf area and dry weights. Vermicompost leachate (1:20; 1:10 and 1:5 dilutions) also significantly increased the number of off-shoots. The positive effect on rooting is beneficial for acclimatization and establishment of tissue-cultured banana plantlets in nurseries and subsequent transfer to the field. However, field trials will be necessary to substantiate the effects demonstrated by these compounds.

In an attempt to contribute to improving banana micropropagation, the current findings provide additional evidence on the increasing advantage of topolins over BA. Nevertheless, some detrimental physiological effects observed with some of the topolins (for example, Me*m*T and Me*m*TR) are clear indication that they should not be taken as a panacea in PTC. Besides optimizing efficient PTC protocols through stringent choice of CKs, other associated physiological and metabolic events taking place in culture during the optimization process need more in-depth investigation. In addition to contributing towards the better understanding of the mode of action of these CKs, such an approach will help solve associated physiological and developmental problems *in vitro*.

Chapter 1: General introduction

1.1 *Musa* spp. and their importance

Musa spp. (bananas and plantains) serve as a staple food for approximately 400 million people in the tropics and an estimated one billion people eat bananas regularly **(Hallam, 1995)**. The species is generally characterized by a high carbohydrate and fibre content with a low protein level and is free of fats. In addition, it is a rich source of potassium, vitamins C, B_6 and A **(Robinson, 1996)**. Botanically, there is no clear-cut difference between bananas and plantains. Bananas are the sweeter and more easily digested forms that can be eaten uncooked when ripe while plantains or cooking bananas are starchier and eaten either ripe or unripe after boiling, roasting or frying **(Heslop-Harrison and Schwarzacher, 2007)**. However, **Dufour et al. (2009)** using thermal and functional characteristics classified cultivated Columbian *Musa* spp. into three subgroups namely: plantains, cooking and dessert bananas. For a better understanding of *Musa* spp., a brief background entailing the history of the species is discussed.

1.2 Origin, distribution and diversity of *Musa* spp.

Edible *Musa* spp. originated in southeast Asian and western Pacific regions where their inedible, seed-bearing, diploid ancestors may still be found in the natural forest vegetation **(Simmonds and Shepherd, 1955)**. As a result, the regions are considered as centres of origin of the edible bananas and plantains **(Robinson, 1996)**. The diploid ancestors *M*. *acuminata* Colla and *M*. *balbisiana* Colla were believed to have originated from the tropical Malay region (humid area) and the more northern Indian (dry monsoon) area, respectively **(Simmonds, 1962)**. As a result of various intra- and inter-specific hybridization as well as human selection for superior qualities, the modern edible banana cultivars were established **(Robinson, 1996)**. Bananas travelled with human populations and early Filipinos probably spread the banana eastward to the pacific islands, including Hawaii. Westward, bananas may have followed the major trade routes that transported other fruits **(Simmonds, 1962)**. According to **Simmonds and Shepherd (1955)**, bananas were taken from Indonesia across the Indian ocean to

Madagascar around 500 AD. Subsequently, it was introduced into east and west Africa. Bananas were not carried to Europe until the 10th century and Portuguese traders were involved in the introduction of bananas from west Africa to the Canary Islands as well as South America in the 16th century **(Simmonds, 1962; Purseglove, 1972)**. As a result of the large number of *Musa* spp. clones that occurred in west and east Africa due to natural somatic mutation, these regions are regarded as the secondary centre of diversity of bananas and plantains, respectively **(Robinson, 1996)**.

1.3 Morphology, taxonomy, and genomics of *Musa* spp.

Musa spp. are large herbaceous plants which commonly grow to about 3 m high, they lack lignification or secondary thickening of stems that is characteristic of trees. The species consist of a branched, underground corm with roots and vegetative buds, as well as an erect pseudostem made up of tightly packed leaf bases. The leaves are spirally arranged extending to about 2.7 m long and 60 cm wide. Foliage leaves form the pseudostem which supports the inflorescence. The corm forms the real stem of the *Musa* spp. and produces 10-15 buds, which may develop into branches called suckers. The apical meristem is located in the centre of the pseudostem and gives rise to a succession of leaf primordia. At the onset of flower initiation, the apical meristem converts into an inflorescence which emerges through the centre of the pseudostem **(Robinson and de Villiers, 2007)**. Female flowers contain both female and male organs with more developed female organs (ovary, style, and stigma) compared to the male organs (stamens). During favourable conditions, edible fruits develop from ovaries of female flowers **(Robinson, 1996)**. The parthenocarpic fruits (i.e. ovaries are un-pollinated and grow into seedless fruit) form hanging clusters with about 20 fruit per hand. The assemblage of hanging clusters is known as a bunch or is commercially called a „banana stem' and weighs an estimated 30-50 kg **(Jones, 2000)**.

Musa spp. are members of the family Musaceae in the order Zingiberales. The family is made up of the two genera, *Ensete* and *Musa* **(Jones, 2000)**. Due to the diverse nature of the genus *Musa*, members are further divided into four sections of unequal hierarchy

which are *Eumusa*, *Rhodochlamys*, *Callimusa* and *Australimusa* **(Simmonds, 1962)**. However, the section *Ingentimusa* was created to accommodate the species *M*. *ingens* with 14 chromosomes (2n = 14), which was found in the highlands of Papua New Guinea **(Argent, 1976)**. The sections *Eumusa* and *Rhodochlamys* have 22 chromosomes (2n = 22) while *Australimusa* and *Callimusa* have 20 chromosomes (2n = 20). These chromosome numbers are not absolute as a number of variations do exist in species such as *M*. *beccarii* which has 18 chromosomes (2n = 18) **(Simmonds and Weatherup, 1990)**.

The taxonomy of *Musa* spp. is difficult, cumbersome and has remained poorly resolved due to the vegetative reproduction mode and natural occurrence of several hybrids **(Heslop-Harrison and Schwarzacher, 2007)**. In 1783, Caroleus Linnaeus made the first scientific classification of bananas, *M*. *sapientium* and *M*. *paradisica* were given to the dessert banana and plantain, respectively **(Robinson, 1996)**. Owing to the shortcoming of Linnaeus's classification, **Simmonds and Shepherd (1955)** devised a modern classification of edible bananas. It was based on the relative expression of *M*. *acuminata* and *M*. *balbisiana* characteristics and the chromosome number of any given cultivar. Although both *M*. *acuminata* and *M*. *balbisiana* are diploid, other ploidy levels do exist among the different *Musa* cultivars. **Stover and Simmonds (1987)** classified many *Musa* cultivars such as the popular Cavendish and east African highland bananas which were grouped as triploid AAA group indicative of the cultivars genetic similarity to *M*. *acuminata*. Although over 50 *Musa* spp. are known, *M*. *acuminata* and *M*. *balbisiana* remain the earliest and largest species. All edible bananas were obtained from these two major groups either singly or in various intra- and inter-specific hybridizations. Accordingly, cultivars having 22, 33 and 44 chromosomes corresponding to diploid, triploid and tetraploid have been reported. However, triploid cultivars are the biggest and sturdiest. In terms of abundance, triploid cultivars remain the most common while tetraploid cultivars are rare **(Robinson, 1996)**. Common examples of the triploid cultivars include: „Williams', 'Gros Michel' and „Grand Naine' bananas.

Attempts to characterize the *Musa* genome chromosomally date back to the early 20th Century **(Cheesman, 1932)**. However, the cytogenetics of *Musa* spp. remained poorly-developed until the mid-1990s when more studies focused on the analysis of ploidy and chromosome number of the wild and cultivated species. *Musa acuminata* (AA genome) and *M*. *balbisiana* (BB genome) with 22 chromosomes (2n = 22) represent the two main progenitors of modern edible banana cultivars. Molecular analyses have suggested the presence of chromosome markers from *Musa* spp. other than the A and B genome. **D'Hont et al. (2000)** used genomic *in situ* hybridization to confirm the presence of complete S and T genomes, from *M*. *schizocarpa* and *M*. *textilis*, respectively. Many of the banana cultivars have various combinations of two or more of these genomes. For example, the diploid cultivar „Wompa' has AS, triploid cultivar „Williams' has AAA genome while AAT and ABBT genomes have also been reported **(D'Hont et al., 2000)**. These genomes A, B, S, and T correspond to the genetic constitutions from the wild *Eumusa* species which include *M*. *acuminata*, *M*. *balbisiana*, *M*. *schizocarpa* and *M*. *textilis*, respectively **(Simmonds, 1962)**.

1.4 Propagation of *Musa* spp.

Due to the high sterile nature and low seed germination rate in *Musa* spp., propagation via seed is almost non-existence and has been restricted to breeding and other improvement activities **(Strosse et al., 2004)**. At present, propagation in *Musa* spp. is predominantly by conventional (suckers and corms) and micropropagation techniques.

1.4.1 Conventional method

Traditionally, *Musa* spp. is propagated using parts such as suckers and bits which are derived from the mother plant. However, suckers is the most common conventional planting material **(Robinson and de Villiers, 2007)**. Suckers are rhizomes in which the central growing point is to be used for regeneration. It first emerges as a conical shoot which opens and releases leaves that are mostly midribs with only vestiges of blade. Suckers used for planting can be either young peepers which have just emerged through the soil surface; large sword suckers which have narrow leaves and a large

rhizome; water suckers which have broad leaves, a narrow pseudostem and a small rhizome; or suckers with the mother rhizome attached for extra sustenance **(Robinson, 1996)**. Often, water suckers are insubstantial, with very little vigour, and are not desirable propagating material. However, the small, medium, or large sword suckers develop thicker stems, and give much higher yields of marketable fruits **(Robinson and de Villiers, 2007)**.

Bits (derived from vigorous healthy rhizomes which are too large to be used as sword suckers) are obtained by selecting a healthy banana plant. After uprooting the bit is carefully cut 10-12.5 cm above the corm. The outer layer of leaf bases is peeled off to expose the buds, leaving just a little to protect the buds during handling and transport. Thereafter, it is divided between the two upper buds and trimmed with square sides, removing the lower, inferior buds and any parts affected by pests or disease, usually indicated by discoloration. Although, bits grow slowly initially, with time they become equal to plants grown from suckers and have been reported to be a more economical planting material **(Robinson, 1995)**

In conventional propagation, genetic improvement is complex and difficult due to low seed viability and the trisomic pattern of gene inheritance. In addition, the plant life cycle is longer and suckers are bulky which are generally of poor sanitary quality, and hinders the distribution of new superior cultivars **(Vuylsteke, 1998)**. As a result of the numerous risks particularly in relation to plant and soil hygiene, the use of conventional planting materials is on the decline. Most commercial banana production currently relies on tissue-cultured planting materials **(Robinson and de Villiers, 2007)**.

1.4.2 Micropropagation of *Musa* spp.

Micropropagation is an invaluable tool which has been useful for the proliferation of several plant species including the elite clones of intractable or recalcitrant species **(Evans et al., 2003; Moyo et al., 2011a)**. Some of the important techniques developed for plant micropropagation include: single node culture; regeneration from callus, cells

and protoplasts; somatic embryogenesis; and enhanced axillary branching/shoot culture **(Pierik, 1987; Thorpe, 1990)**. In bananas, tissue-cultured regenerants produce higher yields than conventional propagules **(Israeli et al., 1995)**. Generally, shoot culture and somatic embryogenesis are considered as the principal micropropagation techniques for many plants including *Musa* spp. **(Vasil, 1994)**. **Cox et al. (1960)** performed the first *in vitro* culture on *M. balbisiana* and successfully cultured zygotic embryos. However, **Ma and Shii (1972)** conducted the first *in vitro* clonal propagation of *Musa* spp. using shoot-tip culture. Although *in vitro* somatic embryo culture system for *Musa* spp. is well-established, the use of shoot-tip culture remains the most widely used technique for the micropropagation of *Musa* spp. **(Vuylsteke, 1998)**.

1.4.2.1 Shoot-tip culture

Shoot-tip culture involves the *in vitro* propagation by repeated formation of axillary shoots from shoot-tips or lateral buds cultured on medium supplemented with plant hormones (e.g. cytokinin, CK) which disrupt apical dominance and enhance axillary shoot production **(George, 1993)**. In *Musa* spp., shoot-tips required as explants can be obtained from any mother plant part such as suckers, peepers, lateral buds or pseudostems containing a shoot meristem and preferably in the flowering period in order to ascertain trueness-to-type **(Cronauer and Krikorian, 1984; Vuylsteke, 1998)**. However, those taken from vigorous sword suckers and peepers are most preferred explants due to their greater ease of handling **(Jarret et al., 1985)**. In addition, there is minimum damage inflicted to the parent „mat' during their removal **(Vuylsteke, 1998)**. The outer leaves, leaf bases and corm tissue of the selected explants are trimmed to 2.5 X 2.5 X 5 cm and surface sterilized using chemicals such as sodium hypochlorite with a surfactant **(Israeli et al., 1995)**. Under aseptic conditions, the shoot-tips are trimmed to cubes with 5 mm sides and transferred to a culture medium. Although several basal medium formulations have been reported to sustain the growth and proliferation of *Musa* spp. **(Vuylsteke, 1998)**, **Murashige and Skoog (1962)** mineral salt mixture with various variations is the most widely used medium. In addition, **Israeli et al. (1995)** reported the inclusion of supplements such as myo-inositol, L-tyrosine,

thiamine-HCl, adenine sulphate, CK, auxin and sucrose. The pH of the medium is adjusted to 5.8 and solidified with a properly dissolved gelling agent. Thereafter, it is sterilized by autoclaving at 121 ºC and a pressure of 103.4 KPa for about 15-20 min **(Vuylsteke, 1998)**.

Inoculated cultures are maintained at a temperature range of 25 to 28 ± 2 ºC and 70% relative humidity in a 16 h light cycle at light intensity of about 42 µmol m^{-2} s^{-1}. At four to six week intervals, subculturing of the small green shoots is done by transferring the propagules onto a fresh CK enriched medium. However, subculture can be done earlier if blackening occurs **(Israeli et al., 1995)**. Blackening occurs due to phenolic oxidation and high concentration of benzyladenine (BA) in the medium has been identified as one of the possible factors responsible for this problem **(Venkatachalam et al., 2007a)**. Blackening can be reduced by dipping the explants in antioxidants (L-cysteine, ascorbic or citric acid) before transferring to the culture medium **(Jarret et al., 1985)**. *Musa* spp. rooting which takes six to eight weeks can be induced by transferring the propagules onto a medium with higher auxin and lower CK concentrations. In preparation for the acclimatization stage, plantlets of approximately 50 mm long with four to five leaves with well-developed root systems are ideal. During acclimatization, plantlets are maintained under greenhouse conditions with temperatures ranging from 25-35 ºC, increased light intensity and reduced humidity. Finally the acclimatized plantlets are transferred to the field for planting **(Israeli et al., 1995; Vuylsteke, 1998)**.

1.4.2.2 Somatic cell culture

Somatic embryogenesis is the *in vitro* production of plants from somatic or vegetative cells **(Gray, 2005)**. Plants are capable of producing offspring from somatic embryos due to their cellular totipotency. This is a highly efficient micropropagation procedure **(Zimmerman, 1993)**. The phenomenon was first demonstrated in carrot callus cells by **Steward et al. (1958)**. Presently, somatic embryogenesis has been reported in many higher plants and is widely used for genetic manipulation such as *in vitro* selection and for transgenic technologies **(Gray, 2005)**. *In vitro* culture of unorganized tissue in

banana is primarily achieved through establishment of embryogenic cell cultures. Basically, an embryogenic callus is induced on solid medium containing high auxin concentration after which it is transferred to a liquid medium where it gives rise to embryogenic cell suspensions **(Strosse et al., 2004)**. Based on the explants from which embryogenesis is induced in *Musa* spp., four major procedures for the development of embryogenic cell suspension have been identified. These include the use of zygotic embryos **(Cronauer-Mitra and Krikorian, 1988)**, rhizome slices and leaf sheaths **(Novak et al., 1989)**, immature male or female flowers **(Escalant et al., 1994)**, and proliferating meristem cultures **(Dhed'a et al., 1991)**. All the aforementioned methods have typical drawbacks that limit their application in banana micropropagation. For instance, zygotic embryos are of limited value as starting materials because most edible banana cultivars are seedless. Successful reports on the efficiency of embryogenesis induction in highly differentiated rhizome and leaf tissue are rare **(Strosse et al., 2004)**. Some cultivars such as „False Horn' and „Harton' plantains do not produce male flowers and the use of female flowers would result in loss of bunches (fruits) which is detrimental for large-scale application. The use of proliferating meristem culture involves an extensive material preparatory phase which can be laborious **(Grapin et al., 1998; Strosse et al., 2004)**.

Although the availability of explants with adequate embryogenic competence is one of the most important limitations in the use of embryonic cell culture for banana micropropagation, it seems to hold great promise as is evident in a study by **Pérez-Hernández and Rosell-García (2008)**. The work successfully induced inflorescence proliferation from male flowers of adult *Musa* spp. which was maintained over time as a continuous source of young flower buds and still retained a high embryogenic competence. In addition, cell cultures are still the most suitable target material for *in vitro* manipulation techniques such as induced mutations and genetic engineering to obtain desired improved clones **(Roux, 2004)**. Embryogenic cell suspensions are ideal for gamma irradiation because of the unicellular origin of regenerants and thus eliminating the occurrence of chimeras **(Strosse et al., 2004)**.

1.5 Significance of the study

No doubt, bananas remain one of the most studied plants in the field of plant biotechnology. The huge economic importance and the inherent problems associated with conventional propagation of bananas makes the species one of the highly prioritized research crops globally **(Nwauzoma et al., 2002)**. In addition, an increase in demand by the world's growing population implies the need for more planting materials of high quality (free of pest and diseases). Even though the use of micropropagation techniques has great benefits such as rapid and mass propagation of plant species, there are some bottlenecks associated with the technique. Often, the tissue culture induced problems cause several morphological and physiological disorders in the regenerants. For instance, the occurrence of somaclonal variations in the micropropagated bananas has remained a major concern to researchers. Other frequently encountered problems during micropropagation include shoot-tip necrosis, hyperhydricity and stunted growth.

In micropropagation, the type and concentration of CK used remains critical. In addition to being the main stimulating factor responsible for the proliferation frequency, CKs have been implicated in the occurrence of several tissue culture-induced physiological problems. Although BA is the most widely used CK in tissue culture systems, hydroxylated analogues of BA named 6-(3-hydroxybenzyl)adenine (*meta*-Topolin, *m*T) and its derivatives have been demonstrated as alternative CKs **(Werbrouck et al., 1996; Strnad et al., 1997; Werbrouck, 2010)**. In the recent past, there has been a surge of promising results with the use of *m*T and its derivatives (topolins) in tissue culture systems. In view of the potential of topolins, a critical assessment of their effect on the physiology and genetic stability on micropropagated „Williams' bananas remains pertinent.

1.6 Aims and objectives:

- To evaluate the effect of topolins on the *in vitro* growth (shoot and root proliferation) of „Williams' banana;

- To determine the effect of topolins on the *in vitro* phenolic content and acclimatization ability of regenerated „Williams' banana;
- To investigate the role of topolins on the photosynthetic pigment contents, foliar and morphometric features as well as the genetic fidelity of regenerated „Williams' banana;
- To compare the effect of smoke technology (smoke-water = SW and Karrikinolide = KAR_1) with *m*T during *in vitro* culture of „Williams' banana;
- To determine the effect on smoke-water and vermicompost leachate during *ex vitro* acclimatization of the micropropagated „Williams' bananas;
- To evaluate the effect of CK analogues (roscovitine and INCYDE = inhibitor of cytokinin dehydrogenase) on the endogenous CK content of micropropagated „Williams' bananas.

1.7 General overview of the thesis

Chapter 2 provides background information reappraising the role of CKs (with emphasizes on topolins) in micropropagation. The review contains numerous studies on the use of topolins in micropropagation. In addition, it discusses aspects of CK metabolism, structure-activity relationships as well as associated physiological problems during *in vitro* plant growth and development. Further detailed background information on the incidence and methods of detecting somaclonal variation are highlighted.

Chapter 3 entails experiments focusing on the shoot and root proliferation in „Williams' bananas. Different concentrations of the topolins were compared with BA. Different parameters including the role of the CKs on phenolic contents in the regenerants were determined. During the rooting phase, the effect of different rooting compounds (auxins, SW and KAR_1) was investigated and will be discussed.

Chapter 4 reports on the effects of the tested CKs on photosynthetic pigment contents. Scanning electron microscope was used to examine the resultant effect of the CKs on the foliar structures and some morphometric features.

Chapter 5 gives an insight on the potential of SW and KAR_1 in „Williams' banana micropropagation. Both compounds were compared to *m*T in terms of their effect on general growth, photosynthetic pigment and phenolic contents.

Chapter 6 focuses on the enhancement of *ex vitro* survival of micropropagated „Williams' bananas. The role of SW and vermicompost leachate at different concentrations was tested under greenhouse conditions.

Chapter 7 includes experiments evaluating the effect of CK analogues (roscovitine and INCYDE) in the presence of BA or *m*T during micropropagation of „Williams' bananas. Their effect on the concentrations of endogenous CK, general growth, photosynthetic pigment and phenolic contents are discussed.

Chapter 8 presents the findings on a comparative experiment on the role of the topolins and BA on the genetic variability of regenerated „Williams' banana.

Chapter 9 provides a summary of the major findings of the research.

References section outlines all the literature and materials cited in the current thesis.

Chapter 2: Literature review

2.1 Introduction

The use of plant biotechnology has been of tremendous benefit to mankind for decades. It remains a handy tool for both fundamental research purposes and in the commercial sector due to its great potential **(Vasil, 2008; Moyo et al., 2011a)**. Despite the numerous inter-related techniques involved in the field of plant biotechnology, micropropagation (*in vitro* propagation or plant tissue culture, PTC) remains one of the most crucial tools. In PTC, clones are produced from explants that are cultured in an artificial medium under controlled physical conditions such as light, temperature and relative humidity while maintaining an aseptic environment. Since the 1970s, the basic principles including the stages and crucial requirements were well-known and documented **(Murashige, 1974)**. Among the numerous benefits of PTC is the potential to mass produce genetically identical plantlets which can be easily acclimatized and established in the field within a relatively short period compared to the conventional cultivation methods. The use of PTC remains crucial to meet the ever-increasing human demands for essential food, medicinal and horticulturaly-important plant species. As highlighted in **Chapter 1**, the technique remains widely-used by most commercial banana farmers globally. Plant growth regulators (PGRs) such as auxins and cytokinins (CKs) constitute essential ingredients during PTC. In the current chapter as well as the thesis as a whole, the major focus is on the CK group of PGRs. Background information that entails the history and application of CKs (including the topolins: a new group of CK) in PTC are provided. In addition, the causes and means of detecting somaclonal variation (a major bottleneck in banana micropropagation), which is partly associated with the type of applied CK in PTC are critically reviewed.

2.2 Cytokinins in plant tissue culture

After the discovery of CKs **(Hall and De Ropp, 1955; Miller et al., 1955; Letham, 1963)**, PTC grew rapidly and exponentially with an equally large expansion in the field of its application **(Vasil, 2008)**. In addition to the valuable contributions of PTC to plant

propagation, improvement and conservation **(Castelblanque et al., 2010; Faizal et al., 2011; Parimalan et al., 2011)**, it has become a fundamental tool to understand various physiological processes which were once explained using speculative hypotheses and growth parameters such as number of regenerated shoots and roots as well as their survival *ex vitro*. The ultimate goal of PTC protocols is the rapid multiplication of uniform and healthy plantlets. During micropropagation of many plant species however, physiological disorders such as stunted growth, epigenetic and somaclonal variation are often encountered **(Hazarika, 2006; Bairu et al., 2011a; Smulders and de Klerk, 2011)**. These problems reduce the commercial application of PTC protocols in these plant species. Therefore, PGRs especially CK, often implicated in the occurrence of these challenges, warrant more stringent studies. Moreover, the choice of CK remains critical to the success or failure of any micropropagation endeavour **(Werbrouck, 2010; Amoo et al., 2011)**.

Presently, information on the structure-activity relationship of CKs and its effect on plant physiology is having great significance in understanding the growth requirements of plant species *in vitro* **(Sakakibara, 2006)**. Most plant species exhibit inherent variation which is translated to their varied responses to the different CKs. As a result, it becomes pertinent to optimize PTC protocols for individual species. Researchers are continuously searching for new as well as superior CKs. Topolins in general and *meta*-topolin (*m*T) in particular are products of such endeavours. Since the discovery of *m*T and its derivatives as naturally occurring aromatic CKs in several plant species **(Jones et al., 1996; Strnad et al., 1997; Tarkowská et al., 2003)**, their use in PTC has increased rapidly **(Werbrouck, 2010)**. Positive reports on important PTC parameters such as higher shoot proliferation, alleviation of commonly observed physiological disorders, better acclimatization and rooting are making topolins popular amongst plant tissue culturists. Despite the use of topolins for more than a decade, a comprehensive and detailed review on the various research outputs is still lacking. **Werbrouck (2010)** highlighted the merits and drawbacks of the new aromatic CKs in PTC. Furthermore, the structure-activity relationships, diverse applications of topolins in agriculture as well as their effect on abiotic stress management has been reviewed **(Subbaraj, 2011)**.

Nevertheless, other important aspects of current research outputs on topolins especially the practical applications remain to be fully exploited.

2.2.1 Cytokinins – Their Discovery, function and diversity

Following the discovery of kinetin (KIN) **(Hall and De Ropp, 1955; Miller et al., 1955)** and later zeatin (Z) **(Letham, 1963)**, many other compounds with CK-like activity have been discovered **(Schmülling, 2004; Sakakibara, 2006)**. At present, there are hundreds of natural and synthetic CKs known to humans. Based on their side chain configuration, the naturally occurring CKs are classified as either isoprenoid or aromatic forms. Urea-based synthetic CKs such as 6-(2-chloro-4-pyridyl)-*N*'-phenylurea (CPPU) and thidiazuron (TDZ) are often recognized as a third group of CKs **(Schmülling, 2004)**. For a long time, the isoprenoid CKs have received more detailed attention than the aromatic CKs which is probably due to their relative higher abundance in higher plants, better knowledge of their biosynthesis and metabolism and the previously-held notion that the aromatic CKs such as benzyladenine (BA) were synthetic **(Van Staden and Crouch, 1996)**. In terms of their functions, **Holub et al. (1998)** postulated that the isoprenoid CKs have a more potent effect on growth processes involving continuation of the cell cycle while aromatic CKs exert a greater influence on developmental processes, especially those involving morphogenesis and senescence. Endogenous CKs are usually present in low concentrations (micro-molar or even lower) and exist in the form of free bases, nucleosides, glucosides and nucleotides in plants **(Schmülling, 2004; Doleța l et al., 2007)**, forming reversible conjugates with sugars and amino acids **(Bajguz and Piotrowska, 2009)**. These conjugates act as storage, transport or biologically inert forms of CKs which are responsible for the physiological and developmental plasticity observed in plants **(Letham and Palni, 1983; Bajguz and Piotrowska, 2009)**. Although adenine-based forms of CKs are mostly preferred in PTC, synthetic CKs such as TDZ and CPPU are also useful, especially in developing protocols for recalcitrant genotypes **(Murthy et al., 1998)**. The effectiveness of these synthetic CKs has been demonstrated in micropropagation of apple **(Fasolo et al.,**

1989), banana **(Makara et al., 2010)**, sugar cane **(Vinayak et al., 2009)** and orchids **(Mata-Rosas et al., 2010)** amongst others.

Despite the presence of numerous natural and synthetic CKs for use in PTC, the search for new CKs has remained active although not many laboratories are committed to it. Major advances in chromatographic and spectrometric techniques has aided and accelerated the search **(Tarkowski et al., 2009)**. Most importantly, the ever growing application of PGRs in plant growth and improvement as well as the physiological and economic (to a lesser extent) limitation of the existing CKs have spurred the active search for new CKs **(Kamínek, 1992; Strnad, 1997; Werbrouck, 2010)**. Consequently, a new class of aromatic CKs, the topolins were discovered and identified. In the topolin basic structure, the presence of an hydroxyl group on the benzyl ring differentiates it from BA **(Figure 2.1)**. Although the first isolation and identification of hydroxylated BA (*o*T) from *Populus robusta* dates back to the early 1970's **(Horgan et al., 1973; Horgan et al., 1975)**, **Strnad et al. (1997)** are credited with the isolation of *m*T [6-(3-hydroxybenzylamino)purine], the highly active hydroxylated BA derivative from mature leaves of *Populus* x *canadensis*. Thereafter, four new methoxy derivatives of the topolins identified as 6-(2-methoxybenzylamino)purine (*ortho*-methoxytopolin, Me*o*T), 6-(3-methoxybenzylamino) purine (*meta*-methoxytopolin, Me*m*T) and their 9-β-D-ribofuranosyl derivatives (Me*o*TR and Me*m*TR) were isolated from *Arabidopsis thaliana* and *Populus* x *canadensis* **(Tarkowská et al., 2003)**. A recent review has subsequently highlighted the identification of different topolins from a few other plant species **(Subbaraj, 2011)**. These major breakthroughs coupled with favourable results from activity evaluation experiments using various bioassays and PTC systems have stimulated the increased use of these new CKs.

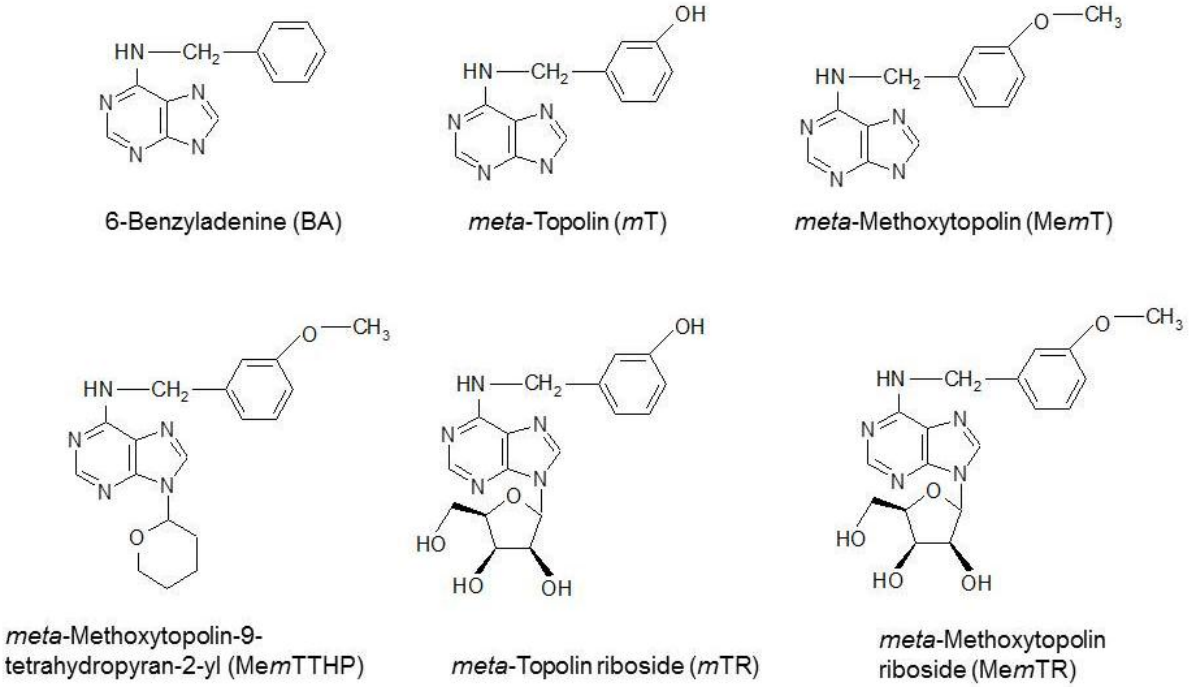

Figure 2.1: Molecular structures of benzyladenine (most widely-used cytokinin) and different topolin derivatives used for micropropagation and investigated in the current study.

Several PTC protocols have been developed and optimized for many plant species. These protocols were developed and optimized based on quantitative and qualitative evaluation of growth parameters such as shoot proliferation, rooting, morphological appearance and acclimatization competence. The choice of medium type such as Murashige and Skoog (MS), gelling agent as well as the type and concentration of PGRs are based on these essential growth parameters. Growth, by and large, is a function of cell division and expansion and it is the factors controlling these processes that receive major emphasis when developing a PTC protocol. With the exception of a few laboratories worldwide, PTC experiments aimed at an in-depth understanding of the physiological and biochemical mechanisms by which PGRs bring about the changes are considered only when things do not work and/or when tissue abnormalities are encountered. As a result, many scientific reports on PTC lack the basic metabolic and physiological explanations of growth. It is important that the type and concentration of PGRs used in the cultivation media are optimized not only based on essential growth and acclimatization parameters but also correlated with optimum levels of active

endogenous PGRs as well as minimization of inactive toxic metabolites **(Malá et al., 2009; Bairu et al., 2011b)**. For a basic understanding of plant hormone physiology in relation to PTC, general overviews on the CK biosynthesis, metabolism and structure-activity relationships are discussed.

2.2.2 Cytokinins biosynthesis and metabolism

Plant tissue culture entails the growth of plants in artificial environments involving the use of externally applied PGRs. This process interferes with, or influences the natural process of CK biosynthesis, a process little understood despite considerable attention and work on plant hormone physiology **(Hutchison and Kieber, 2002; Pan et al., 2008; Jiang and Guo, 2010)**. **Taylor et al. (2003)** identified factors such as the extremely low levels of endogenous CKs, the central role of the likely precursors in cellular metabolism, the existence of numerous native substances with more or less pronounced CK-like activity and the reliance on incorrect or only partially correct assumptions about CK biosynthesis, as obstacles to the understanding of the biosynthetic pathways.

Recent findings on CK analysis of various *in vitro* grown species such as *Watsonia lepida* **(Ascough et al., 2009)** and *Harpagophytum procumbens* **(Bairu et al., 2011b)** indicated variation in type and composition of CK metabolites suggesting that plants vary in their CK metabolism patterns. According to **Palmer et al. (1981)** and **Van Staden and Crouch (1996)**, the variation in the metabolism of applied CKs may be attributed to differences in the stages of development, physiological condition of the plant, organ type, plant species analyzed, concentration of applied PGRs and methods of application. Variation in the composition of CK metabolites of normal and necrotic shoots of *in vitro*-grown *Harpagophytum procumbens* is a notable example of the effect of physiological condition on CK metabolism **(Bairu et al., 2011b)**. This variation therefore makes it difficult to draw general conclusions about the role of CK metabolites in plant growth and development *in vitro*. The variation in the composition of CK

metabolites may also indicate that it is unlikely for all the CKs to be converted to a common metabolite responsible for the growth responses observed.

Plant tissues metabolize exogenous CKs to different types of metabolites such as products of ring substitution (ribosides, nucleotides, *N*-glucosides) and products of side chain substitution (*O*-glucosides) or cleavage (adenine, adenosine, adenosine-5'-monophosphate) **(Letham and Palni, 1983; Van Staden and Crouch, 1996)**. Although the functional significance of these metabolites is not well-understood **(Wagner and Beck, 1993; Van Staden and Crouch, 1996)**, long standing suggestions by **Letham and Palni (1983)** indicates that these compounds could be: active forms of CK, i.e. the molecular form that induces growth or physiological responses; translocation forms; storage forms which would release free (active) CKs when required; detoxification products formed due to the application of exogenous CK at toxic levels; deactivation products formed to lower endogenous (active CK) levels; and post-activation products, the formation of which is coupled with CK action.

The structural variation among CKs is another aspect contributing to the difference in CK metabolism. **Van Staden and Crouch (1996)** emphasized on the absence of a common metabolic pattern for aromatic CKs compared to the knowledge on isoprenoid CK metabolism. The need for better understanding of the pattern of CK metabolism triggered the study of CK action at molecular and cellular levels with emphasis on signal perception, transduction and response **(Kamada-Nobusada and Sakakibara, 2009)**. Initially, these studies were mainly done by searching for proteins which may serve as signal receiving molecules followed by studying the signal transduction pattern(s) **(Strnad, 1997)**. Although very little is known about the molecular mechanism by which target cells for PGRs translate the signals to specific responses, advances in molecular genetics are making such studies at protein and gene level possible **(Kamada-Nobusada and Sakakibara, 2009)**. Earlier studies by **Libbenga and Mennes (1995)** indicated that in a hormonal system, cells of different tissues and organs not only transmit signals, but are also capable of detecting signals which they receive from other parts and respond to those signals in their own characteristic way.

Strnad (1997) discussed the specificity and complexity of CK binding. The existence of two related groups of CK binding proteins (CBPs), one with low affinity to Z but with strong affinity to BA and the other with an opposite character, serves as an indication that there is a distinction between aromatic and isoprenoid CKs with respect to nature of binding and receptor response. As proposed by **Iwamura et al. (1980)**, the differences found in the electron structure and hydrophobicity of the N^6-substituent are probably related to the CK binding site; the role of modified adenines in CK-binding interaction has been elucidated using X-ray crystallographic structural studies **(Strnad, 1997)**.

Mok et al. (2005) reported that topolins and hydroxylated TDZ derivatives are substrates for CK *O*-glucosyltransferase. It was hypothesized that the position specificity may be related to receptor recognition. Evidence indicated that *m*T and *o*T derivatives were the preferred substrates of Z *O*-glucosyltransferase (Z*O*G1; enzyme encoded by *Phaseolus lunatus*) and *cis*-Z*O*G1 (enzyme encoded by *Zea mays*), respectively. There was a direct correlation between the activity of the CKs and their ability to serve as a substrate for glucosyltransferase, thereby suggesting a similarity between CK-binding sites on the enzyme and CK receptors. In addition, studies involving the *Arabidopsis* CRE1/WOL/AHK4 and *Zea mays* ZmHK1 receptors found that the AHK4 receptor responded to *trans*-Z and *m*T while the ZmHK1 receptors responded to *cis*-Z and *o*T **(Mok et al., 2005)**.

Cytokinins, in combination with auxins, are known to affect the basic mechanisms of cell proliferation and differentiation **(Faure et al., 1998)**. **Harrar et al. (2003)** demonstrated that this hormonal control of cell proliferation and differentiation requires PASTICCINO (*PAS*) genes. The authors determined the role of the *PAS* genes by analyzing the expression profiles of several genes involved in cell division and meristem functioning and found that differentiated and meristematic cells of the *PAS* mutants were more competent for ectopic cell division, and were especially enhanced by CKs. They further demonstrated that disorganized cell divisions were associated with the deregulation of cell cycle marker genes like cyclin-dependent kinase A (CDKA) and cyclin B1 (CYCB1).

These variations in CK metabolism, binding and signal perception could possibly explain the inherent variation in response among plants in PTC systems. In addition, the structural variation among CKs is undoubtedly another avenue worth investigating because the literature indicates that a slight variation in structure results in a major change in activity in tissue culture systems. Hence, factors affecting CK structure-activity relationships are discussed below.

2.2.3 Cytokinin structure-activity relationships

The concept of CK structure-activity relationship has attracted the attention of researchers for more than five decades. Today, research interest and focus on the same topic is as topical as earlier, due to the growing application of our knowledge of PGRs. It is well documented that the activity of CKs is highly affected by their structure with the structure-activity relationship of CKs being affected in a number of different ways **(Haberer and Kieber, 2002)**. Different factors such as using: an intact adenine moiety with an N^6-substituent of moderate molecular length **(Skoog and Armstrong, 1970)**, an intact purine ring, unsubstituted 1- and 3- positions, optimum side chain length of five carbon atoms, unsaturation in the side-chain, a substituent which is planar and hydrophobic **(Hecht et al., 1970; Nishikawa et al., 1986)**, an electron rich nitrogen group located opposite to the substituent and linkage atoms or a group that connects the purine ring with the side-chain and restricts the molecular configuration **(Nishikawa et al., 1986)**, 4-hydroxylation of the isopentenyl side chain **(Matsubara, 1980)** have all been mentioned as structural requirements for high CK activity. Alternatively, **Chen and Kristopeit (1981)** suggested that activity depends on the interconversion between the free bases and their ribosides. In addition to the fact that the structure-activity relationships and considerations give an insight on the factors that affect CK activity, these parameters probably provide the basic understanding and/or explain some of the growth related observations in PTC systems. The various roles of topolins in PTC systems in comparison to other CKs are shown in **Table 2.1**.

Table 2.1: The influence of topolins in comparison to other cytokinins on various growth parameters and physiological disorders

Species	Cytokinin(s) tested	Preferred cytokinin(s)	Optimum concentration	Physiological parameters influenced	Reference
Agave cupreata	BA, iP, KIN, *m*T, TDZ	BA	6.7 µM	Shoot multiplication	**Rosales et al. (2008)**
Agave difformis	BA, iP, KIN, *m*T, TDZ	TDZ	0.9 µM	Shoot multiplication	**Rosales et al. (2008)**
Agave karwinskii	BA, iP, KIN, *m*T, TDZ	BA	4.4 µM	Shoot multiplication	**Rosales et al. (2008)**
Agave obscura	BA, iP, KIN, *m*T, TDZ	TDZ	0.9 µM	Shoot multiplication	**Rosales et al. (2008)**
Agave potatorum	BA, iP, KIN, *m*T, TDZ	KIN	13.9 µM	Shoot multiplication	**Rosales et al. (2008)**
Albuca bracteata	BA, *m*T	BA	0.4 µM	Bulb induction, multiplication rate, sizes and masses	**Ascough and Van Staden (2010)**
Aloe ferox	BA, *m*T, *m*TR	*m*T, *m*TR	5.0 µM	Shoot multiplication, abnormality reduction	**Bairu et al. (2009b)**
Aloe polyphylla	BA, *m*T, *m*TR, Me*m*T, Me*m*TR, Z	*m*T	5.0 µM	Shoot multiplication, rooting, acclimatization competence, hyperhydricity alleviation	**Bairu et al. (2007)**
Ananas comosus cv. „Pattawia'	*m*T	*m*T	2.5 µM	Shoot multiplication	**Teklehaymanot et al. (2010)**
Ansellia africana	BA, *m*TR, TDZ, Z	*m*TR	5.0 µM	Shoot length, number of leaves	**Vasudevan and Van Staden (2011)**
Barleria greenii	BA, KIN, *m*T, *m*TR, Me*m*TR	Me*m*TR	7.0 µM	Shoot multiplication, abnormality reduction	**Amoo et al. (2011)**
Beta vulgaris	BA, *m*T, *o*T, Z	*m*T	n/s	Shoot multiplication, rooting	**Kubalákova and Strnad (1992)**
Beta vulgaris cv. „Edda'	*m*TR	*m*TR	3.0 µM	Delayed senescence, increased yield	**Čatský et al. (1996)**
Citrus reticulate x *Poncirus trifolia*	BA, *m*T	BA *m*T	1.0 µM 13.25 µM	Shoot multiplication, length and quality	**Niedz and Evens (2010)**
Curcuma longa cv. „Elite'	Ad, AdS, BA, BAR, iP, KIN, KINR, *m*T, Z	KINR KIN	10.0 µM 10.0 µM	Shoot multiplication, quality of plantlets Rooting	**Salvi et al. (2002)**
Dierama erectum	BA, KIN, *m*T, Z	Z	1.0 µM	Shoot multiplication	**Koetle et al. (2010)**
Eucomis zambesiaca	BA, iP, *m*T, Z	BA	22.19 µM	Bulb induction, multiplication rate	**Cheesman et al. (2010)**

Species	Cytokinin(s) tested	Preferred cytokinin(s)	Optimum concentration	Physiological parameters influenced	Reference
Harpagophytum procumbens	BA, *m*T, *m*TR	*m*TR	5.0 µM	Acclimatization competence, shoot-tip necrosis alleviation	**Bairu et al. (2009a)**
Hibiscus sabdariffa	BA, *m*T, TDZ	BA	17.74 µM	Shoot multiplication, rooting	**Gomez-Leyva et al. (2008)**
Hordeum vulgare	BA, *m*T	*m*T	1.0 µM	Seed germination, rooting	**Huyluoglu et al. (2008)**
Hydrangea macrophylla	BA, *m*T, TDZ	BA	8.9 µM	Shoot multiplication	**Doil et al. (2008)**
Hypericum L. H2003-004-016	BA, *m*T	*m*T	5.0 µM	Shoot quality	**Meyer et al. (2009)**
Lycaste armomatica	BA, KIN, *m*T, TDZ	TDZ	4.4 µM	Shoot multiplication	**Mata-Rosas et al. (2010)**
Malus domestica cv. 'M.26'	BA, BAR, KIN, KINR, *m*T, *m*TR, TDZ, Z, ZR	BAR	18.20 µM	Shoot multiplication	**Dobránszki et al. (2004); Dobránszki et al. (2006)**
Malus domestica „Red Fuji'	BA, BAR, *m*T, BA+KIN	*m*T BA+KIN	20.7 µM (4.4+7.0) µM	Rooting	**Magyar-Tábori et al. (2001)**
Malus domestica cv.„Royal Gala'	BA, BAR, *m*TR, TDZ	BAR	14.0 µM	Rooting, acclimatization competence	**Magyar-Tábori et al. (2011)**
Malus domestica „Royal Gala'	BA, BAR, KIN, KINR, *m*T, *m*TR, TDZ, Z, ZR	TDZ	2.27 µM	Shoot multiplication	**Dobránszki et al. (2004); Dobránszki et al. (2006)**
Malus domestica cv. „Royal Gala'	BA, *m*T	*m*T	2.1-6.2 µM	Shoot multiplication, hyperhydricity alleviation	**Dobránszki et al. (2002)**
Malus domestica cv. „Royal Gala'	BA, KIN, *m*T, BA+KIN, BA+*m*T	*m*T *m*T	2.1 µM 6.2 µM	Shoot multiplication hyperhydricity alleviation	**Dobránszki et al. (2005)**
Malus domestica cv. „Jonagold'	BA, BAR, *m*T	*m*T	20.7 µM	Shoot multiplication	**Magyar-Tábori et al. (2002)**
Musa spp. (AAA)	BA, *m*T, *m*TR, Me*m*T, Me*m*TR	*m*T, *m*TR	22.2 µM	Shoot multiplication, plantlet quality, abnormality index	**Bairu et al. (2008)**
Musa spp. (AAB) cv. „CEMSA 3⁄4'	BA, *m*T	*m*T	4.4 µM	Shoot multiplication, length, rooting	**Escalona et al. (2003)**
Musa spp. (AAB) cv. „CEMSA 3⁄4'	BA, *m*T, TDZ	*m*T	4.4 µM	Shoot multiplication	**Roels et al. (2005)**
Pelargonium × *hortorum* cv. „Katinka'	*m*T, TDZ	*m*T	0.05 mM	Senescence, rooting	**Mutui et al. (2012)**

Species	Cytokinin(s) tested	Preferred cytokinin(s)	Optimum concentration	Physiological parameters influenced	Reference
Pelargonium × *hederaefolium* cv. „Bonete'	BA, *m*T	BA *m*T	2.2 µM 4.1 µM	Shoot multiplication and quality	**Wojtania and Gabryszewska (2001)**
Pelargonium × *hortorum* cv. „Bergpalais'	BA, *m*T	*m*T	4.1 µM	Shoot multiplication and quality	**Wojtania and Gabryszewska (2001)**
Pelargonium x *hederaefolium* cv. „Beach'	BA, *m*T	*m*T	4.1 µM	Shoot multiplication, quality, rooting, delayed senescence	**Wojtania (2010)**
Pelargonium x *hederaefolium* cv. „Luna'	BA, *m*T	*m*T	2.1 µM	Shoot multiplication, quality, rooting, delayed senescence	**Wojtania (2010)**
Pelargonium x *hederaefolium* cv. „Sofie Cascade'	BA, *m*T	*m*T	4.1 µM	Shoot multiplication, quality, rooting, delayed senescence	**Wojtania (2010)**
Pelargonium x *hortorum* cv. „Bergpalais'	BA, *m*T	*m*T	4.1 µM	Shoot multiplication, quality, rooting, delayed senescence	**Wojtania (2010)**
Pelargonium x *hortorum* cv. „Grand Prix'	BA, *m*T	*m*T	4.1 µM	Shoot multiplication, quality, rooting, delayed senescence	**Wojtania (2010)**
Pelargonium x *hortorum* cv. „Jazz Rocky Mountain'	BA, *m*T	*m*T	4.1 µM	Shoot multiplication, quality, rooting, delayed senescence	**Wojtania (2010)**
Pelargonium x *hortorum* cv. „White Rocky Mountain'	BA, *m*T	*m*T	4.1 µM	Shoot multiplication, quality, rooting, delayed senescence	**Wojtania (2010)**
Petunia hybrida	BA, Me*m*TR	Me*m*TR	2.0 µM	Histogenic stability	**Bogaert et al. (2006)**
Pinus pinaster	BA, *m*T, TDZ, Z	BA, Z, *m*T	25 µM	Shoot multiplication	**De Diego et al. (2010)**
Pinus pinea	BA, BPA, *m*T, TDZ	TDZ	2.5 µM	Shoot multiplication	**Cortizo et al. (2009)**
Pinus sylvestris	BA, *m*T, TDZ, Z	*m*T	25 µM	Shoot multiplication	**De Diego et al. (2010)**
Prunus avium	BA, CPPU, *m*T, TDZ	*m*T	4.1 mM	Fruit weight	**Zhang and Whiting (2011)**
Prunus microcarpa	BA, *m*T, TDZ	BA	2.5 µM	Shoot multiplication	**Nas et al. (2010)**

Species	Cytokinin(s) tested	Preferred cytokinin(s)	Optimum concentration	Physiological parameters influenced	Reference
Raphanus sativus	*m*T	*m*T	1.0 mM	Cotyledon growth, chlorophyll content and peroxidase activity	**Palavan-Ünsal et al. (2002a)**
Rosa hybrida cv. „Païline'	BA, F*m*T, F*m*TR, *m*T, Me*m*T, Me*m*TR	F*m*TR Me*m*TR	2.5 µM 2.5 µM	Shoot multiplication Delayed senescence	**Bogaert et al. (2006)**
Saccharum officinarum var. „Gungera'	CPPU, *m*T, TDZ	*m*T	10.0 µM	Shoot multiplication	**Vinayak et al. (2009)**
Saccharum officinarum var. Co 1148	CPPU, *m*T, TDZ	TDZ	0.01 µM	Shoot multiplication	**Vinayak et al. (2009)**
Saccharum officinarum var. CoS 8436	CPPU, *m*T, TDZ	CPPU	5.0 µM	Shoot multiplication	**Vinayak et al. (2009)**
Sclerocarya birrea	BA, *m*T, *m*TR, Me*m*TR	*m*T	8.0 µM	Shoot multiplication, quality and length	**Moyo et al. (2011b)**
Sisyrinchium laxum	BA, *m*T	*m*T	20.7 µM	Shoot multiplication	**Ascough et al. (2011)**
Solanum tuberosum cv „Jaerla'	*m*TR	*m*TR	11.3 nM	Improved root/shoot ratio, acclimatization competence	**Baroja-Fernández et al. (2002)**
Sorbus torminalis	BA, *m*T, Me*m*TR	BA Me*m*TR	0.9 µM 0.8 µM	Shoot multiplication Rooting	**Malá et al. (2009)**
Spathiphyllum floribundum cv. „Petite'	BA, BAR, BPA, *m*T	*m*T	10.0 µM	Rooting, acclimatization competence	**Werbrouck et al. (1996)**
Triticum aestivum	*m*T	*m*T	1.0 mM	Delayed senescence	**Palavan-Ünsal et al. (2002b)**
Uniola paniculata EK 16-3	BA, *m*T	*m*T	20.0 µM 2.2 µM	Shoot multiplication, shoot dry weight, Rooting, acclimatization competence	**Valero-Aracama et al. (2010)**
Uniola paniculata EK 11-1	BA, *m*T	*m*T	10.0 µM 2.2 µM	Shoot multiplication, shoot dry weight, Rooting, acclimatization competence	**Valero-Aracama et al. (2010)**
Vaccinium corymbosum cv. „Highbush blue berry'	*m*T, TDZ, Z	Z	20.0 µM	Shoot multiplication	**Meiners et al. (2007)**
Vaccinium vitis-idaea cv. „Lingonberry'	*m*T, TDZ, Z	Z	20.0 µM	Shoot multiplication	**Meiners et al. (2007)**

Adapted from **Aremu et al. (2012)**

2.2.4 The role of topolins on various parameters in plant tissue culture

As a supplement to **Table 2.1**, the following sections evaluate the potential of topolins as potential substitutes to the commonly used CKs in terms of some important PTC parameters.

2.2.4.1 Shoot proliferation and elongation

In PTC, the production of maximum number of shoots which can be easily rooted, acclimatized and successfully established in the field remains important. Using equimolar concentration (10.0 µM) of either *m*T or BA, **Werbrouck et al. (1996)** observed better shoot-root balance with *m*T-treated *Spathiphyllum floribundum* plantlets. Similarly, *m*T at various concentrations produced more shoots compared to either BA or Z during the micropropagation of *Aloe polyphylla* **(Bairu et al., 2007)**. In banana cv. „Williams' , the use of topolins (*m*T, *m*TR, Me*m*T, Me*m*TR) at 7.5, 15 and 30 µM had higher shoot proliferation rates than BA **(Bairu et al., 2008)**. Studying the effect of nine CKs on shoot production in micropropagated *Curcuma longa*, **Salvi et al. (2002)** obtained more shoots with the use of kinetin ribosides (KINR), iP or *m*T (no significant difference) compared to either BA or other CKs used. However, regenerated shoots from *m*T treatment were greener and stouter than KINR- and iP-treated ones.

Nevertheless, unfavourable responses from the use of topolins for shoot regeneration and proliferation rate have also been observed in certain plant species. In *Rosa hybrida* for example, **Bogaert et al. (2006)** reported that Me*m*TR-treated explants had a lower multiplication rate than the BA treatment. A number of similar responses where other CKs were more effective have been documented by several authors **(Table 2.1)**. There was no definite pattern in the plant species responses to the different CKs; an indication of the interaction of several factors during the shoot regeneration and multiplication phase. Factors such as genotype, CK concentration used and medium type **(Dobránszki et al., 2004; Vinayak et al., 2009; Wojtania, 2010)** remain vital and need to be taken into consideration while optimizing PTC protocols.

2.2.4.2 Rooting and *ex vitro* acclimatization

Lack or inadequate rooting is one of the major problems in PTC. In micropropagated plants generally, there is a positive correlation between good rooting (mainly stimulated by auxins) and their ability to acclimatize faster **(Werbrouck et al., 1996; Koetle et al., 2010)**. Acclimatization of *in vitro*-grown plants to natural conditions is a vital step in the micropropagation of many species especially for large scale application of *in vitro* techniques. As opposed to *ex vitro* conditions, during *in vitro* micropropagation, plants grow under constant temperature, high relative humidity, low irradiance, sugars as carbon source, PGRs in nutrient medium, variable and often insufficient CO_2 concentrations **(Pospíšilová et al., 2007)**. These differences result in variation of factors such as leaf structure, water relations and photosynthetic parameters which are critical for the successful acclimatization of *in vitro* plants **(Pospíšilová et al., 1999)**. In addition, the effect of the applied PGRs such as CKs, auxins and abscisic acid are crucial in the successful and improved acclimatization of *in vitro* plants. Several studies on different species have reported the type and concentration of CKs have a profound effect on *in vitro* plant acclimatization competence **(Moncaleán et al., 2001; Bairu et al., 2008; Valero-Aracama et al., 2010)**. In addition, CKs generally have inhibitory effects on rooting, resulting in poor acclimatization rates afterwards **(Werbrouck et al., 1995; Bairu et al., 2008)**. In particular, BA is often associated with negative side-effects during acclimatization of some plant species. According to **Werbrouck et al. (1995)**, these harmful effects are partly due to the formation of *N*-glucosides (at position 7 or 9) and 9*N*-alanine conjugates, which are biologically inactive (characterized by slow release of active CK free bases) and chemically stable metabolites. More importantly, BA-treated plantlets have the tendency of accumulating these toxic BA metabolites in their basal (rooting zone) portions. Consequently, these metabolites interfere with rooting and acclimatization competence in micropropagated plantlets **(Werbrouck et al., 1995)**. As demonstrated by **Valero-Aracama et al. (2010)**, BA treatment caused detrimental biochemical, physiological and developmental effects in *Uniola paniculata* cultures that resulted in reduced acclimatization competence. Similar problems were absent in *m*T treatments; which is a further indication of the relative lower toxicity of *m*T

over BA and its analogues. **Werbrouck et al. (1996)** postulated that *m*T metabolites are less stable and produce reversibly sequestrated metabolites. The presence of an hydroxyl group in topolins give them a structural advantage over BA in that they can undergo *O*-glucosylation to form storage forms **(Bairu et al., 2011b)**.

In contrast to BA, *m*T stimulated *in vitro* rooting activity in *Spathiphyllum floribundum* **(Werbrouck et al., 1996)**. Similarly, **Bairu et al. (2007)** observed that both *m*T and *m*TR promoted the rooting of *Aloe polyphylla* shoots in the multiplication medium and approximately 90% of plantlets treated with *m*T acclimatized successfully compared to a 65% survival rate recorded with BA-treated plantlets. Low concentrations (5-10 µg l^{-1}) of *m*TR significantly increased the rooting and survival of *Solanum tuberosum* cv. Jaerla plantlets **(Baroja-Fernández et al., 2002)**. However, the authors observed a decrease in the survival rate as the concentration of the *m*TR was increased. The positive effect of topolins on rooting, however, is not universal. *Meta*-topolin and *m*TR at 22.2 µM had an inhibitory effect on rooting of *Musa* spp. (cv. „Williams') compared to BA **(Bairu et al., 2008)**. **Valero-Aracama et al. (2010)** observed that *m*T at 10 µM or higher had inhibitory effects on the *in vitro* rooting of two *Uniola paniculata* genotypes. Similarly, **Escalona et al. (2003)** in a study on plantain cv. „CŒMSA ¾' reported a progressive reduction in root production as the concentrations of BA and *m*T were increased (1.33-22.2 µM). However, 1.33 µM *m*T gave the highest number of roots. The general trend indicates that higher concentrations of most topolins (as is the case with most CKs) were detrimental to the rooting and acclimatization competence of micropropagated plants. Hence, a wide range of concentrations should be incorporated while investigating the use of topolins in PTC. The presence of well-developed roots in multiplication media and inhibition of rooting in some species warrant detailed investigation on the effect of topolins in the physiology of root development.

The interaction of CKs with auxins has always been an important consideration in PTC. Very little published work on topolins is available on this aspect. Recently, the differential effect of the role of CK-auxin interaction on endogenous CK levels in relation to shoot-tip necrosis was observed. The presence of the auxin, indole-3-acetic acid

(IAA) in the culture medium enhanced the formation of 9*N*-glucosides (an undesired and detrimental CK metabolite) in BA-treated cultures but reduced it in topolin-treated cultures. Better *O*-glucosylation was also observed in topolin-treated cultures when IAA was omitted **(Bairu et al., 2011b)**. Similarly, **Malá et al. (2009)** measured lower levels of IAA in BA-treated explants of *Sorbus torminalis* compared to *m*T – a possible explanation for the reduced rooting in BA-treated cultures. Although the structural differences in these CKs will have a role to play, the physiological and biochemical events leading to these effects are yet to be fully understood.

2.2.4.3 Alleviation of *in vitro*-induced physiological disorders

During micropropagation, plantlets develop within the culture vessels under a low level of light, artificial medium formulations containing sugar and other essential nutrients to allow for heterotrophic growth as well as in an atmosphere with high relative humidity **(Hazarika, 2006)**. As a result of these conditions, *in vitro* regenerated plantlets can develop certain characteristic features that are inconsistent with development under greenhouse or field conditions. In most cases, the types and concentrations of the exogenously applied CKs have been implicated as the major contributing factors responsible for these *in vitro*-induced physiological disorders **(Table 2.1)**.

Hyperhydricity is a common morphological and physiological disorder observed in micropropagated plants **(Kevers et al., 1984; Debergh et al., 1992)**. It results from either a passive diffusion of water into the tissue or an active phenomenon related to strong metabolic disturbances **(Pâques, 1991)**. Although the mechanism and interactions of these factors are still not well understood, the effect of PGRs in general and CKs in particular are well documented. **Bairu et al. (2007)** investigated the effect of topolins on hyperhydricity in *Aloe polyphylla*. At an optimum concentration of 5 µM, there were no hyperhydric shoots in topolin (*m*T and Me*m*T) treatments. Although all the CK treatments caused hyperhydricity at higher concentrations, it was most severe with BA treatments. Similarly, drastic alleviation of hyperhydricity was also reported with the use of a low concentration of *m*T for *Beta vulgaris* **(Kubalákova and Strnad, 1992)** and

for several *Malus* x *domestica* cultivars **(Dobránszki et al., 2002; Dobránszki et al., 2004; Dobránszki et al., 2005)**.

Shoot-tip necrosis is an abnormality that is often accompanied by browning of buds and leaves eventually leading to plant death **(Bairu et al., 2009c)**. The symptoms result from the senescence and death of tissues in the apical bud which subsequently proceed basipetally **(Kataeva et al., 1991)**. Shoot-tip necrosis is a consequence of a complex set of factors **(Bairu et al., 2009c)**, the effect of PGRs especially CKs however, remains dominant **(Il'ina et al., 2006)**. Although the rate of shoot-tip necrosis increased with increasing CK concentration, there was significantly lower shoot-tip necrosis in micropropagated *Harpagophytum procumbens* supplemented with *m*TR compared to BA treatment **(Bairu et al., 2009a)**. Me*m*TR alleviated shoot-tip necrosis associated with micropropagated *Barleria greenii* **(Amoo et al., 2011)**.

Early senescence is a common problem in PTC of many plant species e.g. *Pelargonium* cultivars **(Mithila et al., 2001)**. Subsequently, **Wojtania (2010)** reported that *m*T was more effective than BA in the inhibition of early senescence in seven *Pelargonium* cultivars. *Meta*-topolin-treated *Pelargonium* plantlets had higher chlorophyll content. Likewise, the application of *m*T to leaf segments of *Triticum aestivum* retarded senescence by decreasing protease activity and chlorophyll loss **(Palavan-Ünsal et al., 2002b)**. Further studies also established that both nitrogen and polyamine contents increased due to application of *m*T suggesting that increased peroxidase activity, polyamines and nitrogen contents in *m*T-treated excised *Triticum aestivum* was a contributing factor to the overall anti-senescence activity of *m*T **(Palavan-Ünsal et al., 2004)**. When the anti-senescence effect of BA and topolins on *Rosa hybrida* was investigated, no CK treatment prevented the older (lower) leaves from senescing after 6 weeks, Me*m*TR treatment showing the most noteworthy anti-senescing activity. Furthermore, after 18 weeks, 50% of Me*m*TR-treated plants were alive and the next active CK, 6-(3-fluorobenzylamino)purine-9-riboside (F*m*TR) had only 14% survival rate **(Bogaert et al., 2006)**. Foliar application of *m*TR to field grown sugar beet delayed senescence with increased content of natural CKs as well as higher yield compared to

the untreated control **(Čatský et al., 1996)**. These effects were associated with the reduced respiration and/or stimulated membrane transport processes in the *m*TR-treated plants **(Kotyk et al., 1996)**. In view of the importance of early senescence inhibition, molecular approaches have been suggested for better understanding of the process **(Gan and Amasino, 1997)**. In addition, it is recommended that both *in vitro* and *ex vitro* application of these topolins be investigated, to evaluate for their effectiveness in retarding senescence for various plant species.

During micropropagation of ornamentals, it is essential to maintain the pre-existing genetic composition of chimeric plants for their commercial value. Presently, KIN is the only tool to slowly and safely regenerate these valuable ornamentals **(Bogaert et al., 2006)**. However, KIN being a weak CK is often associated with lower shoot multiplication **(Amoo et al., 2011)**. These drawbacks probably stimulated the application of topolins in the micropropagation of chimeras. Preserving the histogenic stability of *Petunia* meristem was improved using Me*m*TR when compared to BA. Furthermore, application of Me*m*TR resulted in a superior visual quality of the axillary shoots **(Bogaert et al., 2006)**.

Histogenic stability is an important factor in commercial micropropagation of plants for two contrasting purposes, namely preserving pre-existing variation (chimeras) and preventing unnecessary (somaclonal) variation (see **Section 2.3** for in-depth review). An increase in BA concentration increased the rate of variation in Cavendish banana cv „Zelig' **(Bairu et al., 2006)** while the absence of significant differences in the occurrence of somaclonal variation was noted when BA, *m*T and *m*TR were compared for „Williams' banana **(Bairu et al., 2008)**. The results could have been influenced by carry-over effects of BA from the initial cultures **(Bairu et al., 2006)**. A similar scenario was observed during the micropropagation of *Barleria greenii* that was initially maintained on BA-supplemented media **(Amoo et al., 2011)**. However, the abnormality index of *m*TR and Me*m*TR treatments were lower than the control treatment, suggesting that the abnormality index in *m*TR and Me*m*TR treatments was a carry-over effect from BA.

2.3 Somaclonal variation in plant tissue culture

Spontaneous heritable variation was known to plant growers before the science of genetics was established and the art of plant breeding practiced. The commencement of the domestication of plants coincided with the occurrence of “sports”, “bolters”, “off-types” and “freaks” in vegetatively propagated plants such as sugarcane, potato and banana. Some of the successful cultivars based on spontaneous mutation such as the naval orange, dwarf bananas, coloured and striped sugarcane as well as several potato cultivars are comparable to somaclonal variants and are frequently cultivated **(Ahloowalia, 1986)**. In the micropropagation of bananas, the incidence of somaclonal variation remains a major problem. In its simplest form, somaclonal variation is defined as all forms of aberration or variation originating during cell and tissue cultures **(Larkin and Scowcroft, 1981)**. Presently, the term somaclonal variants is universally used for all forms of tissue culture derived variants **(Bajaj, 1990)**, however, other names such as protoclonal, gametoclonal and mericlonal variation are often used to describe variants from protoplast, anther and meristem cultures, respectively **(Karp, 1994; Chen et al., 1998)**. The growth of plant cells *in vitro* and their regeneration into whole plants is an asexual process, involving only mitotic division of the cell and theoretically, it should not cause any variation. Ideally, clonal multiplication of genetically uniform plants is the expectation **(Larkin, 1998)**. The occurrence of uncontrolled and random spontaneous variation during the culture process is, therefore, an unexpected and mostly undesired phenomenon **(Karp, 1994)**. Contrary to these negative effects however, its usefulness in crop improvement through creation of novel variants is also well documented **(Bouharmont, 1994; Mehta and Angra, 2000; Predieri, 2001)**. Induced somaclonal variation can be used for genetic manipulation of crops with polygenic traits **(Brar and Jain, 1998; Jain, 2001)**. It can also be an important tool for plant breeding via generation of new varieties that could exhibit disease resistance and improvement in quality as well as better yield **(Karp, 1995; Unai et al., 2004)**. Recently, **Neelakandan and Wang (2012)** highlighted the basic molecular concepts involved in tissue culture-induced genomic changes during micropropagation. In addition, these authors

acknowledged the potential benefits of somaclonal variation especially in recalcitrant species.

2.3.1 Frequency rates, sources and causes of somaclonal variation

In contrast to spontaneous mutations *in vivo*, *in vitro* generated variations seem to occur more frequently **(Yang et al., 2010)**, and are detected easily because variants can be readily spotted in a limited space and within a short time **(Ahloowalia, 1986)**. The exposure of unprotected genetic material to chemicals in the medium and survival of the resulting variants in a non-selective environment increases the mutation rate several fold over that in glasshouse or field-grown plant populations **(Skirvin et al., 1994)**.

In vitro culture of plant material can induce or reveal variation between cells, tissues and organs thereby creating variation within cultures, or among the somaclones. Some, or all, of the somaclones may be physically different from the stock plants from which the culture was derived **(Skirvin et al., 1994)**. Variability of this kind, which usually occurs spontaneously and is largely uncontrolled or not directed, can be of two different kinds: via changes caused by cells having undergone persistent genetic change and those caused by temporary changes to cells or tissues, which are either genetically or environmentally induced **(Pierik, 1987; Karp, 1994)**. Generally though, somaclonal variation *in vitro* can be the result of individuals exhibiting one or more of the following changes; physical and morphological changes in undifferentiated callus; differences in the ability to organize and form organs *in vitro*; changes manifested among differentiated plants; and chromosomal changes **(Skirvin, 1978; Skirvin et al., 1993)**.

Somaclonal variants may differ from the source plant permanently or temporarily. Temporary changes result from epigenetic or physiological effects and are non-heritable and reversible **(Kaeppler et al., 2000; Smulders and de Klerk, 2011; Neelakandan and Wang, 2012)**. However, permanent variants referred to somaclonal variants that are heritable and often represent an expression of pre-existing variation in the source plant or are due to the *de novo* variation via an undetermined genetic mechanism(s)

(Larkin and Scowcroft, 1981). Although it has been studied extensively, the causes of somaclonal variation remain largely theoretical or unknown **(Skirvin et al., 1993; 1994)**. Generally, variation in tissue culture could either be pre-existing or tissue culture induced **(George, 1993)**. The literature to date indicates that this variation could range from a specific trait to the whole plant genome. For instance, **Gengenbach and Umpeck (1982)** demonstrated that somaclonal variation is not limited to nuclear DNA by revealing mitochondrially controlled male sterility using restriction enzyme analysis of isolated mitochondrial DNA.

2.3.1.1 Pre-existing variation

Heritable cellular variation could result from mutations, epigenetic changes, or a combination of both mechanisms **(Kaeppler et al., 2000)**. The distinction between the two mechanisms is an important one because genetic mutations are essentially irreversible and are likely to persist in the progeny of regenerated plants, whereas epigenetic changes are not transmitted by sexual reproduction **(George, 1993; Kaeppler et al., 2000)**. Use of chimeric plants **(McPheeters and Skirvin, 1983; George, 1993)**, variation in ploidy level **(Bright et al., 1983)**, tissue culture-induced chromosome aberrations and rearrangement **(Bryant, 1976; Lee and Phillips, 1988)**, mechanisms regulating the cell cycle **(Kaplan, 1992; Beemster et al., 2003)**, activation of cryptic transposable elements **(Peschke et al., 1987)** are some of the factors known to induce pre-existing variations **(Bairu et al., 2011a)**.

2.3.1.2 Tissue culture induced variation

A comprehensive review with several examples of micropropagated plant species in relation to various tissue culture-induced factors have been documented **(Bairu et al., 2011a)**. **Table 2.2** shows different *Musa* spp. with reported incidence of somaclonal variations. In terms of the types of tissue or starting material used, highly differentiated tissues such as roots, leaves, and stems generally produce more variants than explants from axillary buds and shoot tips which have pre-existing meristems **(Sharma et al., 2007)**. The use of undifferentiated tissue such as the pericycle, procambium and

cambium as starting material for tissue culture reduces the chance of variation **(Sahijram et al., 2003)**. Gross changes in the genome including endo-polyploidy, polyteny and amplification or diminution of DNA sequences could also occur during somatic differentiation in normal plant growth and development **(D'Amato, 1977)**. However, some exceptions where more organized tissues such as shoot-tips cause more variations compared to somatic embryogenesis in micropropagated bananas do exist **(Israeli et al., 1996)**.

Evidence for direct mutagenic action of PGRs remain inconclusive and most evidence points to a more indirect effect through stimulation of rapid disorganised growth **(Karp, 1994)**. The presence of a relatively high concentration (15 mg l^{-1}) of BA was implicated in the increase in chromosome number in a somaclonal variant CIEN BTA-03 derived from the cv. „Williams' **(Giménez et al., 2001)**. High levels of BA (30 mg l^{-1}) also greatly increased the genetic variability of rice callus cultures compared to that found in cultures incubated with 2 mg l^{-1} BA **(Oono, 1985)**. Diphenylurea CK derivatives were implicated in incidences of somaclonal variation in bananas **(Roels et al., 2005)**, calamondin **(Siragusa et al., 2007)** and soybean **(Radhakrishnan and Ranjitha Kumari, 2008)**. Auxins used during cultures of unorganised calli or cell suspensions were found to increase genetic variation by increasing the rate of DNA-methylation **(LoSchiavo et al., 1989)**. Likewise, the synthetic auxin 2,4-D that is frequently used in callus and cell cultures, is often associated with genetic abnormalities such as polyploidy and the stimulation of DNA synthesis that may result in endoreduplication **(Bouman and De Klerk, 2001; Ahmed et al., 2004; Mohanty et al., 2008)**. Researchers have observed that certain levels of PGRs, especially the synthetic ones aggravate the occurrence of somaclonal variation during PTC **(Vidal and De García, 2000; Martin et al., 2006)**. Interestingly, **Bennici et al. (2004)** reported that PGRs did not influence the genetic stability and uniformity of organogenesis and somatic embryogenesis-derived fennel. Similarly, the exposure of the banana cultivar „Nanjanagudu Rasabale' to relatively high concentration of two CKs (BA: 53.28 µM; KIN: 55.80 µM) caused no somaclonal variation **(Venkatachalam et al., 2007a)**. In view of these differing reports, the role of type and concentration of PGRs particularly CKs on incidence of somaclonal variation in

different plant species remains a subject for debate and warrants further stringent experiments.

Increasing the number of subcultures and their duration enhances the rate of somaclonal variations, especially in cell suspension and callus cultures **(Reuveni and Israeli, 1990; Rodrigues et al., 1998; Bairu et al., 2006)**. During micropropagation, a high rate of proliferation is achieved in relatively shorter periods and leads to more frequent sub-culturing. **Rodrigues et al. (1998)** showed that somaclonal variants appeared from the fifth subculture (1.3%) onwards and increased to 3.8% after 11 subcultures. The rapid multiplication of a tissue may affect genetic stability leading to somaclonal variation **(Israeli et al., 1995)**. Studies have shown that somaclonal variation is particularly apparent and higher in plants regenerated from long-term cultures **(Reuveni and Israeli, 1990; Petolino et al., 2003)**. For example, **Bairu et al. (2006)** observed an increase in the rate of occurrence of variants with progressive sub-culturing of micropropagated bananas. On the other hand, shoot culture of pea maintained over a long period (24 years) remained genetically stable and was comparable to the original genotype **(Smýkal et al., 2007)**. Absence of genetic variation was also observed after a long culture period (17 months) of fennel micropropagation **(Bennici et al., 2004)**. The increase of the variant rate as a function of the length of the culture period and observations of different variant rates among lines cultured for the same lengths of time under strictly identical culture conditions are two apparently confusing experimental features often reported in tissue culture **(Müller et al., 1990; Podwyszyńska, 2005)**. For better understanding of this problem as well as the variant rate evolution in PTC, **Côte et al. (2001)** proposed a statistical model for predicting the theoretical mutation rate with the number of cycles as the primary parameter. Two main conclusions derived from the model are that a variant rate increase can be expected as an exponential function of the number of multiplication cycles and secondly, after a given number of multiplication cycles, variable off-types percentages can be expected. Although the statistical approach is useful for a better understanding of experimental features frequently observed in PTC, the model had limited application because of the complexity of biological systems as acknowledged by the authors.

Table 2.2: Examples of *Musa* spp. with incidence of somaclonal variation, possible causes, detection methods and their desirability

Type	Cultivar	Source of variation	#Detection method(s)	Desirability of variants	Reference
Brasilero bananas	Williams	BA	Morphology, chromosome count, RAPD	Yes	**Giménez et al. (2001)**
Dessert banana	Valery	Genotype, explant source, number of subcultures	RAPD	Yes/No	**Sheidai et al. (2008)**
Dessert banana	Williams	Explant, number of subcultures	Morphology	No	**Reuveni and Israeli (1990)**
Dessert banana	New Guinea Cavendish, Williams and Dwarf Parfitt	Gibberellic acid metabolism	Morphology, physiological responses	No	**Damasco et al. (1996)**
Dessert banana	Grand Nain	Embryogenic culture	Morphology	No	**Shchukin et al. (1997)**
Dessert banana	Nanicão	Number of subcultures	Morphology	No	**Rodrigues et al. (1998)**
Dessert banana	Grand Naine	CKs, IAA and abscisic acid metabolism	Morphology, biochemical tests	No	**Zaffari et al. (1998)**
Dessert banana	Grand Naine	IAA, IBA, activation of transposable element	MSAP	No	**Peraza-Echeverria et al. (2001)**
Dessert banana	Grand Naine	Explant	AFLP, MSAP	No	**James et al. (2004)**
Dessert banana	Lakatan	Gamma-rays	Morphology, RAPD, AFLP microsatellite markers	Yes	**Hautea et al. (2004)**
Dessert banana	Grande Naine	BA, number of subcultures	RAPD	Yes/No	**Martin et al. (2006)**
Dessert banana	Zelig	BA, number of subcultures	RAPD	No	**Bairu et al. (2006)**
Dessert banana	Robusta, Giant Governor	Number of subcultures	RAPD, microsatellite markers	No	**Ray et al. (2006)**
Dessert banana	Cachaco, Figure Rose, Prata	Chimeric effect	RDA	No	**Oh et al. (2007)**

Type	Cultivar	Source of variation	#Detection method(s)	Desirability of variants	Reference
Dessert bananas	Rastali, Mutiara	Number of subcultures, activation of transposable element	RAPD, IRAP, susceptibility to fusarium wilt disease	Yes	**Asif and Othman (2005)**
Plantain	Agbagba	Shoot-tip culture	Morphology	No	**Vuylsteke et al. (1988)**
Plantain	French-type	Explant, chimeric effect	Morphology	Yes	**Krikorian et al. (1993)**
Plantain	*Musa* AAB	Explant, chimeric effect	Morphology	No	**Krikorian et al. (1999)**
Plantain	„CEMSA 3⁄4'	TDZ	Morphology	No	**Roels et al. (2005)**
Plantain		Chimeric effect	Morphology, susceptibility to black sigatoka disease	Yes	**Nwauzoma et al. (2002)**

Adapted from **Bairu et al. (2011a)**
#Detection method(s): AFLP = Amplified fragment length polymorphism; IRAP = Inter-retrotransposon amplified polymorphism; MSAP = Methylation-sensitive amplification polymorphism; RAPD = Random amplified polymorphic DNA; RDA = Representational difference analysis

2.3.2 Methods of detecting somaclonal variants

High rates of somaclonal variation during micropropagation of many plants remain a major problem, especially in large-scale commercial operations. Early detection and elimination of variants is therefore, essential to reduce losses to the growers. Efficient detection of variants can also be used to spot variants with useful agronomic traits. Somaclonal variants can be detected using various techniques which are broadly categorized as morphological, physiological/biochemical and molecular detection techniques. Each of these techniques has their peculiar strengths and limitations which are briefly discussed below.

2.3.2.1 Morphological detection

Morphological characters have long been used to identify species, genera, and families in plants. Variants can be easily detected based on characters such as difference in plant stature, leaf morphology and pigmentation abnormality **(Israeli et al., 1991)**. For example, banana off-types can be visually detected during acclimatization in the green house before transplanting to the field. In the field, it is also possible to detect dwarf off-types by observing the stature and leaf index (leaf length/width) 3 to 4 months after establishment **(Rodrigues et al., 1998)**. Similarly, in date palm, the production of bastard off-shoots, excessive vegetative growth, leaf whitening and variegation are common morphological traits used in detecting somaclonal variants **(Zaid and Al Kaabi, 2003)**. However, morphological traits are often strongly influenced by environmental factors and may not reflect the true genetic composition of a plant **(Mandal et al., 2001)**. In addition, morphological markers used for phenotypic characters are limited in number, often developmentally regulated and easily affected by environmental factors **(Cloutier and Landry, 1994)**. **Jarret and Gawel (1995)** raised concerns about the irregular responses of the genomes under *in vitro* manipulation that can result in over or under estimations of the degree of closeness among somaclones. For example, major changes to the genome, as a result of *in vitro* manipulation may not be expressed as an altered phenotype and *vice versa*. Furthermore, the detection of variants using morphological features is often mostly feasible for fully established plants

either in the field or greenhouse which is not an ideal technique for commercial application due to cost implications **(Israeli et al., 1995)**.

2.3.2.2 Physiological/biochemical detection

Relative to morphological detection, the use of physiological responses and/or biochemical tests for detecting variants is faster and can be carried out at juvenile stages to lower the possible economic loss **(Israeli et al., 1995)**. The response of plants to physiological factors such as hormones and light can be used as a basis to differentiate between normal and variant somaclones **(Peyvandi et al., 2009)**. For instance, gibberellic acid regulates growth and influences various developmental processes such as stem elongation and enzyme induction. Therefore, disturbances in gibberellic acid metabolism and levels have been suggested as possible indicators of somaclonal variation in higher plants **(Phinney, 1985; Sandoval et al., 1995)**. Based on response to exogenous gibberellic acid, dwarfs of many plant species have been classified as either gibberellic acid-responsive or gibberellic acid-non responsive **(Graebe, 1987)**. For example, **Damasco et al. (1996)** observed that normal banana plants showed significantly greater leaf-sheath elongation in response to gibberellic acid treatment than in dwarfs and elongation in normal banana plants was 2-fold greater than in the dwarfs. Similarly, **Sandoval et al. (1995)** reported a hormonal analysis based on endogenous gibberellins which could be useful to understand and characterize the inter-varietal differences linked to height in *Musa* spp. This technique is mostly useful (and has been proved to be very effective) for plantlets at the de-flasking stage **(Damasco et al., 1996)**.

Light, as an energy source for photosynthesis, and temperature are essential prerequisites for plant growth and development. Plants have developed mechanisms to adapt and acclimate to variations in light regimes from deep shade to extremely bright light **(Long et al., 1994)**. Photo-inhibition is a state of physiological stress and is expressed as a decline in photosynthetic capability of oxygen evolving photosynthetic organisms due to excessive illumination and plays an important role in plants **(Adir et**

al., 2003). Generally, shade or low-light plants are more susceptible to photo-inhibition than high-light plants **(Long et al., 1994)**. **Senevirathna et al. (2008)** suggested a mild shade environment in which the level of photosynthetic photon flux (PPF) density is high enough to saturate carbon dioxide assimilation and low enough to induce shade acclimation will help to optimize the photosynthetic productivity in plants. Based on photo-inhibition, **Damasco et al. (1997)** evaluated the responses of tissue cultured normal and dwarf off-type Cavendish bananas to suboptimal temperatures under field and controlled environmental conditions. The dwarf off-types showed improved tolerance to low temperature and light compared to the normal ones.

Several authors have used different biochemical tests to distinguish among somaclones. This approach has been very useful to quantify some interesting somaclonal variants **(Daub and Jenns, 1989; Kole and Chawla, 1993; Thomas et al., 2006)**. Callus culture of *Leucaena leucocephala* produced several somaclonal variants, of which the carbon dioxide assimilating potential was highest in the white popinac variants **(Pardha Saradhi and Alia, 1995)**. The synthesis of pigments such as chlorophyll, carotenoids, anthocyanins can be used as a basis for detecting somaclonal variants **(Shah et al., 2003)**. For example, **Mujib (2005)** observed that pineapple somaclonal variants showed significantly lower chlorophyll levels than normal clones. Similarly, the total carotenoid content varied greatly between normal *Ipomoea batatas* and somaclonal variants **(Wang et al., 2007)**. Nevertheless, the application of most biochemical tests is complex and requires high expertise. Beside, most biochemical tests are done on a small scale in the laboratory using *in vitro* techniques and the most favourable results obtained have not been successfully implemented for commercial purposes **(Daub, 1986)**.

2.3.2.3 Molecular detection

Molecular techniques are valuable tools used in the analysis of genetic fidelity of micropropagated plants. At the molecular level, variations in tissue culture-derived plants arise from changes in chromosome number or structure, or from more subtle

changes in the DNA **(Gostimsky et al., 2005)**. Visible morphological variation is known to occur at a much lower frequency than at the DNA level **(Evans et al., 1984)**. As a result, it is necessary to examine for potential variation at the molecular level in order to determine locations and extent of deviance from the true-to-type regenerants **(Cloutier and Landry, 1994)**. This may be conducted at an early growth stage, while in tissue culture and prior to the considerable time and expense of achieving full regeneration. The work by **Botstein et al. (1980)** on the construction of genetic maps using restriction fragment length polymorphism (RFLP) was the first reported molecular marker technique in the detection of DNA polymorphism. Presently, a number of molecular techniques are available to detect sequence variation between closely related genomes including differences between source plants and somaclones. These techniques involve the use of molecular markers which are useful in comparing the DNA from different samples for the differentiation in plants due to sequence variation by identifying random polymorphisms **(Cloutier and Landry, 1994)**. DNA extracts from the leaf part are mostly used in the investigations because of the ease of acquisition and preparation **(Jarret and Gawel, 1995)**. As a result of the high specificity of DNA, molecular markers are able to identify a particular fragment of DNA sequence that is associated to a part of the genome and comparisons are usually made on the basis of the presence or absence of a DNA band. Besides, the use of isozymes which are quicker and cheaper, direct DNA sequencing, single nucleotide polymorphisms and microsatellites are now available for more informative marker systems. RFLPs and isozymes share common advantages over morphological markers such as co-dominance, absence of pleiotropic effects and Mendelian inheritance **(Landry et al., 1987; Havey and Muehlbauer, 1989)**. The underlying principles, similarities and differences as well as strengths and limitations of the various molecular detection methods are briefly discussed below.

2.3.2.3.1 Cytological methods

Variation in ploidy or chromosomal number and structure provides strong evidence of the high likelihood of a change in the genetic composition of an organism **(Kunitake et al., 1995; Al-Zahim et al., 1999)**. Chromosomes as well as other nuclear components

such as RNA and DNA variations are important somaclonal variation detection techniques that have been used widely **(Bogdanova, 2003; Nakano et al., 2006; Fiuk et al., 2010)**. Karyological analysis and observation of chromosomal aberration using light microscopy, oil immersion or other complex microscopy techniques have been successfully employed for detecting somaclonal variation in *in vitro* regenerants **(Al-Zahim et al., 1999; Raimondi et al., 2001; Mujib et al., 2007)**, however, it is time-consuming and often tedious especially where chromosomes are difficult to observe **(Doležel, 1997)**. As a result, the flow cytometry technique is now widely used for counting and examining chromosomes **(Doležel et al., 2004)**. This involves preparation of aqueous suspensions of intact nuclei whose DNA is stained using a DNA fluorochrome. The nuclei are classified according to their relative fluorescence intensity or DNA content. This is achieved by suspending the prepared samples in a stream of fluid under an electronic detecting apparatus **(Pfosser et al., 1995; Doležel and Bartoš, 2005)**. As the sample preparation and analysis is convenient and rapid, this technique has been employed to assess the ploidy stability in cork oak **(Loureiro et al., 2005)**, *Juniperus* **(Loureiro et al., 2007)** as well as the detection of somaclonal variants in strawberry **(Nehra et al., 1992)**, bananas **(Giménez et al., 2001)** and potato **(Sharma et al., 2007)**. Nevertheless, the role of cytosolic compounds interfering with quantitative DNA staining is not well understood as well as the absence of a set of internationally agreed DNA reference standards remain as major limitations on the use of flow cytometry **(Doležel and Bartoš, 2005)**.

2.3.2.3.2 Proteins and isozymes

Proteins are the most abundant organic molecules in cells with diverse functions and isozymes are multiple molecular forms of enzymes. Isozymes are different variants of the same enzymes which differ in amino acid sequence but catalyze the same metabolic reactions **(Hunter and Merkert, 1957)**. It is well known that morphological variation is a result of biochemical variation which is expressed as variation among proteins. As a result, the discriminating property of proteins and isozymes is a function of the number of polymorphic loci that can be identified and genetically characterized in

an organism **(Jarret and Gawel, 1995)**. Until recently, isozymes were one of the most widely used molecular markers for studying genetic variation in most organisms **(Weising et al., 2005)**. Historically, proteins and isozymes such as peroxidase, malate dehydrogenase and superoxide dismutase have been extensively used to study variation in sugarcane **(Srivastava et al., 2005)**, beans **(González et al., 2010)** and *Musa* spp. **(Bonner et al., 1974; Rivera, 1983)**. **Mandal et al. (2001)** used both a salt-soluble polypeptide and four isozymes for varietal identification of various banana cultivars. Variations in somaclones can be detected by analyzing clones for protein and enzyme polymorphism. Freshly prepared tissue extracts mostly from the leaf part are loaded on non-denaturing starch or polyacrylamide gels. Proteins of these extracts are separated by their net charge and size during electrophoresis and incubation with specific isozymes. Thereafter, the position of a particular enzyme in the gel is detected after incubation with a dye **(Jarret and Litz, 1986)**. Depending on the number of loci, their state of homo- or heterozygosity and the specific isozymes used, one or several bands are visualized and the polymorphism of the bands reveals variation **(Weising et al., 2005)**. Although, analyses of isozyme patterns of specific enzymes provide a convenient method for detection of genetic changes, the technique is subjected to ontogenic variations as well as other environmental factors. In addition, the number of isozymes are limited and only DNA regions coding for soluble proteins can be sampled **(Venkatachalam et al., 2007c)**. For instance, total protein and isozymes did not reveal variations between normal and dwarf off-types as well as closely related cultivars of *Musa* spp. **(Bhat et al., 1992)**. **Mandal et al. (2001)** also reported the limitation of salt soluble peptides as molecular markers for varietal identification for banana cultivars. Consequently, many authors no longer rely on this technique to detect variants. Instead, other more sensitive techniques are now employed.

2.3.2.3.3 Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) is a technique used for genome analysis of organisms, thereby providing a molecular basis for any observed differences. It involves the digestion of the crude DNA of the organism with restriction

endonuclease. Restriction endonucleases are enzymes produced by a variety of prokaryotes and are naturally used to destroy invading foreign DNA molecules by recognizing and cutting specific DNA sequence motifs **(Weising et al., 2005)**. Endonuclease recognition sites are usually 4 to 6 base pairs in length and the shorter the recognition sequence, the greater the number of resultant fragments. In RFLP, extracted DNA is digested with restriction enzymes and the resultant fragments are separated by gel electrophoresis according to their sizes **(Karp et al., 1996)**. Molecular variations in the organisms are revealed by the resultant length of the fragments after digesting with the restriction enzymes. This differential profile is generated due to nucleotide substitutions or DNA rearrangements like insertion or deletion or single nucleotide polymorphisms **(Agarwal et al., 2008)**.

Restriction fragment length polymorphism was one of the first techniques used to study somaclonal variation and has been used in several species. For example, **Jaligot et al. (2002)** described methylation-sensitive RFLP markers that differentiated between normal and abnormal embryogenic calli of oil palm. The markers were useful for the early detection of somaclonal variation. Generally, RFLP markers are highly polymorphic, co-dominantly inherited and highly reproducible **(Agarwal et al., 2008)**. Although RFLP markers are useful for sampling various regions of the genome and are potentially unlimited, the technique is time consuming, costly and a large amount of plant tissue is required for analyses **(Piola et al., 1999)**. In addition, it involves the use of radioactive/toxic reagents and is technically demanding. For example, it requires the development of cDNA or genomic DNA probes when heterologous probes are unavailable **(Karp et al., 1996)**. These limitations led to the development of new set of less technically complex methods which are PCR-based.

2.3.2.3.4 Polymerase chain reaction based techniques

Polymerase chain reaction (PCR) was invented by Mullis and co-workers in 1983, and is based on the enzymatic *in vitro* amplification of DNA **(Weising et al., 2005)**. In PCR, a DNA sequence of interest is exponentially amplified with the aid of primers and a

thermostable DNA polymerase. The reaction involves repeated cycles, each consisting of a denaturation, a primer annealing and an elongation step. Usage of random primers eradicated the limitation of prior sequence for PCR analysis **(Agarwal et al., 2008)**. PCR-based analytical techniques using various molecular markers provide an essential tool needed to reveal polymorphism at the DNA sequence level and solved the problems of introgression and lineage **(Gostimsky et al., 2005; Simmons et al., 2007)**.

2.3.2.3.4.1 Random amplified polymorphic DNA

Random amplified polymorphic DNA (RAPD) involves the use of single short primers of arbitrary nucleotide sequences to reproducibly amplify segments of target genomic DNA. These short primers referred to as genetic markers are used to reveal polymorphisms among the amplification products which are seen as visible bands with the aid of ethidium bromide-stained agarose gel electrophoresis **(Williams et al., 1990)**. Arbitrary primed PCR (AP-PCR), arbitrary amplified DNA (AAD) and DNA amplification fingerprinting (DAF) are other variants of RAPD. For example, in AP-PCR, a single primer (10-15 nucleotides long) is used and involves amplification for an initial two cycles at low stringency. Subsequently, the remaining cycles are performed at higher stringency by increasing the annealing temperature **(Welsh and McClelland, 1990)**. Although AP-PCR was not widely accepted because it involves the use of autoradiography, it has been simplified and fragments can now be fractionated with the use of agarose gel electrophoresis **(Agarwal et al., 2008)**. With the DAF technique, shorter single arbitrary primers (less than 10 nucleotides) are used for amplification and the fragments are analyzed using polyacrylamide gel coated with a silver stain **(Caetano-Anollés and Bassam, 1993)**. Technically, RAPD has been described as the simplest version of PCR with arbitrary primers used for detecting DNA variation and for convenience, all RAPD variants are referred to as RAPD **(Weising et al., 2005)**.

Besides providing an efficient technique for detecting polymorphism that allows rapid identification and isolation of chromosome-specific DNA fragment, RAPD markers are also useful for genetic mapping, DNA fingerprinting, plant and animal breeding

(Venkatachalam et al., 2008). The use of RAPD markers are especially beneficial to discriminate between materials that are genetically similar, to evaluate genetic variability within a collection and to choose the components of the core collection **(Piola et al., 1999; Bernardo Royo and Itoiz, 2004)**. The RAPD technique has been successfully used to assess genetic relationship in many plants, for example, sugar cane **(Devarumath et al., 2007)**, sorghum **(Singh et al., 2006)** and apple **(Bernardo Royo and Itoiz, 2004)**. Furthermore, many authors have found the RAPD technique useful in examining tissue culture-induced variation. For instance, it has been used to identify somaclonal variants in peach **(Hashmi et al., 1997)**, sugarcane **(Taylor et al., 1995)**, moth orchids **(Chen et al., 1998)** and bananas **(Bairu et al., 2006)**. Nevertheless, the RAPD technique has been inconclusive or ineffective in some species. For example, RAPD markers did not detect somaclonal variation in Begonia plants regenerated from leaf explants treated with increasing concentrations of nitrosomethylurea **(Bouman and De Klerk, 2001)** as well as in X-ray induced garlic mutants **(Anastassopoulos and Keil, 1996)**. The technique also has a lower reproducibility and reliability **(Jones et al., 1997)** as well as being less informative compared to AFLP markers thereby limiting its application in some species **(Mulcahy et al., 1993; Vos et al., 1995)**. Despite all these limitations, RAPD has remained attractive to researchers when financial investment is limited because the input cost is cheaper than other molecular markers such as AFLP and microsatellites **(Belaj et al., 2003; Weising et al., 2005)**. In addition, the problem of RAPD reliability and transferability among laboratories could be minimized and eliminated by following a standard protocol, replication of amplification reactions and a conservative criterion for band selection **(Belaj et al., 2003)**.

2.3.2.3.4.2 Amplified fragment length polymorphism

Amplified fragment length polymorphism (AFLP) is a PCR-based tool used in genetic research, DNA fingerprinting, and in the practice of genetic engineering. It was developed in the early 1990's to overcome the limitation of reproducibility associated with RAPD. Theoretically, AFLP represent an ingenious combination of the power of RFLP and flexibility of PCR-based technology **(Agarwal et al., 2008)**. It provides a

novel and very powerful DNA fingerprinting technique for DNAs of any origin or complexity. The cDNA-AFLP and three endonuclease AFLP (TE-AFLP) which are used to quantify differences in gene expression levels and to detect transposable element mobility, respectively are variations of AFLP techniques **(Van der Wurff et al., 2000; Weising et al., 2005)**.

In AFLP-PCR, genomic DNA is digested using two restriction endonucleases, typically one with a 6-bp recognition sequence (usually *Eco*RI) and the other with a 4-bp recognition sequence (usually *Mse*I). Thereafter, adapters of known sequence are then ligated to complementary double stranded adaptors of the ends of the restriction fragments. A subset of the restriction fragments are then amplified using two primers complementary to the adaptor and restriction site fragments for two successive rounds of selective PCR amplification. The first round of PCR (preselective or -*f*-1 amplification) uses primers that match the adapters on the *Eco*RI end and *Mse*I end of the fragments plus one extra nucleotide. The second round (selective or +3 amplification) has an additional two nucleotides added to the +1 primer sequences. These rounds of selective amplification reduce the resulting pool of DNA fragments to a size more manageable for analysis **(Vos et al., 1995)**. The fragments are visualized on denaturing polyacrylamide gels either through autoradiography or fluorescence methodologies **(Weising et al., 2005)**. The resultant number of fragments for each AFLP assay depends on the number of selective nucleotides in the AFLP primer combination, the selective nucleotide motif, GC content, and physical genome size as well as complexity **(Agarwal et al., 2008)**.

The AFLP technique generates fingerprints of any DNA from any source even without any prior knowledge of DNA sequence and recent studies showed that it can be used to distinguish closely related individuals at sub-species level **(Althoff et al., 2007)**. For instance, AFLP analysis revealed the values of nucleotide diversity in regenerated *Arabidopsis thaliana* which was 2-3 orders of magnitude smaller than natural variation described for ecotypes of *Arabidopsis thaliana* **(Polanco and Ruiz, 2002)**. This shows that AFLP is a very sensitive and reliable marker technique that could be useful for detecting specific genomic alterations associated with tissue culture variation and

identifying slightly different genotypes. AFLP analysis has also been used to study tissue culture induced somaclonal variation in species such as coffee **(Sanchez-Teyer et al., 2003)**, eastern purple coneflower **(Chuang et al., 2009)**, cork oak **(Hornero et al., 2001)** and bananas **(James et al., 2004)**. However, AFLP often requires more work with optimization and is relatively more expensive than RAPD **(Weising et al., 2005)**. Also, the technique requires high quality DNA samples which are often difficult to obtain in some plants such as conifers **(Piola et al., 1999)**.

2.3.2.3.4.3 Microsatellite markers

In contrast to all the PCR-based techniques explained above which are arbitrarily primed or non-specific, microsatellite-based marker techniques are sequence targeted. They are also known as simple sequence repeats (SSRs), short tandem repeats (STRs), sequence-tagged microsatellite sites (STMS) and simple sequence length polymorphisms (SSLP) **(Albani and Wilkinson, 1998; Hautea et al., 2004)**. Microsatellites consist of tandemly reiterated short DNA (one to five) sequence motifs which are abundant and occur as interspersed repetitive elements in all eukaryotic **(Tautz and Renz, 1984)** as well as in many prokaryotic genomes **(Van Belkum et al., 1998)**. Microsatellite marker techniques use the intra- and inter-individual variation in microsatellites or simple sequence repeat regions for fingerprinting analyses **(Agarwal et al., 2008)**. Polymorphism results from differences in the number of repeat units between individuals at a particular microsatellite locus and is believed to be due to unequal crossing over or slippage of DNA polymerase during replication of repeat tracts **(Levinson and Gutman, 1987; Coggins and O'Prey, 1989)**. However, variation in the number of tandemly repeated units has been reported to be mainly due to strand slippage during DNA replication where the repeats allow matching via excision or addition of repeats **(Schlötterer and Tautz, 1992)**. As slippage in replication is more likely than point mutations, microsatellite loci tend to be hypervariable and are implicated for the extensive inter-individual length polymorphisms observed in microsatellite assays **(Agarwal et al., 2008)**. Most importantly, studies have showed

that the PCR-amplified microsatellite markers are inherited in a Mendelian fashion **(Litt and Luty, 1989)**.

For the microsatellite assay, the sequence information of repeat-flanking regions is used to design locus-specific PCR primer pairs and amplified. These PCR loci-specific can be either unlabelled primer pairs or primer pairs with one radio-labelled or fluoro-labelled primer. Subsequently, amplified PCR products are separated on denaturing polyacrylamide gel and visualized by autoradiography, fluorometry or staining with silver or ethidium bromide **(Weising et al., 2005)**. Although relatively expensive, the use of fluorescent labelled microsatellite primers and laser detection in genotyping procedures has significantly improved the throughput and automation of this technique **(Wenz et al., 1998)**. Microsatellite markers have been found useful in establishing genetic stability of several micropropagated plants such as sorghum **(Zhang et al., 2010)**, trembling aspen **(Rahman and Rajora, 2001)**, rice **(Gao et al., 2009)**, bananas **(Hautea et al., 2004; Ray et al., 2006)**, grapes **(Welter et al., 2007)**, wheat **(Khlestkina et al., 2010)** and sugarcane **(Singh et al., 2008)**. In comparison to AFLP and RFLP, microsatellite markers such as ISSR are cost efficient, overcome the hazards of radioactivity and requires lesser amounts of DNA **(Zietkiewicz et al., 1994)**. In addition, these markers are highly reproducible and are becoming more popular due to their co-dominant inheritance nature, high abundance in organisms, enormous extent of allelic diversity as well as the ease of assessing microsatellite size variation using PCR with pairs of flanking primers **(Li et al., 2002; Weising et al., 2005; Agarwal et al., 2008)**. However, a major drawback for the use of microsatellites is that the development of primers is time-consuming **(Squirrell et al., 2003)**.

2.4 Concluding remarks

In an attempt to develop new protocols, optimize existing ones and alleviate commonly *in vitro*-induced physiological disorders, several studies on CKs have been conducted over the past decades. The majority of the major events and challenges encountered during PTC processes are known. As evident in the current **Chapter**, total elimination of these problems is far from over as most of the challenges are complex and remain to be

fully understood. The choice of CK is amongst the most critical factors in developing a successful PTC protocol. Numerous examples from **Table 2.1** have shown that topolins can successfully replace the commonly used CKs in many PTC protocols. They can also play corrective roles on some physiological disorders. It should however, be noted that there are species that respond better to CKs other than the topolins; hence topolins should not be taken as a panacea and must pass through the routine process of selection. While it is essential to optimize efficient PTC protocols through stringent choice of CKs, tissue culturists should also give emphasis to the associated physiological and metabolic events taking place in culture during the optimization process so that they contribute towards a better understanding of the mode of action of these molecules. Such an approach will help solve associated physiological and developmental problems *in vitro*. Since many tissue culture laboratories are not well equipped for physiological experiments, collaborative efforts could help bridge the gap. It is also recommended that fresh cultures are started to investigate the role of topolins to avoid possible carry-over effects of other CKs and culture additives thereby eliminating reaching incorrect conclusions.

The term somaclonal variation is now universally accepted to represent heritable variations arising in tissue culture. There however, remains some concern in the universal use of the term somaclonal variation especially in polysomatic and chimeric plants. Nevertheless, the causes of somaclonal variations are generally categorized as induced and pre-existing. Visible pre-existing variations such as found in chimeric tissues could theoretically be cultured separately and later manifest themselves phenotypically in somaclones. These may not necessarily represent variations arising from tissue culture. The term should therefore, be restricted to variations that were not visible to the naked eye during the culture initiation stage. In discussing somaclonal variation, both the negative and positive effects need to be treated in parallel. This is due to the potential that induced variation has in crop improvement and it is essential to detect and eliminate variants at early stages to minimize loss. Somaclonal variation can be detected using a wide range of techniques with each having their own strengths and limitations. The choice of detection method therefore, depends on the task at hand.

In the current **Chapter**, the role of different CKs (with emphasizes on the topolins) as well as the incidence of somaclonal variation in PTC has been critically examined. Taken together, the subject matter reviewed clearly indicates the presence of considerable gaps in existing knowledge in the study of CKs. It is conceivable that the use of topolins has great potential in PTC based on the promising results from the micropropagation of several plant species. Bananas, being an important food and fruit, particularly in tropical countries, favourable findings from such stringent study will inevitably contribute to increased production as well as aid in the alleviation of commonly *in vitro*-induced physiological abnormalities. Therefore, the role of topolins in the micropropagation of bananas remains pertinent.

Chapter 3: Micropropagation and *in vitro* phenolic production in 'Williams' bananas

3.1 Introduction

Bananas are among the world's major food crops and an important source of carbohydrate for millions of people in the tropical and subtropical countries **(Hallam, 1995; Vuylsteke et al., 1998)**. Globally, banana production ranks fifth behind cereals and has potential, along with other major crops, to feed the world's increasing population **(Jain, 2004)**. In order to meet the rising food demands, the application of plant tissue culture (PTC) techniques remain a viable means of increasing food production **(Caponetti et al., 2005)**. Based on the various shortcomings including the lack of uniformity, high disease and pest infection rates, as well as the bulkiness of conventional propagation via suckers, the application of various biotechnological approaches has become an integral part of the banana industry **(Vuylsteke et al., 1998)**. In particular, the use PTC via the clonal propagation of superior cultivars has been of immense benefit to commercial banana farmers globally **(Jain, 2004)**.

The plant growth regulators (PGRs), auxins and cytokinins (CKs), remain fundamental for micropropagation as they control several growth and developmental processes such as root and shoot formation in plants **(Nordström et al., 2004)**. In PTC, shoot proliferation largely depends on the type and concentration of exogenously applied CKs **(Gaba, 2005)**. Similarly, the incorporation of auxins into the growth medium is known to stimulate rooting in several species **(De Klerk et al., 1997; 1999)**. Researchers have tested the efficiency of different CK types and concentrations for the micropropagation of several *Musa* spp. **(Vuylsteke, 1998; Strosse et al., 2004; 2008; Makara et al., 2010)**. The desire to improve PTC systems coupled with the deleterious effect of some of the commonly used CKs such as benzyladenine (BA) and zeatin (Z) resulted in the development of a new group of aromatic CKs commonly referred to as topolins **(Strnad et al., 1997)**.

Studies have indicated that CKs regulate the quantity of some secondary metabolites in micropropagated plants **(Ramachandra Rao and Ravishankar, 2002; Quiala et al., 2012)**. In contrast to primary metabolites which are required for basic life processes, plants synthesize and accumulate secondary metabolites in response to ecological and biochemical differentiation **(Lewinsohn and Gijzen, 2009)**. Secondary metabolites are useful for plant interaction and survival in their environment **(Namdeo, 2007)**. The important roles of secondary metabolites especially, the phenolic compounds in PTC as well as subsequent survival in the *ex vitro* environment is well known **(Curir et al., 1990; Wu et al., 2007; Buer et al., 2010)**.

Although some researchers including **Escalona et al. (2003)** and **Bairu et al. (2008)** have evaluated the effect of topolins in the micropropagation of *Musa* spp. especially in comparison to BA, the effect of initial explant which was maintained on BA-supplemented medium remained a major confounding factor. It has been postulated that with the use of the basal and apical meristematic parts of banana plantlets for micropropagation, CKs will inevitably accumulate with each subculture **(Zaffari et al., 2000; Bairu et al., 2006; Makara et al., 2010)**. Consequently, it has been recommended that fresh cultures are used for investigating the role of topolins in order to avoid possible carry-over effects of the preceding different CKs used thereby eliminating incorrect conclusions **(Aremu et al., 2012)**. In addition, reports on the effects of topolins on secondary metabolites are scanty. Findings from such investigations could provide clues and may elucidate additional physiological evidence to support the superiority of topolins over BA in micropropagation of some species.

The current **Chapter** is aimed at further clarifying the role of topolin and its derivatives on the micropropagation of „Williams' bananas, especially with the use of shoot-tip explants with minimum carry-over of exogenously added CKs. In view of the significance of rooting in PTC **(De Klerk et al., 1999)**, the effect of the exogenously applied CKs on root development during the shoot induction phase as well as their possible residual effects on the rooting stage using five rooting compounds was

evaluated. The resultant accumulation of phenolic compounds in the micropropagated „Williams' bananas treated with the different topolins were studied.

3.2 Materials and methods

3.2.1 Chemicals

Indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA) and BA were purchased from Sigma-Aldrich (Steinheim, Germany). The topolins: *meta*-Topolin (*m*T), *meta*-Topolin riboside (*m*TR), *meta*-Methoxy topolin (Me*m*T), *meta*-Methoxy topolin riboside (Me*m*TR), and *meta*-Methoxy topolin 9-tetrahydropyran-2-yl (Me*m*TTHP) were prepared as described previously **(Doleţal et al., 2006; 2007; Szücová et al., 2009)**. Smoke-water (SW) and karrikinolide (KAR_1) were obtained from previous experiments **(Baxter et al., 1994; Van Staden et al., 2004)**.

3.2.2 Source of initial plant material

Rooted tissue-cultured banana (*Musa* spp. AAA cv. „Williams') plantlets were purchased from Du Roi Laboratory, South Africa. For the alleviation of the influence of the *in vitro* history, particularly the exogenously applied CK, the „Williams' banana plantlets were subjected to an *ex vitro* environment for 4 months prior to re-initiation *in vitro*. The plantlets were carefully washed-free of the gelling agent and transferred to 12.5 cm diameter pots containing a sand:soil:vermiculite (1:1:1, v/v/v) mixture, treated with 1% anti-fungal compound (Benlate® Du Pont de Nemour Int., South Africa). Plantlets were transferred to the mist-house having a misting interval of 15 min and 10 s duration, with 80-90% relative humidity **(Figure 3.1A)**. In the mist-house, the day/night temperature was 30/12 ºC and midday photosynthetic photon flux (PPF) ranged from 30-90 µmol m^{-2} s^{-1} under natural photoperiod conditions. After 1 month in the mist-house, the banana plantlets were transferred to a greenhouse with a day/night temperature of approximately 30/15 ºC, average PPF of 450 µmol m^{-2} s^{-1} and 30-40% relative humidity under natural photoperiod conditions **(Figure 3.1B)**.

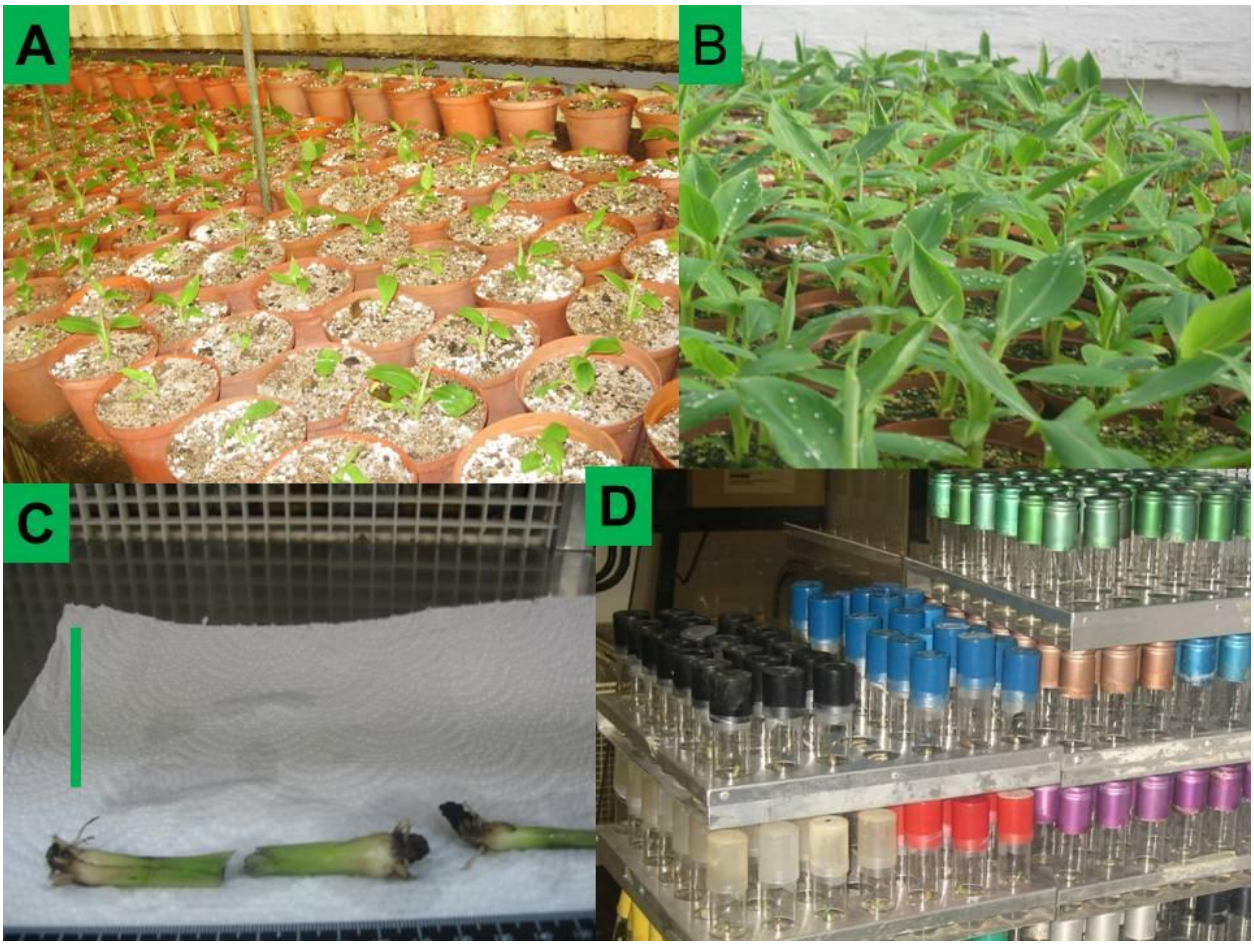

Figure 3.1: Initiation stages of „Williams' banana cultures (A) = one-month-old plantlets in the mist-house (B) = four-month-old greenhouse plantlets; (C) = excised explant used for decontamination and (D) = initiated explants in culture tubes. Bar = 50 mm.

3.2.3 Explant initiation, decontamination, medium composition and growth room conditions

After 4 months, shoot-tip explants were excised from greenhouse-acclimatized banana plantlets **(Figure 3.1B)**. Excised explants of about 40 mm **(Figure 3.1C)** were surface decontaminated using 70% ethanol for 60 s followed by either 3.5% sodium hypochlorite or 0.1% mercuric chloride ($HgCl_2$) solution supplemented with a few drops of Tween 20 for 5, 10, 20 or 25 min. Thereafter, the explants were rinsed thrice in sterile water. The surface decontaminated shoot-tip explants were trimmed to approximately 10 mm and cut in half longitudinally and inoculated in culture tubes (100 x 25 mm, 40 ml

volume **Figure 3.1D**) containing 12 ml of modified Murashige and Skoog (MS) medium **(Appendix 1) (Murashige and Skoog, 1962)** as described by **Vuylsteke (1998)**. The PGR-free medium was supplemented with 30 g l^{-1} sucrose and solidified with 3 g l^{-1} gelrite (Labretoria, Pretoria, South Africa). The pH of the medium was adjusted to 5.8 with either KOH or HCl prior to autoclaving at 121 °C and 103 kPa for 20 min. Filter sterilized ascorbic acid (0.18 g l^{-1}) was added to the medium before solidification (50 °C). Cultures were incubated in a growth room under 16 h light/8 h dark conditions and PPF of 45 µmol m^{-2} s^{-1} at 25 ± 2 °C. Further experiments were done using the resultant aseptic shoot-tip explants which were regularly subcultured at 42 day intervals.

3.2.4 *In vitro* shoot proliferation

Aseptically-obtained explants were transferred onto the modified MS medium with different treatments prepared using the six aromatic CKs at 10, 20 or 30 µM. A control without any PGR was also included in the experiment. The same pH range and growth conditions as described in the preceding section were also applicable during the shoot multiplication experiment. Subculturing was done using the same CK (three concentrations) at a 42 day culture cycle. For the current study (fifth cycle), the treatments were repeated with 12 explants per experiment. Parameters such as the number of leaves, number of shoots, shoot length, shoot fresh and dry weights per explant were recorded and the combined data from the two replicate experiments were used for data analysis. In addition, the abnormality index of the regenerated shoots was evaluated as described by **Bairu et al. (2008)**.

3.2.5 *In vitro* rooting and acclimatization

Based on the maximum number of shoots regenerated from the shoot multiplication experiment, the optimum concentration of each CK treatment (10 µM = Me*m*T, Me*m*TTHP; 20 µM = *m*TR and 30 µM = *m*T, Me*m*TR, BA) was used for the rooting and acclimatization experiments. The MS medium composition, additives and growth conditions were as described for the shooting experiment. However, for each CK-type regenerants, the CK was replaced with a single concentration of one of the five rooting

compounds (IAA, IBA, NAA = 1 µM; KAR_1 = 4.8 X 10^{-22} M or SW = 1:1000 dilution). For all the treatments, three regenerated shoots measuring approximately 40 mm were inoculated in culture jars (110 x 60 mm, 300 ml volume) containing 36 ml of the prepared rooting compound-supplemented growth medium. In addition, the control without any rooting compound was included for the individual CK-type. The experiment was repeated twice with 15 regenerated shoots per experiment. After 30 days, the number and length of the regenerated roots were recorded for each CK-type and the data from the two replicate experiments were combined for analysis. *In vitro* rooted plantlets were carefully washed free of gelrite and transferred to the mist-house and subsequently the greenhouse. The potting mixture, mist-house and greenhouse conditions were as described in **Section 3.2.2**.

3.2.6 *In vitro* phenolic content

In a concurrent experiment, 42-day-old banana plantlets regenerated from the different CK treatments were separated into aerial and underground parts. The plant materials were oven-dried at 50 ± 2 ºC for 7 days and milled into powder form. Ground plant samples were extracted in 50% methanol (MeOH) at 0.1 g per 10 ml in an ultrasonic sonicator (Julabo GmbH, West Germany), that contained ice, for 20 min. The mixture was separated using a Benchtop centrifuge (Hettich Universal, Tuttlingen, Germany) to obtain the supernatant required for the phytochemical quantification.

3.2.6.1 Determination of total phenolic content

The Folin-Ciocalteu (Folin-C) assay as described by **Makkar et al. (2007)** was used to quantify total phenolic content. Briefly, 50 µl of MeOH extracts were added to 950 µl distilled water in glass test tubes. Thereafter, 500 µl Folin-C reagent (1 N) and 2.5 ml of 2% sodium carbonate (Na_2CO_3) were added in succession. The test mixtures were incubated at room temperature for 40 min and the absorbance at 725 nm was measured against a blank solution using a UV-visible spectrophotometer (Varian Cary 50, Australia). Gallic acid (Sigma-Aldrich, USA) at 0.1 mg l^{-1} was used as a standard.

Total phenolic content was expressed as mg gallic acid equivalents (GAE) per g dry weight (DW).

3.2.6.2 Determination of total flavonoid content

Total flavonoid content was determined using the aluminium chloride ($AlCl_3$) colorimetric assay as described by **Zhishen et al. (1999)** with modifications **(Marinova et al., 2005)**. Two millilitre of distilled water were added to 500 µl of 50% MeOH extract followed by 150 µl of 5% sodium nitrite ($NaNO_2$). After 5 min, 150 µl of 10% $AlCl_3$ and 1 ml of 1 M NaOH were added sequentially. The final volume of reaction mixture was made to 5 ml with 1.2 ml of distilled water. The reaction solution was thoroughly mixed and the absorbance at 510 nm was measured using a UV–visible spectrophotometer against a blank consisting of 50% MeOH. Total flavonoid content was expressed as mg catechin equivalents (CE) per g DW.

3.2.6.3 Determination of total proanthocyanidin content

The HCl/Butan-1-ol assay adapted from **Porter et al. (1985)** and outlined by **Makkar et al. (2007)** was used to quantify the total amount of proanthocyanidins. In triplicate, 3 ml of butanol-HCl reagent (95:5 v/v) were added to 500 µl of the 50% MeOH extract in a glass test tube, followed by 100 µl of ferric reagent (2% ferric ammonium sulphate in 2 N HCl). The reaction mixture was thoroughly mixed and incubated in a water bath for 1 h at 100 ºC. Thereafter, the absorbance at 550 nm against a blank standard was measured using a UV-visible spectrophotometer. The amount of proanthocyanidins was expressed as mg of cyanidin chloride equivalents (CCE) per g DW.

3.2.7 Data analyses

For each experiment, data were subjected to one-way analysis of variance (ANOVA) using SPSS software package for Windows (SPSS®, version 10.0, Chicago, USA). Where there was statistical significance (P = 0.05), the mean values were further separated using the Duncan's multiple range test.

3.3 Results and discussion

3.3.1 Explant decontamination frequency

The use of sodium hypochlorite was not effective for the decontamination of the shoot-tip explants producing less than 30% clean cultures. However, the use of 0.1% mercuric chloride ($HgCl_2$) at various time intervals resulted in an approximately 40-70% decontamination rate **(Figure 3.2)**. In view of the better decontamination frequency, explants were treated with 70% ethanol for 60 s followed by 0.1% $HgCl_2$ for 20 min.

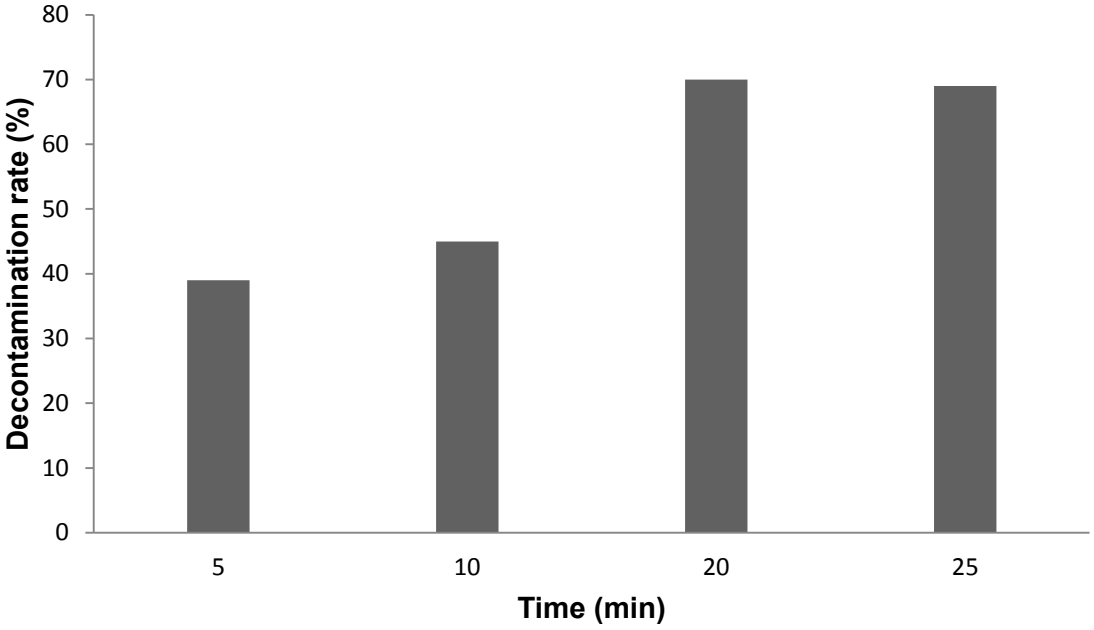

Figure 3.2: Effect of 0.1% mercuric chloride ($HgCl_2$) solution treatment on the decontamination rate (%) of the shoot-tip explants of „Williams' banana.

3.3.2 Effect of the aromatic cytokinins on *in vitro* shoot proliferation

Mean number of shoots per explant was 1.1 and 7.3 for in the control and 30 µM *m*T treatment, respectively **(Table 3.1)**. An increase in concentration of Me*m*TTHP resulted in lower shoot number whereas the highest concentration was required to obtain optimum shoot numbers in *m*T, Me*m*TR and BA treatments. Apart from having a significantly longer shoot, all the growth parameters in control plantlets were clearly

lower compared to the CK treatments. There was generally a decrease in the shoot length in the regenerated plantlets as the concentration of individual CKs was increased, except in *m*TR- and Me*m*TR-treated plantlets where 20 µM gave the optimum shoot length. The abnormality index was lowest in 10 µM *m*TR and highest in 30 µM Me*m*T treatments **(Table 3.1 and Figure 3.3)**. In Me*m*T and BA treatments, the abnormality index increased with an increase in CK concentration while there was no definite pattern with other treatments.

Figure 3.3: Morphological appearance of shoots regenerated from different cytokinins compared to the control. Me*m*T = *meta*-Methoxy topolin and *m*TR = *meta*-Topolin riboside.

Table 3.1: Effect of six aromatic cytokinins at different concentrations on the growth of micropropagated „Williams' bananas

#Cytokinin treatment		Shoot number	Shoot length (cm)	Shoot FW (g)	Shoot DW (mg)	Abnormality index	Leaf number
0	Control	1.1±0.08 d	9.1±1.07 a	0.94±0.190 e	47.0±9.43 h	0.10±0.083 abc	5±0.4 h
10 µM	*m*T	6.3±0.94 ab	4.2±0.53 bc	2.88±0.173 a	135.8±9.18 a	0.33±0.083 abcde	29±3.7 a
	*m*TR	4.0±0.58 bc	4.2±0.33 bc	1.13±0.157 de	60.2±8.25 fgh	0.01±0.014 a	16±2.0 defg
	Me*m*T	7.1±1.17 a	4.7±0.84 b	2.37±0.334 abc	103.8±11.39 abcd	0.17±0.052 abcd	27±3.6 abc
	Me*m*TR	5.8±0.84 abc	3.5±0.35 bc	2.80±0.435 a	125.6±17.75 ab	0.93±0.258 g	23±2.9 abcde
	BA	3.4±0.67 cd	4.7±0.51 b	1.62±0.233 cde	91.2±8.95 bcdef	0.22±0.093 abcde	14±1.7 fg
	Me*m*TTHP	6.4±1.00 ab	3.8±0.35 bc	2.58±0.330 ab	116.7±12.23 ab	0.46±0.130 bcde	25±2.6 abcd
20 µM	*m*T	5.5±0.80 abc	3.7±0.32 bc	1.81±0.287 bcd	94.8±15.27 bcde	0.15±0.070 abcd	21±2.6 bcdef
	*m*TR	5.8±1.04 abc	4.5±0.59 bc	2.14±0.214 abc	93.3±7.79 bcde	0.20±0.097 abcde	23±3.3 abcd
	Me*m*T	5.0±0.58 abc	4.0±0.34 bc	2.69±0.211 a	116.2±8.11 ab	0.52±0.129 cdef	21±1.9 bcdef
	Me*m*TR	5.3±0.79 abc	4.4±0.60 bc	2.55±0.264 ab	112.8±10.26 abc	0.23±0.111 abcde	23±2.7 abcd
	BA	4.3±0.59 bc	4.3±0.32 bc	2.17±0.214 abc	98.4±6.17 bcde	0.43±0.079 abcde	18±2.0 defg
	Me*m*TTHP	5.4±0.96 abc	3.6±0.36 bc	1.67±0.218 cde	77.2±10.33 defgh	0.36±0.109 abcde	20±2.2 cdef
30 µM	*m*T	7.3±1.00 a	2.9±0.17 c	2.79±0.244 a	122.6±10.89 ab	0.40±0.122 abcde	28±3.4 ab
	*m*TR	3.8±0.74 bc	4.2±0.42 bc	1.24±0.188 de	66.4±8.33 efgh	0.04±0.034 ab	15±2.0 efg
	Me*m*T	6.2±0.78 ab	3.5±0.40 bc	2.63±0.198 ab	81.3±6.61 cdefg	0.94±0.235 g	22±2.0 abcdef
	Me*m*TR	7.1±0.65 a	3.0±0.28 bc	2.09±0.150 abc	107.4±7.81 abcd	0.88±0.148 fg	24±2.1 abcd
	BA	5.1±0.92 abc	3.9±0.44 bc	2.33±0.354 abc	105.7±12.40 abcd	0.64±0.172 efg	17±2.2 defg
	Me*m*TTHP	3.4±0.36 cd	3.4±0.31 bc	1.23±0.201 de	53.42±8.72 gh	0.58±0.193 defg	12±1.3 gh

Mean values ± standard error (n = 24) in the same column with different letter(s) are significantly different (*P* = 0.05) based on Duncan's multiple range test.

#Cytokinin: *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Me*m*T = *meta*-Methoxy topolin; Me*m*TR = *meta*-Methoxy topolin riboside; BA = Benzyladenine; Me*m*TTHP = *meta*-Methoxy topolin 9-tetrahydropyran-2-yl.

In view of the diverse response among the regenerants in terms of rooting during the shoot induction phase, the effect of the aromatic CKs on various rooting parameters *in vitro* was measured **(Table 3.2)**. Overall, the use of higher CK concentrations had an inhibitory effect on the number of roots produced among the CK-treated plantlets. Plantlets regenerated from 10 µM Me*m*TTHP had the highest number of roots while the same CK at 30 µM resulted in a drastic reduction in rooting. Similarly, the length of the roots exhibited the same trend but 10 µM *m*T-treated plantlets had the longest roots. The optimum fresh and dry weights were observed in 10 µM Me*m*TTHP regenerated plantlets and the lowest in the control plantlets.

All the CKs at the three tested concentrations (with exception of 10 µM BA and 30 µM Me*m*TTHP) produced a significant increase in the number of regenerated shoots compared to the control, which is a further indication of the importance and requirement of CK for the efficient micropropagation of „Williams' bananas. At equimolar concentrations, the topolins were generally superior to BA for most of the evaluated parameters such as number of shoots and leaves. The superiority of topolins over BA have been mainly linked to their structural advantage (presences of hydroxyl groups) which increases the chances of the formation of *O*-glucosides during CK metabolism **(Bairu et al., 2011b)**. Instead of *O*-glucosides, the *N*-glucosides are the common metabolites in BA-treated plantlets and these have been implicated in the deleterious effect (for example, root inhibition and acclimatization failures) of BA in PTC **(Werbrouck et al., 1995; Valero-Aracama et al., 2010; Bairu et al., 2011b)**. In terms of abnormality index which gives an indication of the CK toxicity to the regenerated plantlets, the use of *m*TR was better than either BA or other topolins at equimolar concentrations. Interestingly, similar effects of *m*TR have been demonstrated in the micropropagation of other species such as *Barleria greenii* **(Amoo et al., 2011)** and *Aloe polyphylla* **(Bairu et al., 2007)**.

Among the numerous CKs that have been used for the micropropagation of bananas, only a few have been effective for induction and high shoot proliferation rate. For instance, the use of CKs such as Z, kinetin (KIN) and isopentenyladenine (iP) are not

recommended as they are characterized by weak CK-activity in different banana cultivars **(Arinaitwe et al., 2000; Strosse et al., 2008)**. Inevitably, the commonly used CKs for the micropropagation of bananas are BA and thidiazuron (to a lesser extent). In view of the detrimental effects and limitations of both CKs, the suitability of topolins as a replacement in the micropropagation of bananas **(Bairu et al., 2008)** and plantain **(Escalona et al., 2003; Roels et al., 2005)** have been investigated. The potential of the topolins in *Musa* spp. micropropagation has been highlighted by the aforementioned researchers. As a continuation of a previous study **(Bairu et al., 2008)**, the efficacy of *m*T and its derivatives on fresh „Williams' banana cultures was verified in this study. In addition, Me*m*TTHP (a new derivative) and Me*m*T exhibited a relative better shoot proliferation compared to BA at 10 and 20 µM. The new derivative was synthesized in an attempt to improve the biological activity of the parent structure and was demonstrated to either maintain or increase CK activity when tested in three classical CK bioassays **(Szücová et al., 2009)**. Consequently, the authors attributed the better efficiency of Me*m*TTHP to its longer lifespan as well as availability in the plant tissues. When a high concentration of Me*m*TTHP was used in the current study, there was a reduction in the shoot number which could be due to inhibitory effects of the excess CK pool in the plant tissue. Although high concentrations of adenine-based CKs are commonly required for efficient shoot multiplication in bananas **(Vuylsteke, 1998; Venkatachalam et al., 2007b; Strosse et al., 2008)**, optimum mean shoot number per explant with Me*m*T and Me*m*TTHP was attained using the lowest tested concentration (10 µM) in the present study.

Table 3.2: Effect of six aromatic cytokinins at different concentrations on the rooting parameters of micropropagated „Williams' bananas during the shoot proliferation phase

Concentration	#Cytokinin	Number	Length (cm)	Fresh weight (g)	Dry weight (mg)
0	Control	7.7±0.88 fghi	7.8±1.31 ab	0.5±0.08 e	23.4±3.69 e
10 µM	*m*T	25.3±4.98 ab	8.3±0.83 a	1.7±0.25 ab	86.6±11.27 abc
	*m*TR	8.4±1.73 fgh	3.0±0.51 fg	0.5±0.08 e	37.3±6.21 e
	Me*m*T	19.8±2.61 bc	7.0±0.56 abcd	1.2±0.15 bc	67.3±6.74 bcd
	Me*m*TR	10.8±2.07 defg	4.9±0.82 def	1.5±0.25 abc	80.3±10.67 abc
	BA	19.0±2.74 bc	7.1±0.64 abc	1.3±0.13 abc	74.7±7.73 abc
	Me*m*TTHP	26.9±3.30 a	7.9±0.88 ab	1.8±0.20 a	95.9±12.30 a
20 µM	*m*T	9.3±2.62 defgh	3.2±0.66 fg	1.1±0.17 cd	60.6±8.33 cd
	*m*TR	4.8±1.30 ghi	1.9±0.56 gh	0.7±0.09 de	48.1±5.63 de
	Me*m*T	10.7±2.44 defg	4.1±0.63 ef	1.2±0.12 bc	69.3±6.96 bcd
	Me*m*TR	13.8±2.55 cdef	4.1±0.48 ef	1.3±0.13 bc	72.6±7.51 abc
	BA	16.7±1.85 cd	6.5±0.53 abcd	1.5±0.14 abc	83.4±5.63 abc
	Me*m*TTHP	9.9±2.34defgh	3.3±0.91 fg	1.2±0.15 bc	78.6±9.13 abc
30 µM	*m*T	16.3±2.49 cde	5.2±0.75 cdef	1.5±0.15 abc	80.7±8.07 abc
	*m*TR	3.6±0.69 ghi	1.6±0.23 gh	0.5±0.06 e	35.6±4.14 e
	Me*m*T	8.8±2.22 efgh	3.4±0.55 fg	1.4±0.17 abc	30.0±2.80 e
	Me*m*TR	2.9±0.79 hi	1.3±0.31 gh	0.6±0.05 e	84.5±9.49 abc
	BA	14.4±2.13 cdef	5.9±0.77 bcde	1.7±0.21 ab	92.1±8.09 ab
	Me*m*TTHP	1.0±0.33 i	0.6±0.26 h	0.6±0.15 e	34.7±7.63 e

Mean values ± standard error (n = 24) in the same column with different letter(s) are significantly different (*P* = 0.05) based on Duncan's multiple range test.
#Cytokinin: *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Me*m*T = *meta*-Methoxy topolin; Me*m*TR = *meta*-Methoxy topolin riboside; BA = Benzyladenine; Me*m*TTHP = *meta*-Methoxy topolin 9-tetrahydropyran-2-yl.

Table 3.3: Effect of five rooting compounds on the rooting and production of new off-shoots frequencies (%) of different cytokinin-derived shoots of „Williams' bananas

Type and concentration of cytokinin used for shoot regeneration	#Rooting compound	Rooting frequency (%)	New off-shoots regenerated (%)
meta-Topolin	Control	73	0
(*m*T) – 30 μM	IAA	100	20
	IBA	69	8
	NAA	87	40
	KAR_1	93	33
	SW	85	0
meta-Topolin riboside	Control	100	0
(*m*TR) – 20 μM	IAA	87	7
	IBA	100	33
	NAA	87	0
	KAR_1	83	8
	SW	83	8
meta-Methoxy topolin	Control	69	15
(Me*m*T) – 10 μM	IAA	67	7
	IBA	73	7
	NAA	93	13
	KAR_1	73	13
	SW	60	0
meta-Methoxy topolin riboside	Control	58	17
(Me*m*TR) – 30 μM	IAA	93	13
	IBA	75	33
	NAA	67	33
	KAR_1	79	14
	SW	87	13
Benzyladenine	Control	83	0
(BA) – 30 μM	IAA	83	8
	IBA	73	0
	NAA	83	0
	KAR_1	60	0
	SW	87	13
meta-Methoxy topolin 9-tetrahydropyran-2-yl	Control	92	8
(Me*m*TTHP) – 10 μM	IAA	100	8
	IBA	83	0
	NAA	93	13
	KAR_1	80	7
	SW	83	0

#Rooting compound: IAA = Indole-3-acetic acid; IBA = Indole-3-butyric acid; NAA = Naphthalene acetic acid; KAR_1 = Karrikinolide and SW = Smoke-water.
Concentration used: IAA, IBA and NAA = 1 μM; KAR_1 = 4.8 X 10^{-22} M and SW = 1:1000 dilution.

3.3.3 Effect of the aromatic cytokinins on *in vitro* rooting and acclimatization

Overall, the type of CK from which the shoots were generated as well as the type of rooting compound had a diverse effect on the various rooting parameters recorded. A high root regeneration rate that ranged from 60-100% was observed across all the treatments **(Table 3.3)**. In addition, the controls, which were devoid of any exogenously applied rooting compound had frequencies that ranged from 58-100%. During the rooting phase, shoots regenerated from *meta*-topolin and its derivatives had more off-shoots compared to those from BA. Particularly, the production of new off-shoots was more prominent in the presence of IBA in Me*m*TR- and *m*TR-derived shoots, KAR_1 in *m*T-derived and NAA in *m*T- and Me*m*TR-derived shoots.

Figure 3.4 illustrates the effect of the tested rooting compounds on the number of roots produced in the micropropagated „Williams' bananas. In most cases **(Figure 3.4A, B, C and E)**, SW and KAR_1 stimulated the production of more roots compared to the tested auxins. Apart from *m*T- and BA-obtained shoots **(Figure 3.4A and E)**, the different rooting compounds had no significant effect on increasing the quantity of roots observed on shoots regenerated from other CKs compared to the control. In *m*T-derived shoots for instance, the number of roots were significantly higher in IBA, SW and KAR_1 treatments compared to the control **(Figure 3.4A)**. Similarly, BA-derived plantlets produced more roots in the presence of the rooting compounds (IAA, SW and KAR_1) compared to the control treatment **(Figure 3.4E)**. Generally, there was no significant increase in the length of the regenerated roots across all the treatments **(Figure 3.5)**. In Me*m*TR-derived plantlets, the root length in the control treatment was significantly higher than the five different rooting compound treatments **(Figure 3.5D)**. Conversely, the roots in KAR_1-treated (BA- and Me*m*TTHP-derived) plantlets were significantly longer than with some rooting compound-treated and control plantlets **(Figure 3.5E** and **F)**. Overall, more than 90% of the *in vitro* rooted-plantlets were successfully acclimatized under the *ex vitro* conditions.

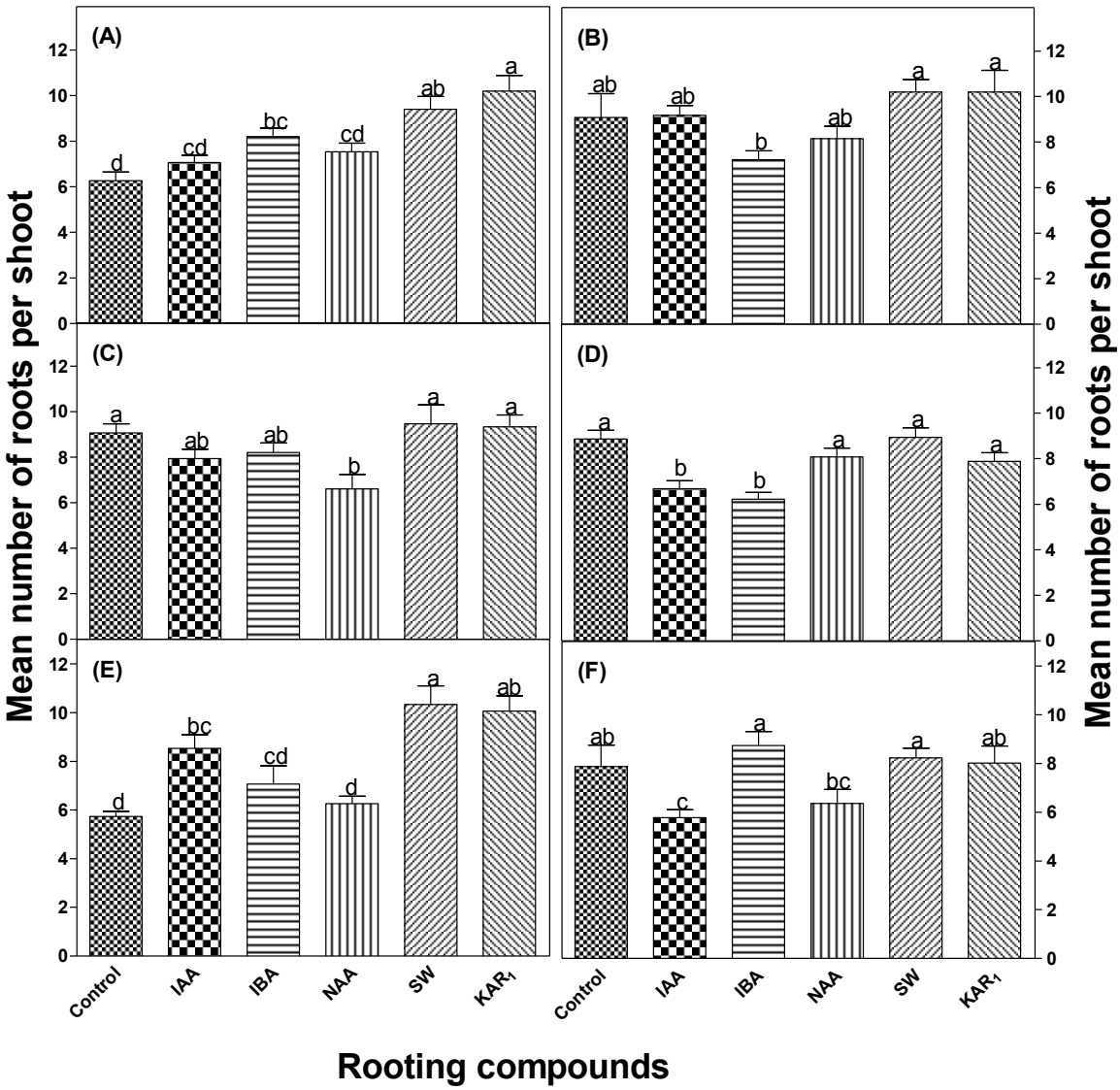

Figure 3.4: Effect of rooting compounds on the number of regenerated roots. A= *meta*-Topolin; B = *meta*-Topolin riboside; C = *meta*-Methoxy topolin; D = *meta*-Methoxy topolin riboside; E = Benzyladenine and F = *meta*-Methoxy topolins 9-tetrahydropyran-2-yl. IAA = Indole-3-acetic acid; IBA = Indole-3-butyric acid; NAA = Naphthalene acetic acid; SW = Smoke-water; KAR_1 = Karrikinolide. Concentrations of root inducing compounds: IAA, IBA and NAA = 1 µM; KAR_1 = 4.8 X 10^{-22} M and SW = 1:1000 dilution. In each graph, bars with different letter(s) are significantly different (*P* = 0.05) according to Duncan's multiple range test.

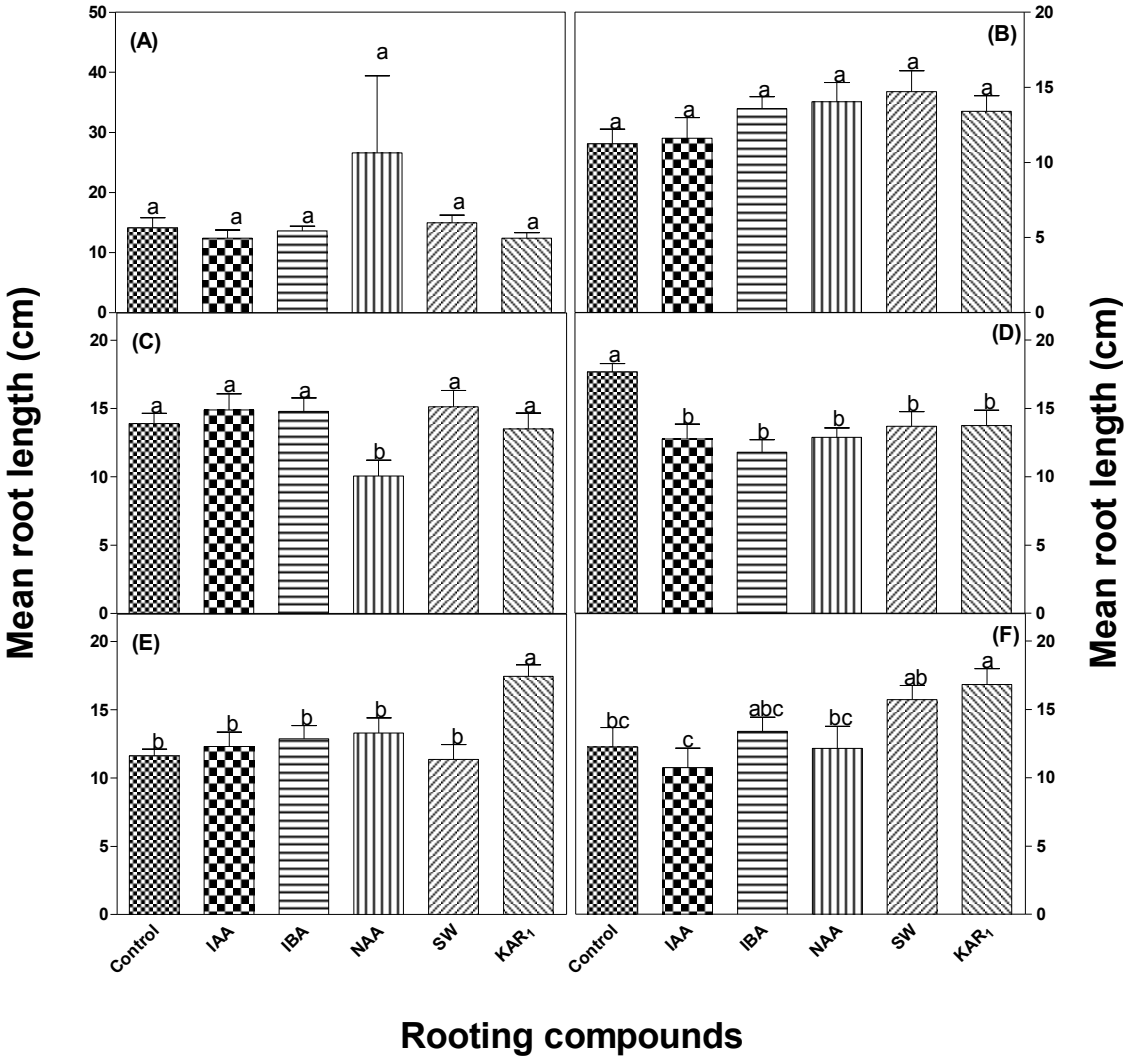

Figure 3.5: Effect of rooting compounds on the length of regenerated roots. A= *meta*-Topolin; B = *meta*-Topolin riboside; C = *meta*-Methoxy topolin; D = *meta*-Methoxy topolin riboside; E = Benzyladenine and F = *meta*-Methoxy topolin 9-tetrahydropyran-2-yl. IAA = Indole-3-acetic acid; IBA = Indole-3-butyric acid; NAA = Naphthalene acetic acid; SW = Smoke-water; KAR_1 = Karrikinolide. Concentrations of root inducing compounds: IAA, IBA and NAA = 1 µM; KAR_1 = 4.8 X 10^{-22} M and SW = 1:1000 dilution. In each graph, bars with different letter(s) are significantly different (*P* = 0.05) according to Duncan's multiple range test.

In vitro rooting is mainly controlled by the levels of both exogenous and endogenous auxins in plant tissue **(De Klerk et al., 1999)**. Despite the absence of exogenous auxins in the shoot multiplication medium, the CK-treated plantlets still produced roots. In fact, 10 µM Me*m*TTHP-treated plantlets had approximately 3.5-fold more roots than the control. It is possible that the addition of CKs to the medium stimulated the production of endogenous auxins in the regenerated plantlets resulting in the observed roots. However, higher concentrations of CK in the plantlets were detrimental to rooting as observed in the drastic reduction in number of produced roots across all the treatments. Rooting inhibition at higher concentrations for both BA and topolins in several micropropagated species is well documented **(Escalona et al., 2003; Bairu et al., 2008; Valero-Aracama et al., 2010)**. Even though the existence of synergistic, antagonistic and additive interactions between auxin and CK (endogenous and exogenous) is well-known, the underlying mechanisms are complex and remain to be fully understood **(Nordström et al., 2004)**.

During the rooting stage, the presence of different rooting compounds produced diverse responses in the evaluated rooting parameters of the „Williams' banana plantlets. Although rooting frequency was high irrespective of the type of CK from which the shoots were obtained or the type of rooting compounds in the medium, the topolins (particularly, Me*m*T and Me*m*TR treatments) had higher off-shoot production frequency compared to BA-derived shoots. Clearly, the topolins maintained a stronger positive carry-over effect that resulted in production of more shoots in the rooting medium which was devoid of any exogenous CK. The production of the extra off-shoots during the rooting stage inevitably contributes to the overall shoot multiplication potency of the topolins for the micropropagation of „Williams' bananas.

In addition to the exogenously applied auxins, the rooting ability of *in vitro* plantlets is influenced by the endogenous auxin levels as well as the presence of an efficient transport mechanism **(De Klerk et al., 1999; Fogaça and Fett-Neto, 2005)**. Furthermore, the efficiency of different rooting compounds depends on their specific affinities for auxin receptor molecules and the concentration of free auxin reaching

target cells **(De Klerk et al., 1999)**. In this study, the five rooting compounds generally had no significant effect on the number of roots produced in *m*TR-, Me*m*T-, Me*m*TR- and Me*m*TTHP-derived shoots compared to the control. In fact, some of the auxins such as IAA and NAA slightly inhibited the number and length of roots produced when compared to the control treatment. Similarly, **Ascough et al. (2011)** observed that the presence of different auxins (IAA, IBA and NAA) at varying concentrations inhibited root elongation in micropropagated *Sisyrinchium laxum*. In particular, the negative effect of NAA on rooting has been attributed to its longer persistence in plant tissue where it remains in free form and blocks root emergence **(Fogaça and Fett-Neto, 2005)**. On the other hand when compared to the control, current findings indicate a significant increase in number of roots due to IBA, SW and KAR_1 treatments in *m*T-derived shoots as well as IAA, SW and KAR_1 treatments in BA-derived shoots. In addition to IBA and IAA which are frequently used in PTC **(De Klerk et al., 1999)**, the auxin-like activity of SW and KAR_1 is a further indication of the great potential of both compounds as a substitute for commonly used rooting compounds. Evidence demonstrating the auxin- and CK-like activity of both SW and KAR_1 in PTC have been reported **(Jain et al., 2008)**. A significant increase in root length was achieved with the use of KAR_1 on BA- and Me*m*TTHP-derived shoots.

3.3.4 Effect of the cytokinins on phenolic compound accumulation

Overall, total phenolics and flavonoids were higher in the aerial parts compared to underground parts **(Table 3.4)**. However, total phenolic content was higher in the underground parts in 30 µM Me*m*TTHP treatment. In terms of the proanthocyanidin levels, the underground parts generally had an higher content with the exception of 20 and 30 µM *m*TR regenerants. Treatments with *m*T and Me*m*TTHP indicated that increasing the concentration of these CKs decreased the quantity of total phenolic and flavonoid contents in aerial parts. On the other hand, an increase in quantity of total phenolics and flavonoids was observed with higher concentrations of Me*m*TR (aerial and underground parts). Likewise, higher concentrations of Me*m*T produced more proanthocyanidins in the underground parts.

Table 3.4: Effect of aromatic cytokinins on the accumulation of phenolic compounds in micropropagated „Williams' bananas

[#]Cytokinin treatment		[1]Total phenolics (mg GAE /g DW)		[2]Total flavonoids (mg CE /g DW)		[3]Proanthocyanidins (µg CCE /g DW)	
		Aerial part	Underground part	Aerial part	Underground part	Aerial part	Underground part
0	Control	25.9±0.73 de	7.5±0.54 i	25.5±0.18 d	5.67±0.55 l	448.0±21.56 def	617.3±41.87 fg
10 µM	*m*T	33.7±2.10 a	16.6±0.53 e	25.4±0.53 d	12.9±0.18 g	643.8±21.12 abcd	3363.5±13.38 a
	*m*TR	28.5±1.01 c	11.4±.18 g	24.8±0.20 d	8.0±0.08 j	495.6±12.81 bcdef	711.2±11.13 efg
	Me*m*T	14.4±0.61 i	10.6±0.20 gh	12.1±0.18 k	8.1±0.07 j	335.5±32.34 ef	707.2±13.52 efg
	Me*m*TR	17.5±0.40 gh	14.4±0.55 f	13.9±0.14 j	11.7±0.14 h	603.1±10.20 bcd	947.1±39.34 def
	BA	31.7±0.64 ab	17.7±1.25 de	29.07±0.22 b	15.1±0.04 ef	484.7±89.53 cdef	1269.0±273.99 cd
	Me*m*TTHP	33.4±1.01 a	21.7±1.36 b	30.1±0.24 a	18.8±0.39 b	555.0±15.56 bcdef	1827.4±156.13 b
20 µM	*m*T	27.0±0.37 cd	12.3±0.24 fg	22.8±0.34 e	9.0±0.05 i	656.86±67.19 abcd	745.7±13.38 efg
	*m*TR	16.5±0.55 hi	12.2±0.23 fg	11.2±0.07 l	9.5±0.10 i	478.6±25.19 cdef	384.2±21.61 g
	Me*m*T	20.7±0.56 f	17.9±0.19 de	15.5±0.68 i	14.9±0.08 f	702.68±86.83 abc	781.3±11.44efg
	Me*m*TR	23.6±0.35 e	19.7±0.74 bcd	20.3±0.26 f	15.9±0.08 d	425.5±1.50 def	659.7±49.91 efg
	BA	24.2±0.97 e	8.0±0.58 i	20.68±0.10 f	4.0±0.05 m	327.58±3.39 f	562.4±7.22 fg
	Me*m*TTHP	30.9±0.13 b	11.8±0.61 g	26.6±0.45 c	7.4±0.10 k	832.8±12.6 a	1028.6±39.77 def
30 µM	*m*T	19.4±1.16 fg	18.9±0.24 cde	16.7±0.16 h	14.7±0.14 f	360.4±4.83 ef	1119.1±47.05 cde
	*m*TR	19.2±0.18 fg	7.7±0.20 i	14.7±0.04 ij	4.5±0.05 m	617.25±21.74 abcd	576.5±23.28 fg
	Me*m*T	11.8±0.27 j	8.5±0.39 hi	9.25±0.20 m	5.2±0.12 l	722.49±240.55 ab	1537.8±510.55 bc
	Me*m*TR	24.3±1.43 e	21.2±0.26 bc	20.26±0.38 f	17.2±0.15 c	549.36±6.30 bcdef	807.9±10.23 efg
	BA	25.1±0.39 de	19.7±0.50 bcd	17.9±0.42 g	15.5±0.13 de	563.5±6.86 bcde	1271.85±41.59 cd
	Me*m*TTHP	24.8±0.46 de	27.9±2.14 a	22.4±0.04 e	21.6±0.14 a	648.9±81.60 abcd	772.27±55.22 efg

Mean values ± standard error (n = 6) in the same column with different letter(s) are significantly different (*P* = 0.05) based on Duncan's multiple range test.

[#]Cytokinin treatment: *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Me*m*T = *meta*-Methoxy topolin; Me*m*TR = *meta*-Methoxy topolin riboside; BA = Benzyladenine; Me*m*TTHP = *meta*-Methoxy topolin 9-tetrahydropyran-2-yl.

[1]GAE = Gallic acid equivalents; [2]CE = Catechin equivalents; [3]CCE = Cyanidin chloride equivalents.

Generally, *m*T and Me*m*TTHP stimulated higher phytochemical production compared to BA-treated and control plantlets. Notably, *m*T and Me*m*TTHP at 10 µM increased the proanthocyanidin content in the underground part by 5- and 3-fold, respectively, compared to the controls. Furthermore, the same concentration of BA yielded approximately 2.7-fold lower content compared to *m*T. Similarly, Me*m*TTHP plantlets had 1.4- and 3.7-fold more total phenolics than the corresponding BA and control ones, respectively, in the underground parts.

The regulatory role of CKs on different physiological and developmental processes is well documented **(Werner et al., 2001; Criado et al., 2007)**. Studies have shown that PGR's signaling pathways are not isolated, they are interconnected with a complex regulatory network involving various defence signalling pathways and developmental processes **(Bari and Jones, 2009)**. In recent times, there have been a great deal of evidence on the increasing superiority of topolins over the commonly used CKs such as BA, Z and KIN in PTC **(Aremu et al., 2012)**. In addition, this study demonstrated the better stimulatory effects of topolins (*m*T and Me*m*TTHP) on phenolic content in „Williams' banana compared to BA. Generally, secondary metabolites such as phenolics and flavonoids play vital roles during the micropropagation of several species. The diverse functions of these compounds have been investigated in detail by several researchers in PTC **(Wu et al., 2007; Buer et al., 2010; De Klerk et al., 2011)**. As postulated by **Curir et al. (1990)** and **Buer et al. (2010)**, rooting is enhanced in the presence of certain types of flavonoids. The high accumulation of flavonoids could have accounted for the observed better *ex vitro* rooting in *m*T and Me*m*TTHP regenerated plantlets. The current finding demonstrates the advantage of Me*m*TTHP over BA in that the formation of the metabolite 6-benzylamino-9-*β*-D-glucopyranosylpurine, known for inhibition of rooting and associated abnormalities **(Bairu et al., 2011b)**, is blocked due to the conjugation of this molecule at the N^9 position **(Szücová et al., 2009)**. In the current study, BA negatively affected rooting as indicated in the number and length of the roots compared to the topolins. The detrimental effect of BA during the acclimatization of micropropagated plants is well-documented **(Werbrouck et al., 1995; Baroja-Fernández et al., 2002; Valero-Aracama et al., 2010; Bairu et al., 2011b)**.

Apparently, the accumulation of the more stable and toxic metabolite 6-benzylamino-9-*β*-D-glucopyranosylpurine mainly in the underground or basal regions results in these acclimatization problems in several plant species. Among the tested CKs, Me*m*TTHP consistently remained the most promising by stimulating rooting and subsequently better acclimatization in the micropropagated „Williams' bananas. If confirmed with more similar studies using different plant species, Me*m*TTHP would be an ideal CK for other plants with rooting and acclimatization problems.

The numerous advantages associated with the use of PTC for production of secondary metabolites remain of major interest to researchers **(Ramachandra Rao and Ravishankar, 2002)**. These authors highlighted the regulatory role of different CKs in the accumulation of secondary metabolites. The type and concentration of CKs affect the quantity of secondary metabolites produced. In *Hypericum perforatum* for example, BA-treated plantlets accumulated more hypericins compared to zeatin-treated ones **(Liu et al., 2007)**. On the other hand, there was a reduction in the phenolic content of micropropagated *Tectona grandis* with an increase in BA concentrations **(Quiala et al., 2012)**. The current findings demonstrate varying levels of the quantified phenolic compounds due to different CK types as well as concentration in micropropagated bananas. The topolins particularly *m*T and Me*m*TTHP increased total phenolics, flavonoids and proanthocyanidins in both underground and aerial parts of regenerated banana plantlets.

Plant survival depends on their interaction with the environment aided by their secondary metabolite content. During stress, the phenylpropanoid pathway is of critical importance as its products (phenolic compounds) protect the plant against abiotic and biotic factors **(Dixon and Paiva, 1995)**. Evidence has shown that phenylpropanoid-based polymers such as lignin, and proanthocyanidin, contribute substantially to the stability and robustness of higher plants towards mechanical or environmental damage **(Vogt, 2010)**. Generally, phenolic compounds are essential in UV protection and modulating the levels of reactive oxygen species in plants **(Peer and Murphy, 2007; Buer et al., 2010)**. Flavonoids have been shown to be polar auxin transport modulators

via different regulatory pathways **(Peer and Murphy, 2007)**. Recently, **De Klerk et al. (2011)** also reported that the presence of certain phenolic compounds protect auxins from decarboxylation which inevitably stimulated better rooting in *Malus* spp. Banana being a large herbaceous plant requires firm attachment thereby necessitating the development of a good root system. Plants regenerated from Me*m*TTHP had the best shoot/root ratio, an indication of the plantlet survival potential during unfavourable conditions such as drought **(Bernier et al., 1995)**. Furthermore, a positive correlation has been established between the host resistance and products (mainly phenolic compounds) of the phenylpropanoid pathway **(Binks et al., 1997; Collingborn et al., 2000)**. The biochemical basis for secondary metabolites and degree of resistance is not fully elucidated **(Wuyts et al., 2007)**. In addition, different PGRs play positive or negative roles against various biotrophic and necrotrophic pathogens depending on the type of plant-pathogen interactions **(Bari and Jones, 2009)**. Unfortunately, most of the underlying molecular mechanisms involved in these interactions remain poorly understood.

3.4 Concluding remarks

In conclusion, when compared to BA, the use of topolins (particularly, Me*m*TTHP) demonstrated several advantages such as higher mean shoot number per explant recorded at low CK concentrations and the ease of rooting during the shoot proliferation phase. In addition, the topolins easily produced additional off-shoots during the rooting phase compared to BA. Overall, rooting was easily achieved among the regenerated shoots from the six CK at their optimum multiplication concentrations. However, the use of rooting compounds such as IAA, IBA, SW and KAR_1 were required to significantly increase the number of roots in *m*T- and BA-derived shoots compared to the control devoid of rooting compounds. Karrikinolide was effective as it significantly improved the root length in BA- and Me*m*TTHP-regenerated shoots. In addition to *m*T and Me*m*TTHP increasing the phenolic compounds in micropropagated bananas, plantlets regenerated from most of the topolins acclimatized better than the BA-treated plantlets. Clearly, Me*m*TTHP, which is a new topolin derivative, has great promise as a CK which could be

useful to circumvent rooting inhibition and acclimatization failure in PTC. Findings from the current **Chapter** provide further evidence on the increasing importance of topolins as potential and reliable alternatives to commonly used CKs (for example, BA, KIN and Z) in PTC.

Chapter 4: Influence of *meta*-topolins on photosynthetic pigment profiles and foliar structures of micropropagated 'Williams' bananas

4.1 Introduction

The application of micropropagation techniques has witnessed major advances and numerous benefits over the last few decades **(Vasil, 2008)**. However, it is still beleaguered by a number of challenges. Researchers have continuously reviewed the subject matter and suggested means of tackling the recurrent problems that include shoot-tip necrosis **(Bairu et al., 2009c)**, hyperhydricity **(Rojas-Martínez et al., 2010)** and somaclonal variation **(Bairu et al., 2011a)**. The majority of these physiological disorders are not only limited to the period of *in vitro* growth but become more apparent during acclimatization **(Kozai, 1991; Pospíšilová et al., 2007)**. The success of plant tissue culture (PTC) particularly, on a large scale depends on how these challenges can be alleviated or possibly eradicated **(Kozai et al., 1997; Hazarika, 2006)**. Evidence has directly or indirectly implicated the photomixotrophic nature (addition of sugar into the culture medium to serve as the main source of carbon and energy for growth and development) of plant growth *in vitro*, as the major contributing factor in *in vitro*-induced abnormalities **(Kozai, 1991; Kozai et al., 1997)**. Several authors have also postulated that the presence of exogenous sugars limits photosynthesis and prevents the proper development of the photosynthetic apparatus **(Hdider and Desjardins, 1994; Koch, 1996; Rybczyński et al., 2007)**. Consequently, the use of photoautotrophic micropropagation has been suggested as an alternative means of enhancing the physiology of *in vitro* regenerants **(Xiao et al., 2011)**. Despite the substantial potentials of photoautotrophic micropropagation, the higher cost and technical complexity involved have remained major concerns and a bottleneck **(Kitaya et al., 2005; Xiao et al., 2011)**. Moreover, the addition of sugars during the culturing of some species remains critical as its exclusion from medium may be detrimental under certain *in vitro* growth conditions **(Kadleček et al., 2003; Eckstein et al., 2012)**. For instance, **Eckstein et al. (2012)** observed that sugar especially at higher concentrations act as photoprotectants for overcoming the damaging effects of strong light on the

photosynthetic apparatus. Furthermore, it may also influence photosynthesis by enhancing the growth and development of regenerants under *in vitro* conditions.

Globally, researchers have extensively reported on the various factors that facilitate better *ex vitro* acclimatization **(Hazarika, 2006; Pospíšilová et al., 2007)**. For instance, **Valero-Aracama et al. (2006)** correlated acclimatization failures to the poorly-developed photosynthetic apparatus coupled with the induced stress from the artificial environment during the micropropagation of *Uniola paniculata*. In view of the importance of sugar in the medium, it becomes pertinent to seek other ways to circumvent photomixotrophic-induced physiological problems, particularly the acclimatization failure which is partially associated to abnormalities of the photosynthetic apparatus. Thus, improving the photosynthetic parameters such as chlorophyll content and the appearance of stomata structure would ultimately enhance the *ex vitro* survival of micropropagated plants **(Van Huylenbroeck et al., 2000; Pospíšilová et al., 2007)**.

Chlorophylls and carotenoids are the basic pigments required for photosynthesis in plants **(Fraser et al., 2001)**. Although chlorophyll a and b are the major and accessory pigments, respectively, carotenoids are essential for the protection of the reaction centres from detrimental effects of excess light and oxygen **(Lichtenthaler, 1987; Young and Frank, 1996)**. As one of the important plant growth regulators (PGRs) required during PTC, cytokinins (CKs) are involved in processes such as shoot and pigment production as well as delaying senescence **(Stirk and Van Staden, 2010)**. However, the type and concentration of CKs affects the photosynthetic potential *in vitro* **(Stetler and Laetsch, 1965; Genkov et al., 1997)**. In fact, senescence, a process mainly manifested as plant pigment disintegration is highly sensitive to PGR (mainly ethylene and CKs) perturbation **(Gan and Amasino, 1997)**. The leaves are the main organs where senescence is initially prominent as a result of the gradual and significant change in cell structure including the breakdown of the chloroplast, the organelle that contains up to 70% of the leaf protein **(Gan and Amasino, 1997)**. The effect of the classical CKs such as benzyladenine (BA) and zeatin (Z) on delaying senescence has been studied in detail **(Gan and Amasino, 1996)**.

Currently, the use of topolins, especially the *meta*-forms, in PTC is increasing due to their ability to enhance general plant growth and alleviate various physiological disorders **(Aremu et al., 2012)**. The role of topolins in photosynthesis which is a fundamental physiological parameter has however, not received much attention. The few available studies examined the effect of the exogenous application of a few topolins on plant species such as wheat **(Palavan-Ünsal et al., 2002b; Palavan-Ünsal et al., 2004; Vlčková et al., 2006)**, cucumber **(Çağ et al., 2003)** and sugar beet **(Čatský et al., 1996)**. However, the existence of wide disparities between the *in vitro* and *ex vitro* environments resulting in varied structural and functional changes in regenerated plants is well-known **(Dobránszki et al., 2005)**. Consequently, stringent studies on the role of topolins on the photosynthetic pigment contents *in vitro* remain essential. Undoubtedly, such studies could provide more evidence on the beneficial effects of topolins in PTC. The current **Chapter** investigated the effect of topolins on the photosynthetic pigment contents in micropropagated „Williams' bananas over 70 days compared to BA. In addition, the resultant structural variations on the foliar surface of the micropropagated banana were examined using scanning electron microscopy (SEM).

4.2 Materials and methods

4.2.1 Cytokinin and explant sources, medium composition and growth conditions

The same types and concentrations of aromatic CKs listed in **Section 3.2.1** were tested. Sterile *in vitro* regenerated „Williams' banana plantlets obtained as described in **Section 3.2.2** and regularly subcultured at six-weekly intervals were used. Plantlets at the seventh subculture cycle were used for the current experiments. Medium composition and growth conditions were as described in **Section 3.2.2**. In addition to the control devoid of PGR, media for the treatments had CKs at 10, 20 or 30 µM.

4.2.2 Determination of photosynthetic pigment content

Chlorophyll a and b as well as the total carotenoids were quantified as outlined by **Lichtenthaler (1987)**. In brief, leaves from the micropropagated plantlets were

harvested at day 10, 20, 30, 40, 50, 60 and 70. At each interval, 0.1 g leaf samples from the individual treatments were macerated in 5 ml ice-cold acetone using a mortar and pestle with the addition of a few pinches of acid-washed sand (BDH Chemicals Ltd, England). The use of ice-cold acetone and a relative short grinding period of approximately 20-30 s were to minimize the conversion of chlorophylls to pheophytins **(Lichtenthaler, 1987)**. Thereafter, the solution was filtered through Whatman No. 1 filter paper and separated using a Benchtop centrifuge (Hettich Universal, Tuttlingen, Germany) at 5 000 rpm for 5 min at room temperature. The absorbance of the supernatant was measured at 470, 645 and 662 nm using a UV-visible spectrophotometer (Varian Cary 50, Australia) against a blank consisting of acetone. Pigment contents were evaluated and expressed in µg per g fresh weight (FW) using the formulae outlined by **Lichtenthaler (1987)**.

$$\text{Chlorophyll a } (C_a) = 11.24A_{662} - 2.04A_{645}$$
$$\text{Chlorophyll b } (C_b) = 20.13A_{645} - 4.19A_{662}$$
$$\text{Total chlorophylls} = 7.05A_{662} + 18.09A_{645}$$
$$\text{Total carotenoids} = (1000A_{470} - 1.90C_a - 63.14C_b)/214$$

4.2.3 Scanning electron microscopy of *in vitro* regenerated banana leaves

For SEM and morphometric analysis, 40-50 days old plantlets were used because micropropagated banana plantlets are usually subcultured or transferred to the rooting medium during this period. In addition, most of the CK-treated plantlets had their optimum pigment contents at this stage as evident in the current study. Leaves from individual treatments were harvested during the light period and cut into discs of 1 cm^2. The leaf discs were immediately fixed in cold 3% (v/v) glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2) for 2 h at 4 °C. After incubation, the samples were washed twice at 30 min intervals in 0.05 M sodium cacodylate buffer before dehydration in a graded ethanol series (10, 30, 50, 70, 80, 90 and 100%, v/v). The leaf discs were dried in CO_2 in an HCP-2 critical point drier (Hitachi, Japan), then mounted on aluminum SEM stubs with the abaxial side up and coated with a 20 nm layer of gold-palladium in an Eiko IB-3 ion coater (Ibaraki, Japan). Coated leaf discs were visualized in a ZEISS EVO® MA 15 SEM (Carl Zeiss SMT, Germany) fitted with

a digital imaging system driven by SmartSEM software and operating at 10.0 kV and a working distance ranging from 9.5-11.5 mm. Photomicrography of the foliar surfaces were taken at 500x and 5000x magnification.

Morphometric parameters such as stomatal density and pore area were electronically measured using the analySIS® software (Soft Imaging System, Münster, Germany). The number of stomata was expressed per unit area (mm^2). The abaxial surface was used for the examination as a result of evidence of the higher abundance and distribution of stomata **(Miguens et al., 1993; Yang and Yeh, 2008)**. Five leaf discs with 3 replicates (n = 15) were used to determine the stomatal density while the stomatal size and pore area were estimated from 14 replicates (n = 70) per treatment.

4.2.4 Data analyses

Data were subjected to one-way analysis of variance (ANOVA) using SPSS for Windows (SPSS®, version 10.0 Chicago, USA). Where there was statistical significance (P = 0.05), the mean values were further separated using Duncan's multiple range test.

4.3 Results and discussion

4.3.1 Effect of cytokinins on the photosynthetic pigment content

The patterns of chlorophyll a content for the 10, 20 and 30 µM CK treatments are presented in **Figures 4.1A, B and C,** respectively. Higher CK levels generally reduced the quantity of chlorophyll a, with the exception at day 10 (all CKs) and day 70 (Me*m*TTHP) when 20 µM treatments were higher. In addition, a prolonged culture period (after 50 days) had a detrimental effect on the chlorophyll a content. In contrast to the other CKs such as *m*T, *m*TR and BA which maintained fairly constant pigments after 50 day, Me*m*TTHP-regenerants had increased pigment content at day 70. At day 50, the control plantlets had the highest chlorophyll a content (1150 µg/g FW), which was 4.6-fold higher than at day 10. In the current study, chlorophyll a was lowest with 30 µM Me*m*TTHP (95 µg/g FW) at 10 day. With the exception of

10 and 20 µM Me*m*TTHP, chlorophyll a content in all the CK-treated plantlets was less than 1000 µg/g FW.

Chlorophyll b, total chlorophyll and carotenoid contents are depicted in **Figures 4.2 4.3** and **4.4,** respectively. Similar trends as described for chlorophyll a were also observed for chlorophyll b, total chlorophyll and carotenoids. Chlorophyll b content ranged from 22-550 µg/g FW as observed in the 30 µM Me*m*TTHP-treated (day 10) and control plantlets (day 50), respectively. In terms of total chlorophyll, plantlets cultured on 30 µM Me*m*TTHP (10 day) had the lowest pigment content (118 µg/g FW) while the highest level (1700 µg/g FW) was detected in the controls after 50 days in culture. Similarly, total carotenoids were highest in control at day 50 (310 µg/g FW) and lowest in 30 µM *m*TR (28 µg/g FW) regenerants at day 10. Generally, the quantities of both pigments were lower compared to chlorophyll a content in all the treatments.

Figure 4.5 depicts the chlorophyll a/b ratio for the CKs at the three concentrations tested. Among the CK-treated and control plantlets, the highest chlorophyll a/b ratios were observed at day 10 with the use of 10 µM CKs **(Figure 4.5A)**. Notably, all the tested CKs (with the exception of *m*TR) stimulated a distinctly higher chlorophyll a/b ratio than the control plantlets at day 10. Thereafter, chlorophyll a/b ratio in the regenerants fluctuated between 2.1 in the control (day 50) to 3.4 in 20 µM *m*TR (day 50) during the current experiment. The greenness of the regenerated plants as indicated by the total chlorophyll/carotenoid ratio is presented in **Figure 4.6**. At the initial stage (10 days), majority of the CK-treated plantlets were greener than the control plantlets, as indicated by the higher ratios (greater than 4). After 10 days and beyond, both the control and CK-treated plantlets had a ratio above 4. Even though the ratio varied throughout the culture period, the majority of the topolin (for example, *m*T, Me*m*T at 10 and 20 µM) and BA treatments were greener than the control at day 70. Overall, CKs at the highest concentrations did not improve the total chlorophyll/carotenoid ratio.

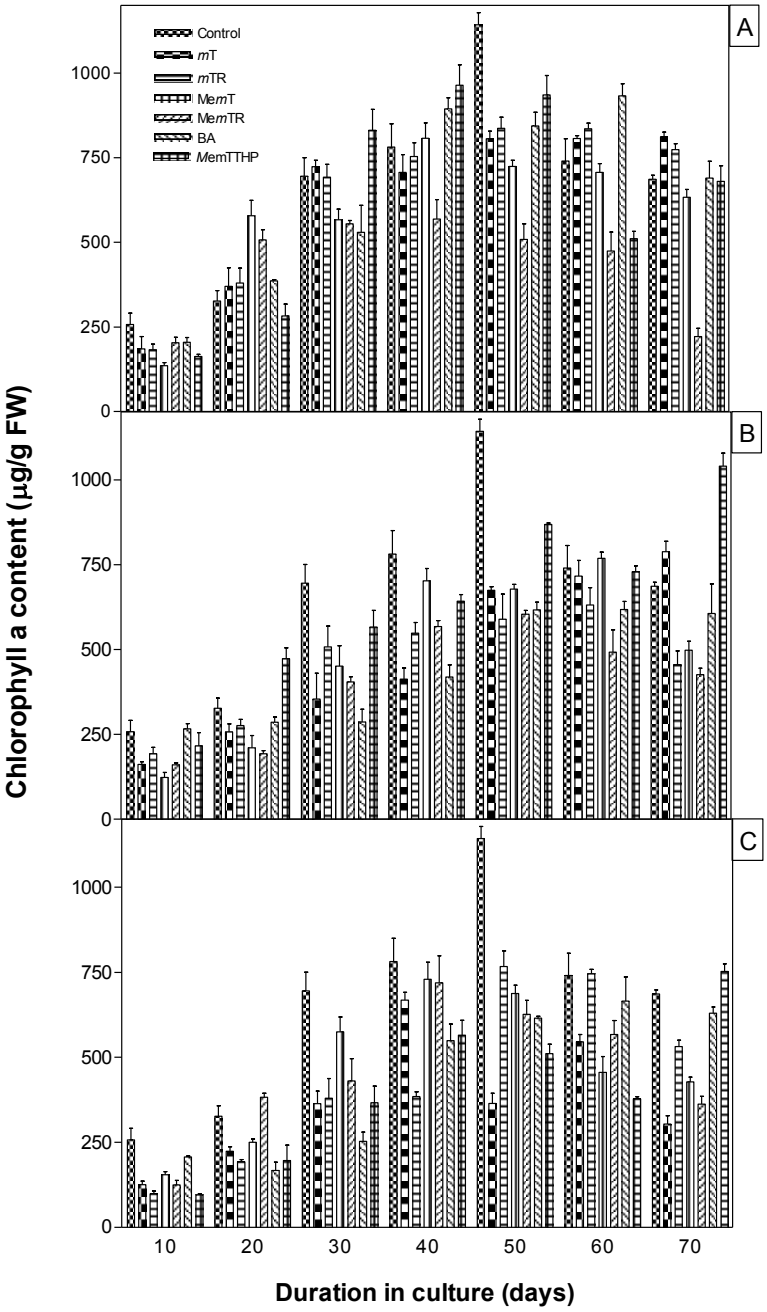

Figure 4.1: Effect of different cytokinins on the chlorophyll a content (µg/g FW) of micropropagated „Williams' bananas. A = 10 µM; B = 20 µM and C = 30 µM cytokinin treatments with each bar representing mean value (± standard error, n = 6) *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Me*m*T = *meta*-Methoxy topolin; Me*m*TR = *meta*-Methoxy topolin riboside; BA = Benzyladenine; Me*m*TTHP = *meta*-Methoxy topolin 9-tetrahydropyran-2-yl.

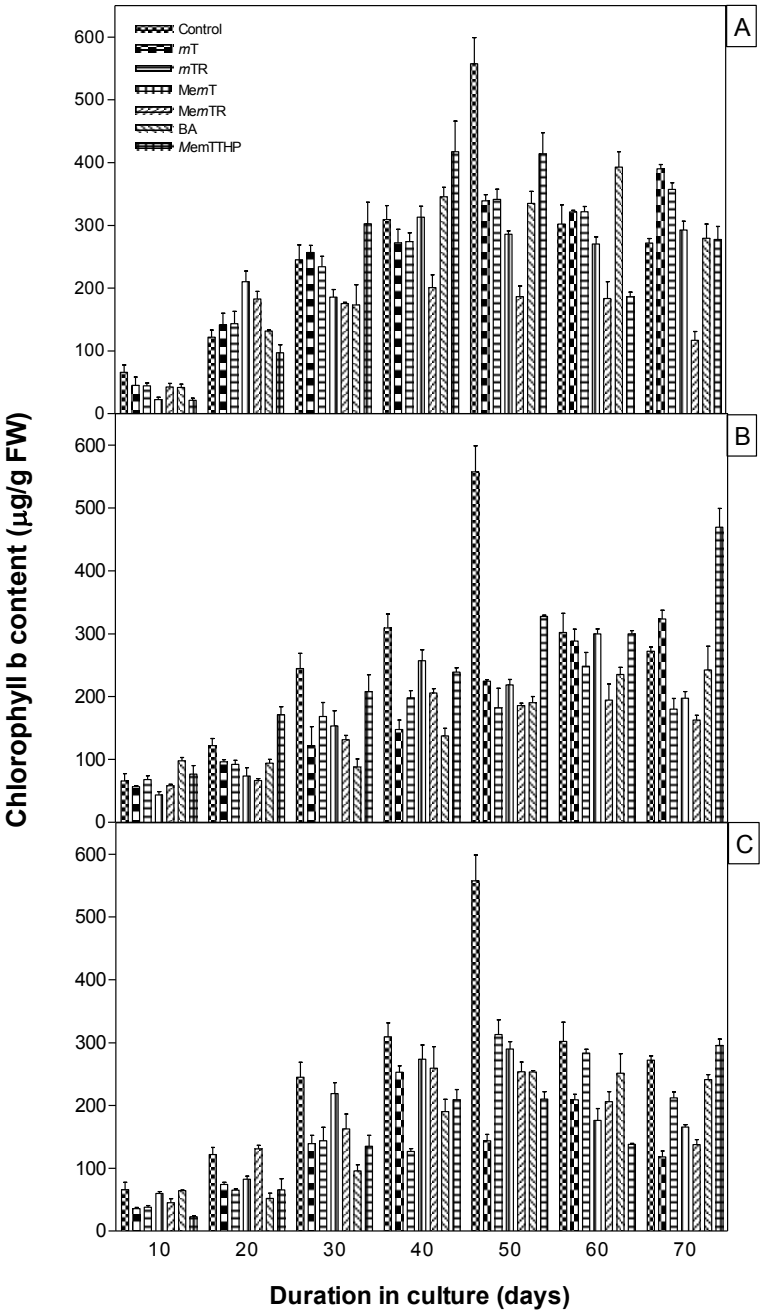

Figure 4.2: Effect of different cytokinins on the chlorophyll b content (µg/g FW) of micropropagated „Williams' bananas. A = 10 µM; B = 20 µM and C = 30 µM cytokinin treatments with each bar representing mean value (± standard error, n = 6). *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Me*m*T = *meta*-Methoxy topolin; Me*m*TR = *meta*-Methoxy topolin riboside; BA = Benzyladenine; Me*m*TTHP = *meta*-Methoxy topolin 9-tetrahydropyran-2-yl.

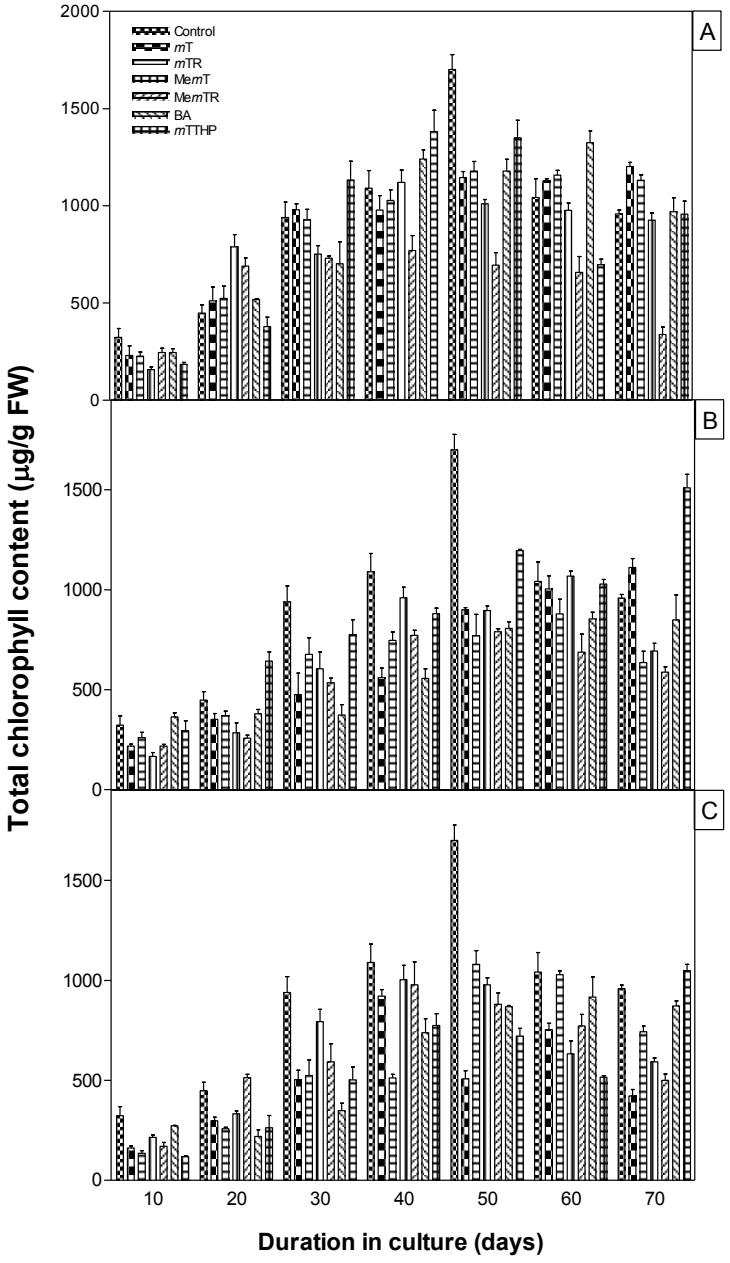

Figure 4.3: Effect of different cytokinins on the total chlorophyll content (µg/g FW) of micropropagated „Williams' bananas. A = 10 µM; B = 20 µM and C = 30 µM cytokinin treatments with each bar representing mean value (± standard error, n = 6). *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Me*m*T = *meta*-Methoxy topolin; Me*m*TR = *meta*-Methoxy topolin riboside; BA = Benzyladenine; Me*m*TTHP = *meta*-Methoxy topolin 9-tetrahydropyran-2-yl.

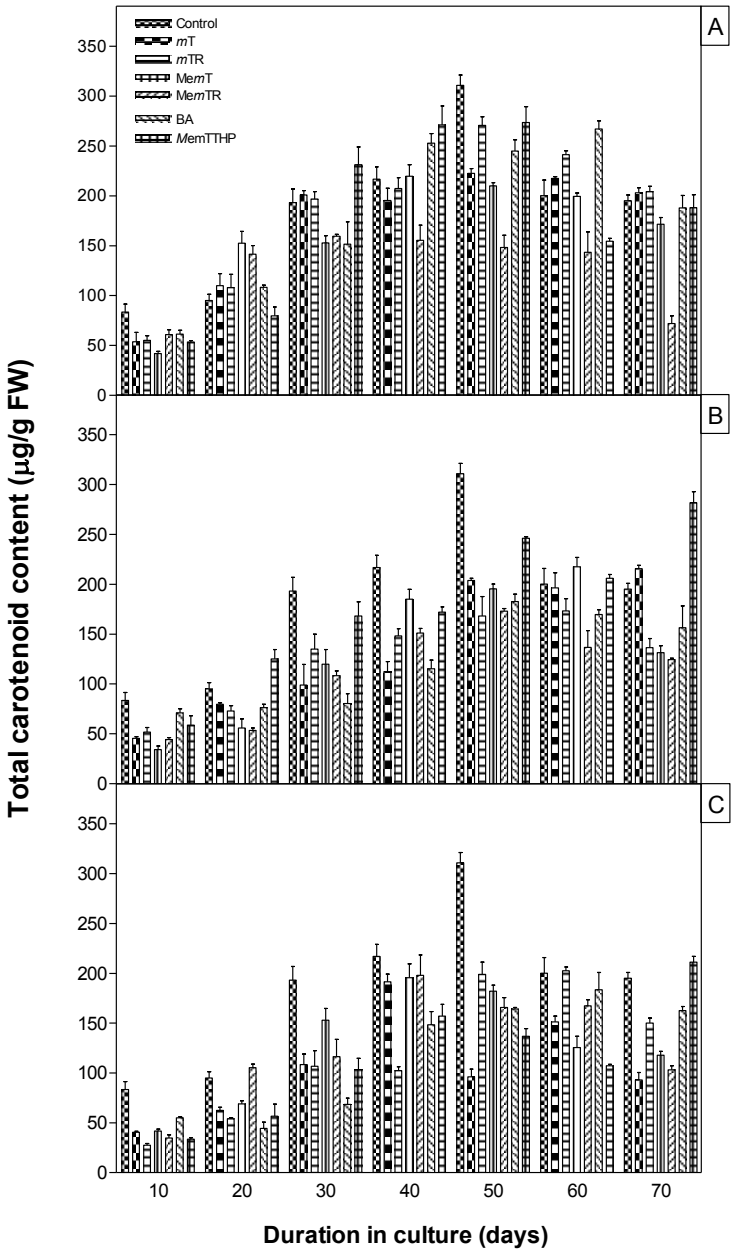

Figure 4.4: Effect of different cytokinins on the total carotenoid content (µg/g FW) of micropropagated „Williams' bananas. A = 10 µM; B = 20 µM and C = 30 µM cytokinin treatments with each bar representing mean value (± standard error, n = 6). *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Me*m*T = *meta*-Methoxy topolin; Me*m*TR = *meta*-Methoxy topolin riboside; BA = Benzyladenine; Me*m*TTHP = *meta*-Methoxy topolin 9-tetrahydropyran-2-yl.

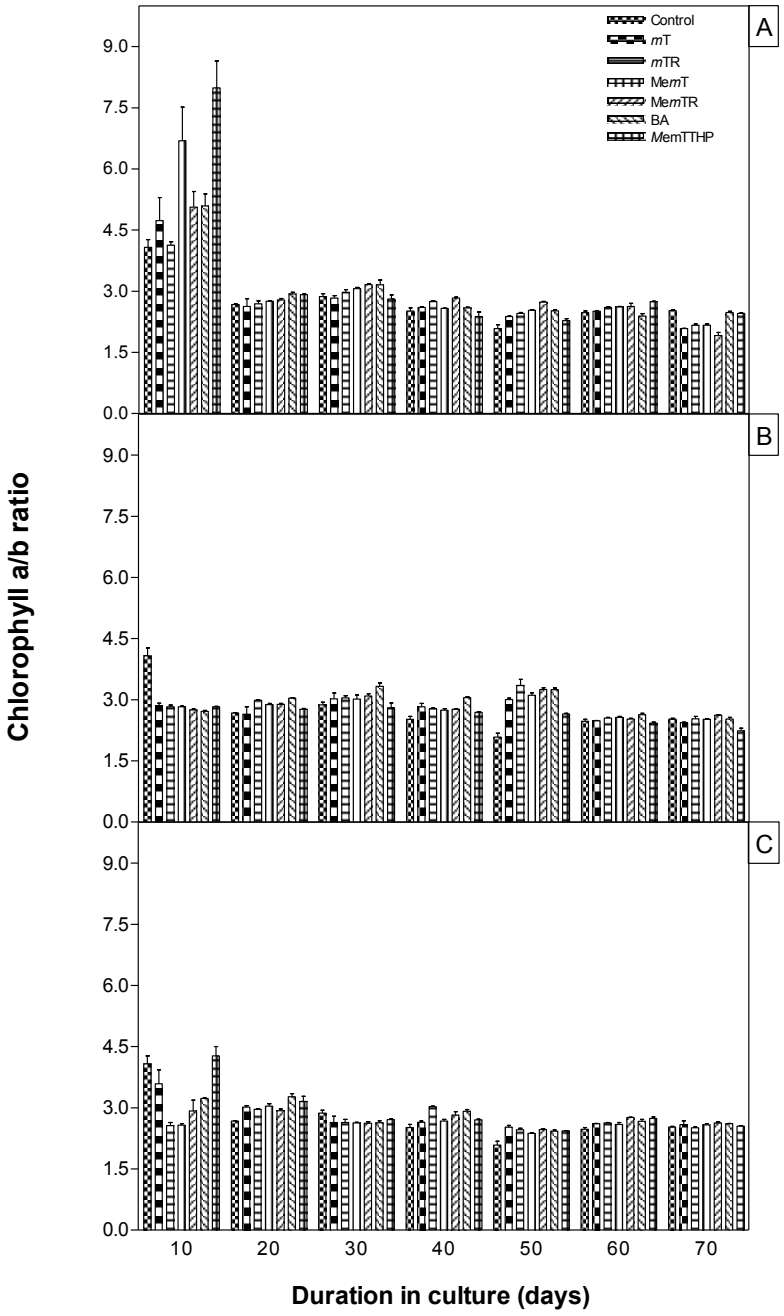

Figure 4.5: Effect of different cytokinins on the chlorophyll a/b ratio of micropropagated „Williams' bananas. A = 10 µM; B = 20 µM and C = 30 µM cytokinin treatments with each bar representing mean value (± standard error, n = 6). *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Me*m*T = *meta*-Methoxy topolin; Me*m*TR = *meta*-Methoxy topolin riboside; BA = Benzyladenine; Me*m*TTHP = *meta*-Methoxy topolin 9-tetrahydropyran-2-yl.

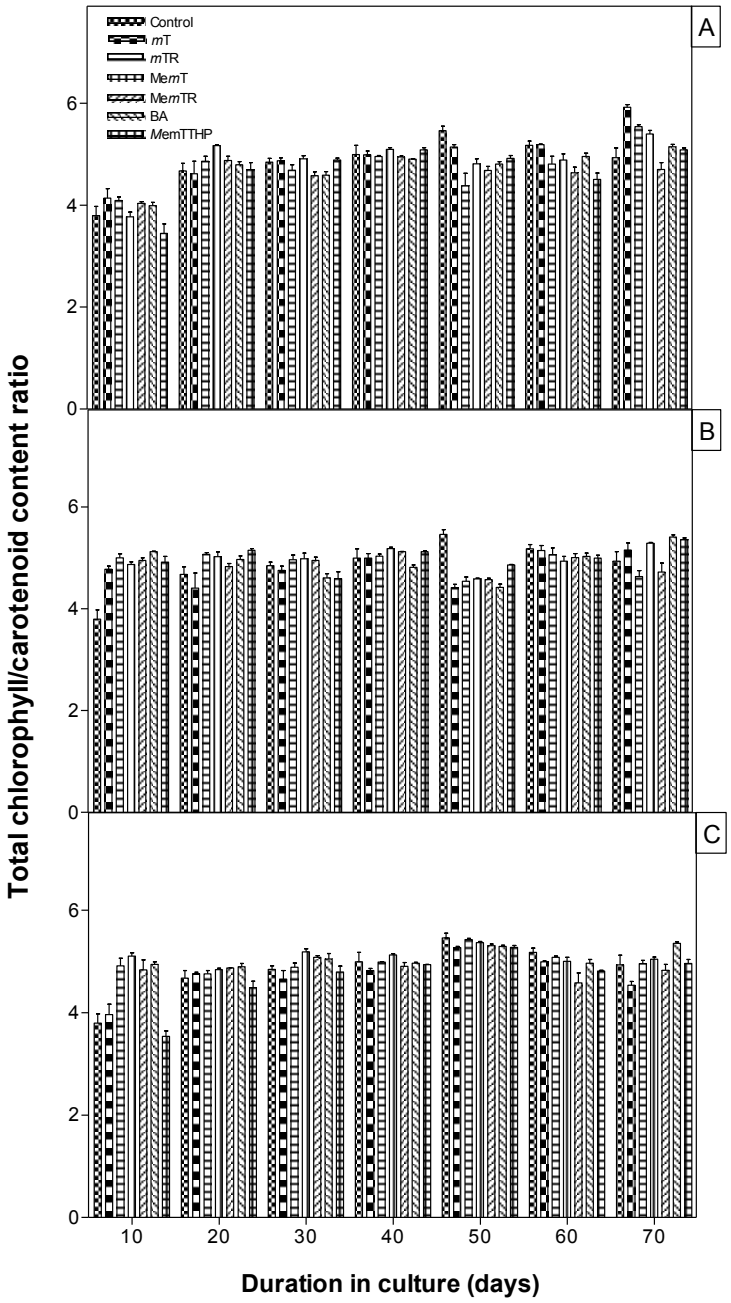

Figure 4.6: Effect of different cytokinins on the total chlorophyll/carotenoid ratio of micropropagated „Williams' bananas. A = 10 µM; B = 20 µM and C = 30 µM cytokinin treatments with each bar representing mean value (± standard error, n = 6). *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Me*m*T = *meta*-Methoxy topolin; Me*m*TR = *meta*-Methoxy topolin riboside; BA = Benzyladenine; Me*m*TTHP = *meta*-Methoxy topolin 9-tetrahydropyran-2-yl.

Cytokinins are generally known for their anti-senescence properties which is accomplished by various mechanisms at the molecular level as well as their interaction with other PGRs **(Gan and Amasino, 1997)**. However, some negative responses have also been reported. Both stimulatory and inhibitory effects of different CKs on photosynthetic pigment content have been reported in species such as *Dianthus caryophyllus* **(Genkov et al., 1997)**, *Arabidopsis thaliana* and *Daucus carota* **(Carimi et al., 2004)**. In the current study, higher CK levels reduced the pigment contents. In addition, the type of CK played a crucial role in pigment stimulation. **Szücová et al. (2009)** reported the better stability and lifespan of Me*m*TTHP due to its greater resistance to enzymatic degeneration by CK oxidase, inevitably contributing to the higher pigment content as well as stomatal frequency observed in Me*m*TTHP-treated plantlets. Contrary to the better anti-senescing activity that was observed with Me*m*TR-treated *Ros*a plants compared to *m*T or BA treatments in a study by **Bogaert et al. (2006)**, the same CK had the least pigment stimulatory effect in the current study. Thus, the type of plant species remains crucial to the effectiveness of any CK during *in vitro* propagation. As observed in this study, similar detrimental effects of higher concentrations of CKs on photosynthesis have been reported by other authors **(Carimi et al., 2003; 2004)**.

At day 10, lower quantities of chlorophyll a and total carotenoids were observed with the CK-treated plantlets compared to the control. As postulated by **Yokoyama et al. (1980)** in BA-treated *Phaseolus vulgaris*, the lower photosynthetic pigment and higher DNA contents compared to the control was associated with the preferential promotion of cell division over plastid proliferation during certain stages of plant growth. The lower amount of pigments in the CK-treated plantlets especially at day 10 could be explained thus: (i) the CKs stimulated other physiological processes such as cell division and shoot regeneration at the expense of pigment accumulation and (ii) since all the cultures were initiated at the same period, the CK-treated cultures synthesized the pigments at a slower rate than the control. The lower quantity of photosynthetic pigments after 50 days is clearly a response to depletion of medium nutrients. As commonly observed during micropropagation, the down-regulation of photosynthesis *in vitro* is probably due to depletion of CO_2 in the culture vessels **(Kozai, 1991)**. Similarly, the effect of stress on photosynthetic pigment content is well known. As recently demonstrated in nitrogen-stressed *Chlorella*

minutissima cultures, depletion of nitrogen resulted in the breakdown of the photosynthetic pigments **(Ördög et al., 2012)**.

Despite the higher amount of pigments accumulated in the control plants, the chlorophyll a/b and total chlorophyll/carotenoid ratios from all the treatments were relatively comparable. Clearly, this is an indication of distinct regulation of the amount of chlorophyll a, b and carotenoids with the individual CK treatments. Chlorophyll a is found in the reaction centre of photosystem I and II as well as in pigment antenna while chlorophyll b is mostly confined to the pigment antenna system. As a result, the chlorophyll a/b ratio gives an indication of a functional photosynthetic apparatus **(Lichtenthaler et al., 1981; Senevirathna et al., 2008)**. Photosynthetic rate depends on the amount of chlorophyll a in the plant. However, the importance of chlorophyll b cannot be under-estimated. Generally, higher chlorophyll b levels allow light interception over wider wavelength bands and improved photosynthesis via the transfer of a larger amount of energy to reaction centres **(Kitajima and Hogan, 2003)**. Consequently, the chlorophyll a/b ratio has been frequently used as an indicator of plant response to shading **(Lichtenthaler et al., 2007)**. After the prolonged culture incubation, 20 µM *m*TR treatments retained an higher chlorophyll a/b ratio compared to the control and other CK treatments.

The total chlorophyll/carotenoid ratio is directly related to the greenness of the leaves and serves as a sensitive indicator of photo-oxidative damage **(Lichtenthaler, 1987)**. Lower ratios suggest promotion of senescence and possible damage to the photosynthetic apparatus **(Lichtenthaler et al., 2007)**. As light-harvesting pigments, the synthesis and accumulation of carotenoids are vital protective mechanisms for attenuating stress caused by high irradiance **(Basanti, 1995; Veres et al., 2006)**. Carotenoids act as accessory light harvesting pigments, in which case they transfer excitation energy to chlorophyll thereby playing a crucial role during photosynthesis **(Young and Frank, 1996)**. Current findings indicate that the lowest CK concentration (10 µM) of some topolins, for example, *m*T and *m*TR had higher total chlorophyll/carotenoid ratios. It is a demonstration of the better anti-senescence ability of the tested topolins compared to BA treatment or the control at 70 day. However, BA-treated plantlets were better at higher concentrations (20 and 30 µM) during the same 70 day period.

4.3.2 Effect of cytokinins on the morphometric features of the foliar surfaces of the regenerated banana plantlets

Table 4.1 shows the effect of CKs on frequency and dimension of the stomata. An increase in the concentrations of *m*T, BA and Me*m*TTHP resulted in lower stomatal density and area. Conversely, more stomata were produced by increasing the concentration of Me*m*TR while 20 µM was the optimum concentration for maximum number of stomata for *m*TR and Me*m*T treatments. At 10 µM, *m*T and Me*m*TTHP had higher stomatal density than the control and BA regenerants **(Table 4.1** and **Figure 4.7)**. The control plantlets **(Figure 4.7A)** had fewer open stomata compared to the CK-treated ones **(Figures 4.7B, C** and **D)**. In addition, the type of CKs had a significant effect on the stomatal structures. For instance, there were major differences in the shape of the guard cells as well as pore sizes of BA, Me*m*T and Me*m*TTHP treatments compared to the control **(Figure 4.8)**. Higher concentrations of CKs resulted in bigger stomatal openings/pores in some CK treatments such as *m*TR and Me*m*T **(Table 4.1)**. Conversely, the lowest CK concentrations (for example, 10 µM Me*m*T) generally tended to have smaller pore areas **(Table 4.1** and **Figure 4.8B)**. In terms of stomatal area, 10 µM BA-treated plantlets were the largest **(Table 4.1** and **Figure 4.8A)**.

Stomata regenerated from the topolin-treated plantlets such as Me*m*TTHP and Me*m*T appear more elliptical than BA-treated plantlets. Even though culture conditions such as sugar content, temperature as well as light intensity and duration are major causes of variation in the anatomical structures of organs such as leaves **(Dobránszki et al., 2005; Vlčková et al., 2006)** and roots **(Moyo et al., 2012)**, the type and concentration of CKs remain equally critical **(Bairu et al., 2011a; Aremu et al., 2012)**. For example, **Quiala et al. (2012)** observed that increased BA concentration in the medium, induced abnormal development of the stomatal complex. The morphometric properties of the stomata and the photosynthetic apparatus are vital for survival of the *in vitro* regenerants upon transfer *ex vitro* **(Yang and Yeh, 2008)**. Generally, the abnormal stomata are often ring-shaped, raised and wide open compared to the normal ones which are elliptically-shaped. More so, the stomata remain open leading to rapid dehydration because of the excessive and uncontrolled transpirational water loss during *ex vitro* acclimatization

(Pospíšilová et al., 1999). However, it has been suggested that micropropagated plants have the potential to develop a functional photosynthetic apparatus after acclimatization under good climatic control during the initial critical days of acclimatization **(Van Huylenbroeck et al., 2000)**.

Table 4.1: Effect of type and concentration of cytokinins on morphometric parameters of foliar surfaces of regenerated banana plantlets

Conc.	#Cytokinin	ΨStomatal density (mm^2)	ΨΨStomatal area (μm^2)	ΨΨΨPore area (μm^2)
0	Control	96.9±4.73 cdefg	831.8±17.99ab	83.1±4.68 c
10 µM	*m*T	110.4±3.99 bc	517.3±26.36 e	56.4±2.90ef
	*m*TR	79.2±4.84 hijk	282.4±13.20 g	44.7±2.44fg
	Me*m*T	89.2±4.60 efghi	279.0±16.78 g	55.3±3.24ef
	Me*m*TR	64.9±2.14 k	757.6±41.46 c	48.1±3.21 fg
	BA	106.3±4.37 bcd	890.0±18.72 a	89.5±4.39 c
	Me*m*TTHP	151.0±9.80 a	713.5±21.16 c	64.7±3.53 de
20 µM	*m*T	81.9±5.67 ghi	258.1±20.16 gh	57.1±3.64 ef
	*m*TR	98.3±4.69 cdef	213.6±10.76 hi	53.9±3.04ef
	Me*m*T	110.4±4.57 bc	266.9±12.51gh	122.8±9.58 a
	Me*m*TR	92.7±4.07 defgh	149.2±6.71 j	119.0±6.13 a
	BA	91.7±7.38 defghi	752.4±18.11 c	104.6±5.22 b
	Me*m*TTHP	120.5±3.75 b	596.6±17.80 d	83.5±4.54 c
30 µM	*m*T	76.4±3.82 ijk	190.4±10.95 ij	34.2±1.94 g
	*m*TR	70.5±4.02 jk	855.1±25.27ab	127.1±6.25 a
	Me*m*T	101.4±3.01 cde	820.8±2578 b	125.1±6.61 a
	Me*m*TR	103.8±5.61 cde	586.8±15.93 d	84.2±4.22 c
	BA	83.0±3.22 fghij	496.8±13.62ef	87.7±4.63 c
	Me*m*TTHP	92.4±4.41 defgh	448.1±20.19 f	77.5±4.95 cd

#Cytokinin treatment: *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Me*m*T = *meta*-Methoxy topolin; Me*m*TR = *meta*-Methoxy topolin riboside; BA = Benzyladenine; Me*m*TTHP = *meta*-Methoxy topolin 9-tetrahydropyran-2-yl.
Mean values ± standard error (Ψn = 15, ΨΨn = 70, ΨΨΨn = 70) in the same column with different letter(s) are significantly different (*P* = 0.05) based on Duncan's multiple range test.

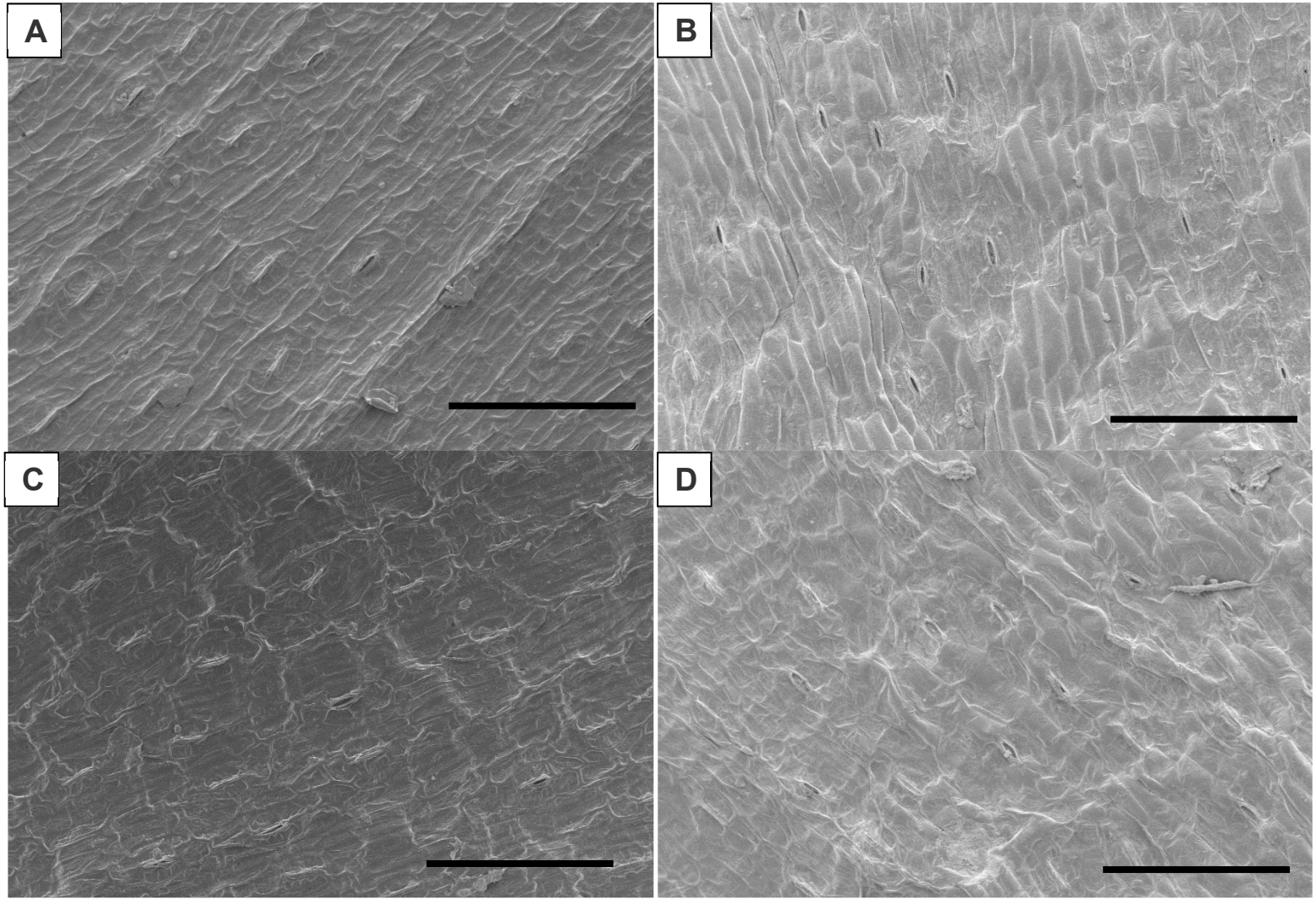

Figure 4.7: Effect of 10 µM cytokinins on the foliar surface of micropropagated „Williams' bananas. A = Control; B = *meta*-Methoxy topolin 9-tetrahydropyran-2-yl; C = Benzyladenine and D = *meta*-Topolin. Magnification = 500x, Bar = 100 µm.

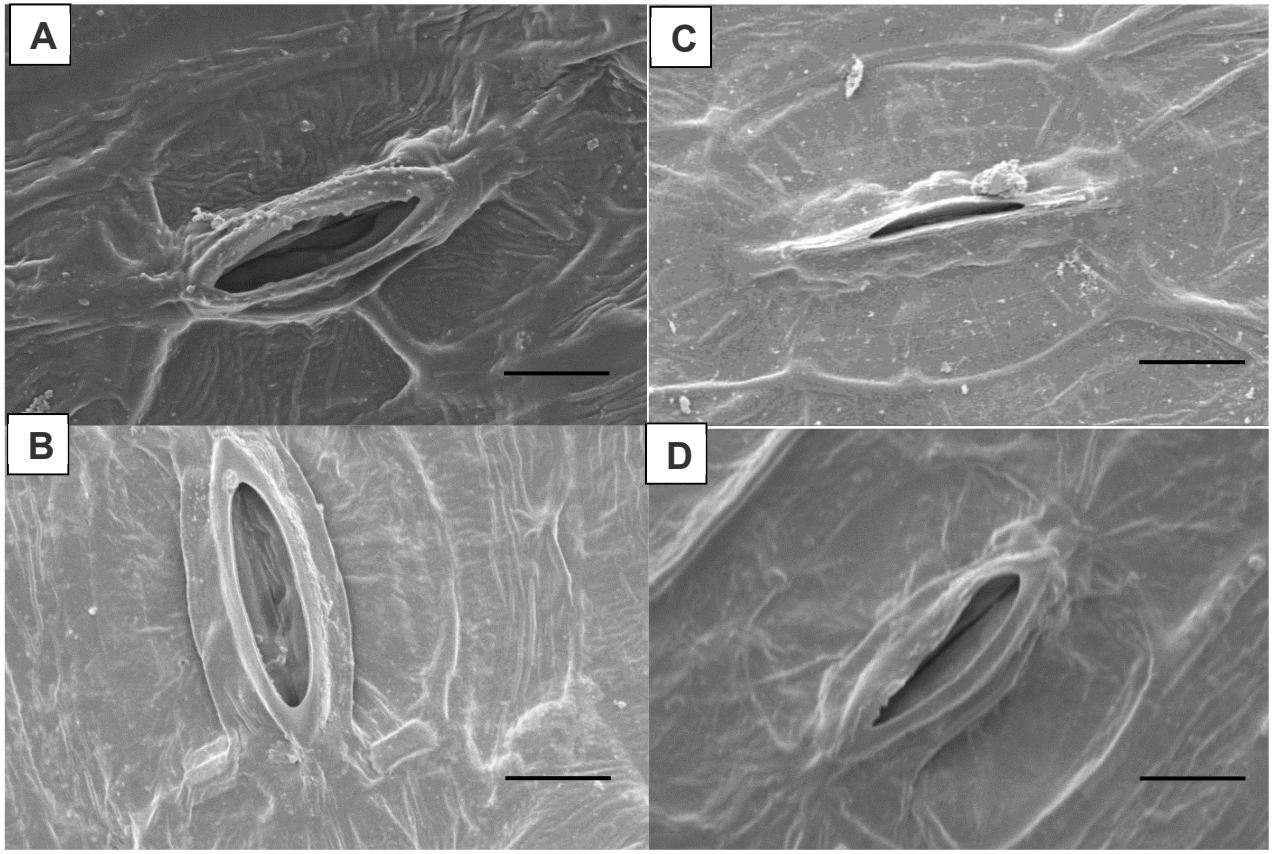

Figure 4.8 : Effect of 10 µM cytokinins on the stomata structure of micropropagated „Williams' bananas. A = Benzyladenine; B = *meta*-Methoxy topolin; C = *meta*-Methoxy topolin 9-tetrahydropyran-2-yl and D = Control. Magnification = 5000x, Bar = 10 µm.

4.4 Concluding remarks

Although the control had an higher pigment content compared to the CK-treated plantlets, *in vitro* propagation of „Williams' banana in the absence of CKs is not a practical option due to low multiplication rates **(Bairu et al., 2008)**. In addition, topolin-treated banana plantlets have demonstrated better acclimatization compared to BA-treated or control plantlets (data not shown). Current findings further confirm the potential anti-senescence activity of the topolins such as *m*T, *m*TR and Me*m*TTHP under *in vitro* conditions. Interestingly, Me*m*TTHP showed strong correlation of higher chlorophyll a and stomata density, and this happens to be the first report of the effect of the compound on photosynthetic parameters *in vitro*. This study articulates that the right choice and concentration of CKs applied during *in vitro* propagation may alleviate photomixotrophic-induced physiological stress that usually accompanies the transfer of plantlets to *ex vitro* conditions. Nevertheless, more stringent experiments focusing on the effect of the studied CKs on essential photosynthetic functionality such as chlorophyll fluorescence and quantity of evolved oxygen is required.

Chapter 5: Application of smoke-water and karrikinolide in *in vitro* propagation compared to *meta*-topolin

5.1 Introduction

Globally, it is well-known that for centuries fire and smoke have been employed for various practices in traditional agricultural systems **(Kulkarni et al., 2011)**. Researchers have indicated that smoke from burning plant material stimulates a significant increase in seed germination under field conditions in over 1200 species from 80 genera **(Dixon et al., 2009)**. In addition to the various benefits derived from use of smoke technology, the details of this phenomenon and its potential application, especially in seed technology have been extensively discussed in a number of review papers **(Brown and Van Staden, 1997; Van Staden et al., 2000; Dixon et al., 2009; Light et al., 2009; Kulkarni et al., 2011)**.

Although smoke-technology has been used in traditional farming practices for centuries, the active compound (karrikinolide = KAR_1) was only identified about eight years ago **(Flematti et al., 2004; Van Staden et al., 2004)**. Today, karrikins (including karrikinolide KAR_1, 3-methyl-2*H*-furo[2,3-c]pyran-2-one previously termed butenolide) refers to a new family of plant growth regulator's (PGR's) identified in smoke from burning plant material **(Dixon et al., 2009)**. The isolation and identification of KAR_1 was a major breakthrough as it has eliminated certain problems associated with use of crude smoke-water (SW). For instance, the disparity in SW (no two batches contain exactly the same balance or concentration of compounds) can be eliminated by using pure KAR_1 allowing for valid comparison of biological activities during experiments **(Chiwocha et al., 2009; Light et al., 2009)**. Although the poor yield of KAR_1 during synthesis was a major concern **(Light et al., 2010)**, a more efficient synthesis was recently described **(Matsuo and Shindo, 2011)**. The authors demonstrated a method that effectively increased the yield of KAR_1 to approximately 7.3%.

The potential of KAR_1 at extremely low concentrations have been mainly demonstrated by both South African **(Jain et al., 2008; Kulkarni et al., 2008)** and Australian **(Rokich and Dixon, 2007; Dixon et al., 2009)** researchers. For instance,

the application of either 2 or 20 g/ha butenolide promoted *Brassica tournefortii* germination up to 7- and 9-fold, respectively compared to the control **(Stevens et al., 2007)**. Using tomato seedlings, **Kulkarni et al. (2008)** demonstrated interesting activity of KAR_1 at 1 nM under greenhouse conditions. As indicated by **Merritt et al. (2005)**, KAR_1 promoted seed germination at remarkably low concentrations (parts per trillion). Studies have indicated that smoke extracts exhibit PGR-like responses and interact with some PGRs **(Chiwocha et al., 2009)**. Using the dwarf rice micro-drop assay, **Gardner et al. (2001)** observed an increase in putative gibberellin measured in smoke-treated lettuce seeds compared to red light-treated seeds. In another study, KAR_1 exhibited cytokinin- (CK) and auxin-like activity, stimulating cell division in the soybean callus bioassay and rooting in the mungbean bioassay respectively **(Jain et al., 2008)**. However, the author postulated that the response could be due to the interaction of KAR_1 with endogenous PGR and not necessarily substituting for PGR. For some unknown reason, till date, the potential of smoke technology remains to be fully exploited in micropropagation.

From the available literature, it is clear that majority of the studies on the effect of SW and KAR_1 have focused on seed germination as well as general growth and yield of several plant species. Experiments on vital physiological components such as photosynthetic pigments and secondary metabolites remain neglected. In spite of the traditional evidence of the significant effects of smoke, only a few agricultural and horticultural-important species have received deserved attention. In the current **Chapter**, the effect of different concentrations of SW and KAR_1 on *in vitro* growth, photosynthetic pigment and phenolic contents of „Williams' bananas was compared to *meta*-topolin (*m*T). Furthermore, synergetic effects of either compound when used in conjunction with *m*T on the aforementioned growth and physiological parameters were examined.

5.2 Materials and methods

5.2.1 Sources of materials used for *in vitro* propagation and growth conditions

Sterile banana obtained by shoot-tip culture **(Section 3.2.3)** and maintained on *m*T were used for the current experiments. Explants from plantlets produced in the fourth

subculture cycle were used. Smoke-water and KAR_1 were obtained from laboratory stock and preparation methods have been fully-described previously **(Baxter et al., 1994; Van Staden et al., 2004)**. After carefully peeling the sheath, shoot-tip explants measuring approximately 10 mm were cut in half longitudinally and inoculated in screw-cap jars (110 x 60 mm, 300 ml volume) containing 36 ml modified Murashige and Skoog (MS) medium **(Murashige and Skoog, 1962)** as described by **Vuylsteke (1998)**. Multiplication medium was supplemented with varying concentration of either SW (1:125; 1:250; 1:500; 1:1000; 1:2000 dilutions) or KAR_1 (3.3×10^{-12}; 7.8×10^{-17}; 1.0×10^{-19}; 4.8×10^{-22} M). Hormone-free and 3.0×10^{-5} M (30 µM) *m*T media were included in the experiment. The same quantity of sucrose, gelrite and other additives as well as growth conditions as indicated in **Section 3.2.3** were applicable for the current experiments. Individual treatments had 12 explants and the experiments were conducted twice.

5.2.2 Growth, *in vitro* phenolic and photosynthetic pigment quantification

After 42 days, regenerated plantlets from the treatments were removed from culture media and the growth parameters such as number and length of the leaves, shoot (pseudostems) and roots were measured. Thereafter, plantlets were divided into either aerial or root parts and oven-dried at 70 ± 2 ºC for 7 day. Dried plant materials were ground and extracted in 50% methanol (MeOH: 0.1 g per 10 ml) in an ultrasonic sonicator (Julabo GMBH, Germany) containing ice for 20 min. The extracts were centrifuged and the resultant filtrate used for *in vitro* phenolic quantification. Details on the methods applied for the quantification of the total phenolic content, flavonoid and proanthocyanidin contents were as outlined in **Sections 3.2.6.1**, **3.2.6.2** and **3.2.6.3**, respectively. The levels of photosynthetic pigment were determined as described in **Section 4.2.2**.

5.2.3 Synergistic effect of *meta*-topolin with either smoke-water or karrikinolide

Based on initial results, further studies evaluating the effects of the interaction of *m*T (3.0×10^{-5} M) with either KAR_1 (4.8×10^{-22} M) or SW (1:500) were conducted. The synergistic effect of these compounds on the growth, phenolic and photosynthetic pigments were determined.

5.2.4 Data analyses

Data were subjected to one-way analysis of variance (ANOVA) using the SPSS software package for Windows (SPSS®, version 10.0 Chicago, USA). Where there was statistical significance (P = 0.05), the mean values were further separated using Duncan's multiple range test.

5.3 Results and discussion

Smoke-water and KAR_1 produced more leaves and shoots (pseudostems) per explant than the control, although not significant in most cases **(Table 5.1)**. Nevertheless, aforementioned parameters were highest with the *m*T treatment. Rooting performance (number, length and biomass) was similar for SW, KAR_1 as well as the control plantlets but was significantly (P = 0.05) lower in the *m*T treatment. The use of either SW (1:500) or KAR_1 (4.8×10^{-22} M) with *m*T resulted in better shoot proliferation rate than the control **(Table 5.2)**. In addition, rooting inhibition observed in *m*T treatment was slightly reversed by combining *m*T and SW (1:500).

Optimum growth *in vitro* and improved acclimatization competence constitutes basic requirements for any successful tissue culture protocol. The levels and types of photosynthetic pigments and phenolic compounds are among the several factors that may make or mar the proper growth and development of plants. Although KAR_1 was first identified as a potent germination stimulant present in plant-derived smoke **(Flematti et al., 2004; Van Staden et al., 2004)**, the compound promotes other growth factors such as yield and seedling vigour **(Light et al., 2009)**. Recently, low concentrations of SW and KAR_1 have also been associated with auxin and CK-like activities *in vitro* **(Jain et al., 2008)**. Some authors postulated that PGR-like responses of SW and KAR_1 are probably due to their interaction with the endogenous PGRs **(Jain et al., 2008; Chiwocha et al., 2009)**.

Table 5.1: Effect of different concentrations of smoke-water (SW) and karrikinolide (KAR_1) on the growth of micropropagated „Williams' bananas

*Treatment		#Leaf	#Pseudostem				#Roots			
		Number	Number	Length (cm)	Fresh weight (g)	Dry weight (mg)	Number	Length (cm)	Fresh weight (g)	Dry weight (mg)
Control		6.2±0.36 c	1.3±0.12 c	9.9±0.75 a	1.4±0.14 bc	63.0±6.75 cd	10.2±0.67 ab	12.5±0.73 a	1.0±0.14 ab	39.3±5.49a
*m*T	3.0×10^{-5}	15.9±1.60 a	4.1±0.50 a	3.4±0.25 c	1.8±0.19 a	92.5±9.58 a	0 d	0 b	0 d	0 e
KAR_1	3.3×10^{-12}	6.7±0.49 bc	1.4±0.13 bc	8.5±0.63 ab	1.2±0.13 bcd	70.8±9.04 abcd	10.4±0.59 ab	11.3±0.67 a	0.8±0.11 abc	33.0±4.90 abc
	7.8×10^{-17}	6.6±0.53 bc	1.6±0.18 bc	7.9±0.58 b	0.9±0.08 d	52.8±4.77 cd	7.7±0.57 c	12.2±0.79 a	0.7±0.17 bc	19.5±2.77 d
	1.0×10^{-19}	7.5±0.64 bc	1.6±0.24 bc	9.7±0.63 ab	1.5±0.16 ab	89.8±9.61 ab	11.0±0.79 a	12.8±0.90 a	1.0±0.14 a	36.7±4.86 ab
	4.8×10^{-22}	5.9±0.43 c	1.3±0.09 c	10.1±0.45 a	1.2±0.09 bcd	62.6±4.74 cd	8.9±0.51 abc	12.7±0.54 a	0.7±0.07 abc	22.8±2.19 cd
SW	1:125	7.1±0.44 bc	1.5±0.15 bc	9.6±0.72 ab	1.3±0.09 bcd	74.6±5.92 abc	9.5±0.52 abc	12.0±0.66 a	0.8±0.09 abc	24.9±3.72 cd
	1:250	7.6±0.69 bc	1.5±0.18 bc	9.9±0.63 a	1.3±0.11 bcd	71.7±6.17 abcd	9.1±0.77 abc	12.3±0.51 a	0.8±0.09 abc	27.8±3.57 bcd
	1:500	8.8±1.14 b	2.2±0.33 b	7.9±0.76 b	1.3±0.17 bcd	69.6±8.41 bcd	8.5±0.84 bc	11.1±0.57 a	0.8±0.12 abc	26.7±5.01 bcd
	1:1000	6.4±0.53 c	1.5±0.13 bc	7.9±0.57 b	1.0±0.12 d	51.4±6.28 d	8.4±0.81 bc	10.8±0.66 a	0.6±0.07 c	20.0±2.57 d
	1:2000	7.6±0.65 bc	1.8±0.19 bc	8.8±0.62 ab	1.3±0.10 bcd	71.7±5.26 abcd	9.5±0.83 abc	12.0±0.91 a	0.7±0.09 bc	24.3±3.72 cd

#Parameter was evaluated per single explant cultured.

*Treatment: *m*T = *meta*-Topolin; KAR_1 = Karrikinolide; SW = Smoke-water. *m*T and KAR_1 concentrations values were in Molar while SW was in dilution.

Mean values ± standard error (n = 24) in the same column with different letter(s) are significantly different (*P* = 0.05) based on Duncan's multiple range test.

Table 5.2: Synergistic effect of *meta*-topolin with either smoke-water (SW) or karrikinolide (KAR_1) on the growth of micropropagated „Williams' bananas

*Treatment	#Leaves	#Pseudostem				#Roots			
	Number	Number	Length (cm)	Fresh weight (g)	Dry weight (mg)	Number	Length (cm)	Fresh weight (mg)	Dry weight (mg)
Control	6.7 ± 0.50 c	1.3±0.55 c	9.6±0.56 a	1.2±0.12 b	71.8±7.12 ab	10.5±0.82 a	11.4±0.79 a	579.0±74.12 a	17.8±2.70 a
*m*T	15.9±1.60 a	4.1±0.50 a	3.4±0.25 b	1.8±0.20 a	92.5±9.58 a	0 c	0 c	0 c	0 c
*m*T + KAR_1	15.6± 1.85 a	3.2±0.40 ab	4.0±0.36 b	1.1±0.14 b	66.4±8.31 b	0 c	0 c	0 c	0 c
*m*T + SW	11.6±1.01 b	2.5±0.35 b	3.9±0.35 b	1.0±0.13 b	66.6±7.40 b	0.3±0.17 b	0.1±0.06 b	5.17±0.04 b	0.5±0.004 b

#Parameter was evaluated per single explant cultured.
*Treatment: *m*T = *meta*-Topolin (3.0×10^{-5} M); KAR_1 = Karrikinolide (4.8×10^{-22} M); SW = Smoke-water (1:500).
Mean values ± standard error (n = 24) in the same column with different letter(s) are significantly different (*P* = 0.05) based on Duncan's multiple range test.

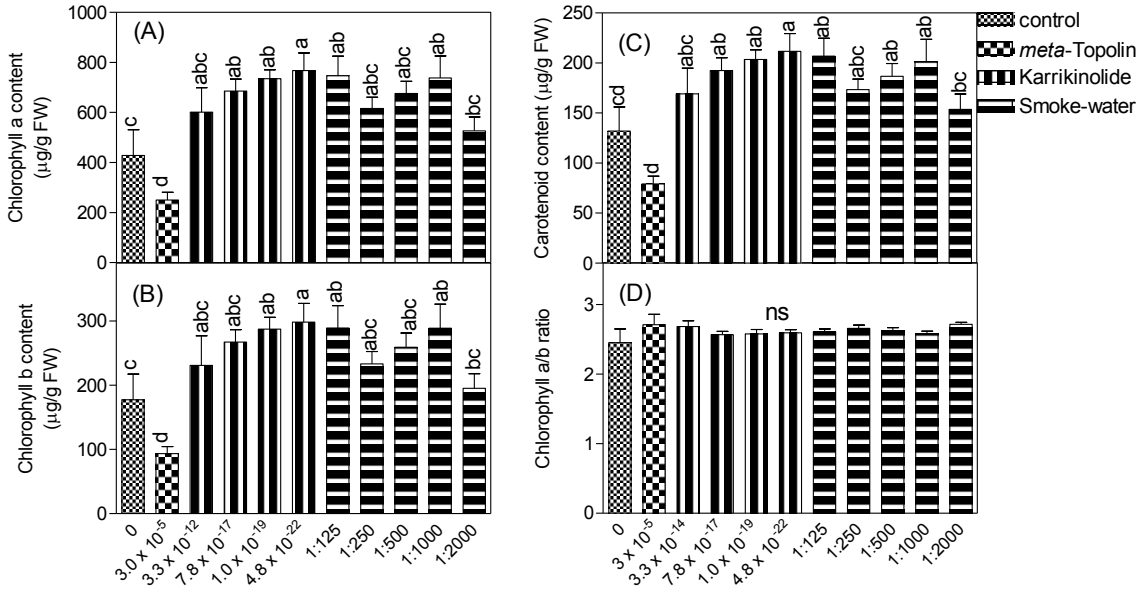

Figure 5.1: Effect of different concentrations of karrikinolide and smoke-water on micropropagated „Williams' banana photosynthetic pigment contents (A) Chlorophyll a (B) Chlorophyll b (C) Carotenoid and (D) Chlorophyll a/b ratio. In each graph, bars with different letter(s) are significantly different (P = 0.05) according to Duncan's multiple range test. n/s = Not significant.

Table 5.3: Synergistic effect of *meta*-topolin (*m*T) with either smoke-water (SW) or karrikinolide (KAR_1) on photosynthetic pigment content of micropropagated „Williams' bananas

*Treatment	Chlorophyll a (µg/g FW)	Chlorophyll b (µg/g FW)	Total chlorophyll (µg/g FW)	Chlorophyll a/b ratio	Carotenoids (µg /g FW)
Control	299±32.5 a	112±16.8 a	412±48.9 a	2.7±0.11 b	240±29.0 a
*m*T	89±9.60 b	30±3.4 b	119±13.0 b	3.0±0.04 a	80±9.2 b
*m*T + KAR_1	118±12.0 b	43±4.2 b	162±16.1 b	2.7±0.04 b	100±10.1 b
*m*T + SW	134±16.0 b	49±6.4 b	183±22.4 b	2.8±0.05 b	115±14.3 b

*Treatment: *m*T = *meta*-Topolin (3.0 x 10^{-5} M); KAR_1 = Karrikinolide (4.8 x 10^{-22} M); SW = Smoke-water (1:500).
Mean values (± standard error, n = 6) in the same column with different letter(s) are significantly different (P = 0.05) based on Duncan's multiple range test.

Plantlets treated with KAR_1 at 4.8 x 10^{-22} M had the highest level of chlorophyll a, b and carotenoids **(Figure 5.1)**. The application of KAR_1 at 4.8 x 10^{-22} M resulted in approximately 2- and 3-fold higher pigment content than the control and *m*T, respectively. Smoke-water treatment at 1:125 and 1:1000 dilutions also significantly

improved the pigments when compared to both the control and *m*T. In terms of the chlorophyll a/b ratio **(Figure 5.1D)**, all the treatments were rather similar with values ranging from 2.4 to 2.9 in the control and *m*T-treated plantlets. Using *m*T in combination with either SW (1:500) or KAR_1 (4.8 x 10^{-22} M) reduced the photosynthetic pigment contents **(Table 5.3)**. Furthermore, the combinations of these compounds did not improve the chlorophyll a/b ratio; instead, plantlets regenerated from *m*T alone had a significantly higher chlorophyll a/b ratio.

The effect of CKs on the production of photosynthetic pigments is generally stimulatory **(Fletcher and McCullagh, 1971; Goltsev et al., 2001; Mutui et al., 2012)** even though there is lack of conclusive evidence in micropropagated plants. In this study, treatment with SW and KAR_1 generally induced more pigment content than the control whereas *m*T-treated ones were lower than the control. As indicated in **Table 5.3**, a similar tendency was apparent when *m*T was used in combination with either SW (1:500) or KAR_1 (4.8 x 10^{-22} M). This unusual effect observed with *m*T treatment is possibly due to: (1) preferential resource mobilization and utilization in favour of shoot proliferation, and (2) the presence of sugar and essential nutrients in the medium. Thus, there is little or no need for photosynthesis. Furthermore, **Vlčková et al. (2006)** observed a switch from a protective to a damaging action of *m*T during senescence of detached wheat leaves in continuous light. The authors postulated that the damaging effect of *m*T during artificial senescence could be due to the over excitation of the photosynthetic apparatus resulting in oxidative damage. Here, in the presence of a poorly developed photosynthetic apparatus *in vitro*, an increase in excitation pressure by *m*T might have caused damage/modification to chloroplast ultrastructure thereby reducing the ability to synthesize photosynthetic pigments. Despite the wide variation in the quantity of pigments, the plantlets from all the treatments still maintained a similar chlorophyll a to b ratio, presumably as an adaptive response to maximize the light harvesting capability of the chloroplast apparatus **(Chow et al., 1990)**.

In order to improve secondary metabolite content in *in vitro* plants, the effects of different explants **(Zhang et al., 2011)**, manipulating the culture medium **(Abdullahil Baque et al., 2010; Savio et al., 2012)** and varying CK types as well as concentrations **(Liu et al., 2007)** have been reported as potential elicitors. A recent

study reported that the indigo content of a Chinese medicinal plant was significantly improved with SW at 1:500 dilution **(Zhou et al., 2011)**. In the current experiment, total phenolics and flavonoids were more abundant in the leaves whereas proanthocyanidins were generally higher in the roots **(Table 5.4)**. Treatment with KAR_1 (1.0×10^{-19} M) produced a significantly higher total phenolic content in the leaves and proanthocyanidins in the roots. The highest amount of total flavonoids was measured in treatments with SW (1:500) for leaves and KAR_1 (1.0×10^{-19} M) for the roots. The combination of *m*T with either SW (1:500) or KAR_1 (4.8×10^{-22} M) generally increased the accumulation of the secondary metabolites in the leaves **(Table 5.5)**. Evidence from a recent study indicated that the phenylpropanoid-pathway and flavonoid-related genes were up-regulated following SW and KAR_1 treatments **(Soós et al., 2010)**. In the current study, a significant stimulatory effect of both compounds on the concentration of total phenolics and flavonoids was observed. The finding are in agreement with **Soós et al. (2010)** which postulated that smoke compounds apparently caused a gene expression shift towards enhanced phenolic biosynthesis. Interestingly, even in the absence of roots, due to inclusion of *m*T, the secondary metabolite content remained relatively high in the leaves **(Table 5.5)**. Flavonoid accumulation was correlated with adventitious root formation in *Eucalyptus gunnii* micropropagated by axillary bud stimulation **(Curir et al., 1990)**. In this study, plantlets accumulated more flavonoids even when rooting was completely inhibited. The relationship between rooting phenomena and flavonoids has been a subject for debate. Opinions have been divided along the line of the flavonoids being either modulators or regulators of auxin transport **(Jacobs and Rubery, 1988; Brown et al., 2001; Peer and Murphy, 2007; Buer et al., 2010)**. **Peer and Murphy (2007)** viewed flavonoids as modulators of transport processes; they are synthesized in response to auxin accumulation presumably to prevent the plant from oxidative damage resulting from auxin catabolism. This hypothesis could explain the high flavonoid content detected in plantlets devoid of roots.

Table 5.4: Effect of *meta*-topolin (*m*T), smoke-water (SW) and karrikinolide (KAR_1) on secondary metabolite accumulation in micropropagated „Williams' bananas

*Treatment – concentration		[1]Total phenolics (mg GAE /g DW)		[2]Total flavonoids (mg CE /g DW)		[3]Proanthocyanidins (µg CCE /g DW)	
		Leaves	Roots	Leaves	Roots	Leaves	Roots
Control	0	24.8±1.07 bcd	11.8±0.36 def	15.0±0.14 cd	6.2±0.05 cd	469.9±57.48 a	395.4±26.92 de
*m*T (M)	3.0×10^{-5}	22.66±1.62 cd	ND	12.7±0.26 f	ND	454.0±13.77 ab	ND
KAR_1(M)	3.3×10^{-12}	27.3±075 bc	12.7±0.76 abcd	15.0±0.07 cd	7.0±0.25 ab	379.5±24.37 bcde	346.0±22.67 e
	7.8×10^{-17}	28.1±2.53 b	13.7±0.34 a	15.7±0.17 c	7.1±0.06 ab	381.2±14.38 bcde	402.3±12.34 de
	1.0×10^{-19}	34.1±2.17 a	12.5±0.37 bcde	17.2±0.04 b	7.3±0.14 a	444.5±9.31 abc	732.4±5.39 a
	4.8×10^{-22}	21.4±1.14 d	10.9±0.14 fg	11.1±0.74 g	5.5±0.01 e	350.3±41.69 de	475.9±19.64 cd
SW (v/v)	1:125	26.3±2.02 bcd	13.6±0.58 ab	15.0±0.09 cd	6.8±0.06 b	321.0±7.04 e	457.0±4.89 cd
	1:250	27.2±1.04 bc	13.1±0.23 abc	14.8±0.40 d	7.0±0.03 ab	368.3±6.21 cde	568.4±73.53 b
	1:500	24.2±2.11 bcd	11.5±0.15 efg	18.7±0.19 a	5.9±0.00 d	429.0±7.83 abcd	455.3±7.90 cd
	1:1000	24.6±0.32 bcd	12.3±0.23 cde	13.7±0.04 e	6.4±0.15 c	408.4±18.65 abcd	490.5±6.62 c
	1:2000	23.4±1.17 bcd	10.4±0.23 g	13.7±0.18 e	5.6±0.00 e	401.0±18.05 abcd	341.7±7.97 e

*Treatment: *m*T = *meta*-Topolin; KAR_1 = Karrikinolide; SW = Smoke-water.
[1]GAE = Gallic acid equivalents; [2]CE = Catechin equivalents; [3]CCE = Cyanidin chloride equivalents.
Mean values ± standard error (n = 6) in the same column with different letter(s) are significantly different (*P*= 0.05) based on Duncan's multiple range test.
ND = Not determined due to rooting inhibition by the treatment.

Table 5.5: Synergistic effect of *meta*-topolin (*m*T) with either smoke-water (SW) or karrikinolide (KAR_1) on secondary metabolite accumulation in micropropagated „Williams' bananas

*Treatment	[1]Total phenolics (mg GAE /g DW)		[2]Total flavonoids (mg CE /g DW)		[3]Proanthocyanidins (µg CCE /g DW)	
	Leaves	[#]Roots	Leaves	[#]Roots	Leaves	[#]Roots
Control	57.0±1.38 b	34.0±0.66	19.3±0.24 c	13.7±0.17	463.5±53.88 d	476.7±15.12
*m*T	31.4 ±1.88 c	ND	16.3±0.13 d	ND	612.2±12.98 c	ND
*m*T + KAR_1	83.4±3.18 a	ND	26.8±0.21 a	ND	941.9±16.96 b	ND
*m*T + SW	87.7±1.51 a	75.9±2.84	23.3±0.23 b	ND	1201.2±23.07 a	ND

[1]GAE = Gallic acid equivalents; [2]CE = Catechin equivalents; [3]CCE = Cyanidin chloride equivalents.
*Treatment: *m*T = *meta*-Topolin (3.0×10^{-5} M); KAR_1 = Karrikinolide (4.8×10^{-22} M); SW = Smoke-water (1:500).
Mean values ± standard error (n = 6) in the same column with different letter(s) are significantly different ($P = 0.05$) based on Duncan's multiple range test.
[#]ND = Not determined due to rooting inhibition by the treatments and mean values in these columns were not compared.

Another important observation was that the site of synthesis for the secondary metabolites analysed appear to be the leaves or more correctly the above ground parts. In the absence of *m*T and presence of roots for instance, the highest amount of proanthocyanidins was detected in roots. However, in the presence of *m*T (characterized by rooting inhibition), higher amounts of proanthocyanidins were detected in the leaves suggesting that the roots serves as storage sites. For total phenolics and flavonoids the leaves may serve both functions due to the higher amount that was detected in the leaves in the presence or absence of roots. These observations are in agreement with **Peer et al. (2001)**. The authors indicated that flavonoids are localized in the apical and nodal parts of the *Arabidopsis* inflorescence stem whereas other metabolites such as anthocyanins decreased from basal sections upwards.

5.4 Concluding remarks

Overall, the use of SW and KAR_1 had a negative effect on shoot proliferation compared to *m*T treatment. Therefore, their inclusion may be more valuable at the rooting phase of micropropagation. Perhaps, these compounds could be used in conjunction with auxin to improve the rooting performance prior to acclimatization. On the other hand, SW and KAR_1 improved chlorophyll a and b as well as the carotenoid contents of the regenerated banana plantlets compared to the control or *m*T treatment. The enhanced photosynthetic pigment level could play a vital role during the acclimatization stage. A similar stimulatory effect was observed with the levels of phenolic compounds detected. In terms of the secondary metabolites, the present finding could be part of different approaches employed by researchers to improve the quantity in micropropagated plants. In the absence of previous studies on the role of *m*T or KAR_1 on the production of secondary metabolites *in vitro*, these findings could serve as an alternative approach. The fact that smoke solution and KAR_1 are active at extremely low concentrations and have no toxic effects on crops **(Verschaeve et al., 2006; Kulkarni et al., 2010)** makes them attractive options. In order to derive untapped potential of SW and KAR_1, researchers need to perform more studies that will elucidate the fundamental physiological mechanisms.

Chapter 6: Enhancement of the acclimatization potential of *in vitro*-cultured 'Williams' bananas in the greenhouse

6.1 Introduction

The human population is continuously increasing and by the year 2050, the global population is anticipated to be 50% larger than the current figure. The increasing populations, especially in developing countries, will require increased food production **(Welch and Graham, 1999)**. Therefore efforts to increase production of essential crops such as banana are pertinent. In addition, the reduction in quality and yield of bananas often due to soil moisture deficits and declining soil fertility are of paramount concern **(Kahangi, 2010)**. Plant biotechnological techniques including micropropagation remain an essential approach for increasing the sustainability and profitability of food production. It is a valuable aid in the multiplication of many plant species **(Caponetti et al., 2005)**. In banana, micropropagation has played a significant role for the mass propagation of clonal material, conservation and breeding **(Vuylsteke, 1998; Jain, 2004)**. Evidence has shown that micropropagated banana establish faster, produce higher yield and grow taller with more uniform and shorter production cycles compared to conventionally grown plants **(Israeli et al., 1995; Kahangi, 2010)**. However, the acclimatization and field establishment of micropropagated plants is critical to derive the aforementioned benefits, particularly for large scale application of micropropagation techniques **(Pospíšilová et al., 1999)**.

Prior to field establishment, nutrient deficient soils need to be supplemented with fertilizers to promote growth and yield. In most developing countries, inorganic fertilizers are expensive and not easily accessible to small-scale farmers. Furthermore, increased use of inorganic fertilizers for improved crop production is associated with increased health hazards to humans as well as causing severe environmental problems such as water and soil pollution **(Alam et al., 2007)**. Hence, researchers are continuously devising means of improving crop yield and quality without compromising environmental integrity or public health. The use of sustainable agricultural practices which entails the

conservation of resources and the environment remain a viable option to increase agricultural product output **(Lazcano et al., 2011)**. The use of organic fertilizers, in the form of vermicompost and smoke technology, are practical examples of attempts geared towards sustainable agricultural farming. Vermicompost including their leachate, tea and extracts are produced by the activity of earthworms from a wide range of organic wastes **(levinsh, 2011)**. Fire and smoke have found useful application for various practices in traditional agricultural systems for centuries **(Kulkarni et al., 2011)**. In recent times, there has been an increase in demand for naturally derived agro-chemicals for sustainable farming systems **(Suthar, 2010)**.

Bananas are large herbaceous plants which rely heavily on the anchoring ability of their roots to support the plant and bunch weight. Hence, well developed root systems during field planting are essential. Cultural practices enhancing root and shoot development during acclimatization and nursery management prior to field planting are therefore, worth studying. Previous studies on the use of smoke-water (SW) have reported improved growth and vigour in various crops **(Sparg et al., 2005; Kulkarni et al., 2010)** as well as medicinal plants **(Van Staden et al., 2006)**. The studies reported superior rooting and seedling vigour compared to the controls. Similarly, the use of vermicompost is gaining more popularity due to the numerous benefits attributed to its usage **(Tomati et al., 1990; Bachman and Metzger, 2008)**. For instance, some of these benefits include improved plant health, yield and nutritive quality in *Brassica rapa* **(Pant et al., 2009)**. Data on the role of these growth promoting substances during acclimatization and/or prior to field establishment of micropropagated bananas remain scanty. The effect of SW and vermicompost leachate on the growth of micropropagated „Williams' bananas in the greenhouse was investigated.

6.2 Materials and methods

6.2.1 Source of 'Williams' banana plantlets, smoke-water and vermicompost leachate solutions

Rooted tissue-cultured banana (cv. „Williams') plantlets (5-7 cm) were purchased from Du Roi Laboratory, South Africa. The micropropagated plantlets were planted in 12.5 cm pots containing sand, soil, vermiculite (1:1:1, v/v/v), treated with 1% Benlate® (Du Pont de Nemours Int., South Africa) and acclimatized in the mist-house for one month. Thereafter, the acclimatized plantlets were transferred to the greenhouse for the experiment. Smoke-water solution was prepared as described by **Baxter and Van Staden (1994)** while the vermicompost leachate was purchased from Wizzard Worms commercial company Ltd, Greytown, South Africa.

6.2.2 Experimental design and growth conditions

Smoke-water treatments included - soil drenching and foliar application. The SW solutions were prepared by diluting the crude smoke solution with distilled water to obtain three concentrations (1:250; 1:500 and 1:1000 v/v). For foliar treatment, the solutions were prepared in spray bottles with the addition of a few drops of surfactant (Tween 20) and sprayed directly onto the leaves. Soil drenching treatments with different concentrations of SW solutions (100 ml/pot) were applied directly to the soil containing the potted plants. Stock vermicompost leachate was diluted with distilled water to three different concentrations (1:5; 1:10 and 1:20 v/v). All diluted solutions were prepared on the day of application and 100 ml/pot of each concentration applied directly to the soil. Vermicompost leachate requires further bioremediation making it unsuitable as a foliar spray **(ROU, 2006)** hence, it was not used as foliar spray in this study. All the treatment solutions using either soil drenching (SW and vermicompost leachate) or foliar application (SW) were repeated twice weekly and the plants were irrigated with tap water between these treatments.

In the mist-house, the day/night temperature was 30/12 °C, relative humidity between 80 and 90%, a misting interval of 15 min and a misting duration of 10 s. The

greenhouse had 30-40% relative humidity, day/night temperature of 30/15 ºC with an average photosynthetic photon flux density of 450 µmol m^{-2} s^{-1}. During the experiment, photoperiod was that of prevailing conditions during summer (12 h). Ten plants were used for the soil drenching experiment while five plants were utilized for foliar application treatments. After two months, plants were transferred into bigger containers (17.5 cm) with the same potting media and grown for a further month prior to harvesting and data collection. The experiment was initiated in November 2010 and terminated in March 2011.

6.2.3 Data collection and analyses

After three months, the plants were harvested, washed and air-dried. Subsequently, the growth parameters such as number (leaf, shoot and root), length (shoot and root) and fresh weights (shoot and root) were measured. The shoot/root ratio was calculated as the ratio of the dry weights of the aerial part over that of the root. The leaf area was determined using an L1-3100 area meter (Li-Cor Inc., Lincoln, Nebraska, USA). Dry weights of the harvested plants were determined after the fresh materials were oven-dried at 70 ± 2 ºC for 7 days. For the analysis, the treatments in each application method were compared with their corresponding controls. Data were subjected to one-way analysis of variance (ANOVA) using SPSS software package for Windows (SPSS®, version 10.0 Chicago, USA). Where there was statistical significance (P = 0.05), the mean values were further separated using the Duncan's multiple range test.

6.3 Results and discussion

Overall, „Williams' banana responded positively to the SW and vermicompost leachate treatments. In terms of mode of application, soil drenching with SW was more effective than foliar application. The effect of these treatments was most pronounced on the morphology of the roots **(Figure 6.1A and B)**. Furthermore, massive hairy roots were most conspicuous following soil drenching with SW solutions **(Figure 6.1B)**.

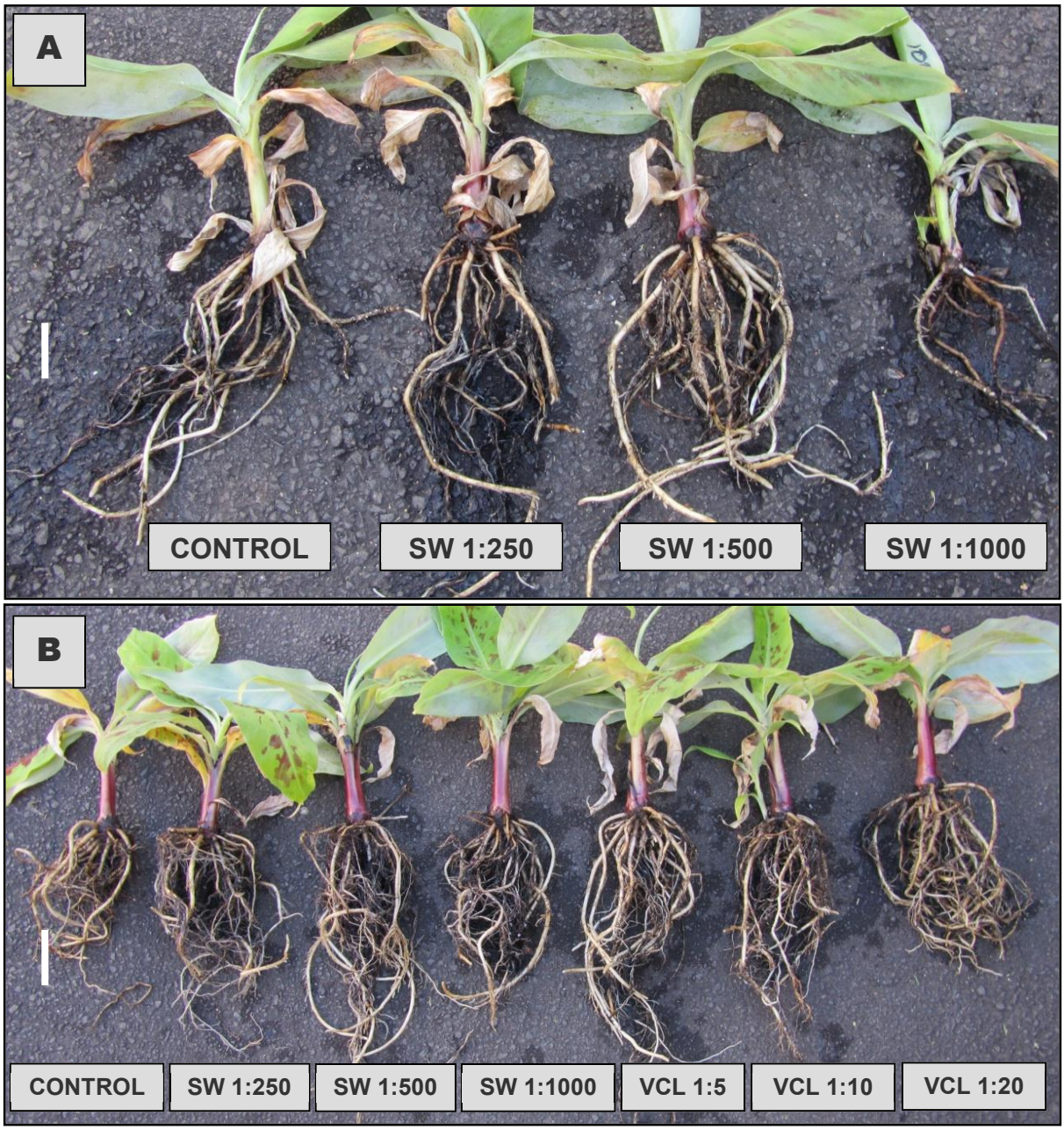

Figure 6.1: Effect of smoke-water and vermicompost leachate on growth of „Williams' bananas (A) Foliar application using smoke-water and (B) Soil drenching using smoke-water and vermicompost leachate. SW = Smoke-water; VCL = vermicompost leachate; scale bars = 5 cm.

Soil drenching of both treatments significantly improved the mean dry weight of the root (P = 0.05) compared to the control **(Table 6.1)**. Smoke-water drenching treatment (1:500) produced the highest fresh and dry weights (although not significant from other treatments). In addition, there was no significant (P = 0.05) difference in the number of roots between the control and vermicompost leachate treatments, whereas, SW treatments significantly (P = 0.05) reduced the number of roots **(Table 6.1)**. However, the reduction in root number was compensated by the higher root length observed in SW (1:1000 and 1:500).

For the regenerated off-shoots, vermicompost leachate (1:5) produced the longest shoots which was significantly (P = 0.05) different from all other treatments **(Table 6.2)**. Conversely, other growth parameters evaluated (leaf number and area as well as dry weights for shoots and roots) were similar amongst the other treatments and the control.

The shoot/root ratio which is a vital measure to predict seedling survival potential during transplanting was evaluated. The use of SW (1:250 and 1:500 dilutions) treatments gave the optimum mean shoot/root ratio among the regenerated off-shoots. Even though these aforementioned ratios were better than the control, they were still similar to the majority of the other treatments **(Figure 6.2A)**. Using soil drenching application, the use of vermicompost leachate and SW positively affected shoot/root ratios compared to the control plants **(Figure 6.2B)**. Among the drenching application experiments, vermicompost leachate (1:10) stimulated the production of the highest number of off-shoots from the parent plantlets **(Figure 6.2C)**.

Using foliar application, a dilution of 1:500 v/v SW was the optimum concentration for use on the basis of the evaluated parameters **(Figure 6.3B, C, E and F)**. However, these parameters were not significantly (P = 0.05) different from the 1:250 dilution and the control. Generally, a 1:1000 dilution was clearly too diluted as indicated by leaf area **(Figure 6.3A)** as well as fresh and dry weight of the shoots and roots **(Figure 6.3C and F)**. In terms of the mean shoot/root ratio, both control and the treatments were not significantly different **(Figure 6.3D)**.

Table 6.1: Effect of soil drenching of different concentrations of vermicompost leachate (VCL) and smoke-water (SW) on the growth parameters of „Williams' bananas

*Treatment	Leaves		Shoots			Roots			
	Number	Area (cm^2)	Length (cm)	Fresh weight (g)	Dry weight (g)	Number	Length (cm)	Fresh weight (g)	Dry weight (g)
Control	9.8 ± 0.33 a	70.5 ± 2.1 b	10.2 ± 0.11 cd	60.4 ± 2.73 a	8.1 ± 0.55 a	28.2 ± 1.88 a	33.1 ± 1.4 b	39.8 ± 2.8 c	3.3 ± 0.32 b
SW 1:1000	9.8 ± 0.33 a	69.5 ± 3.8 b	10.7 ± 0.41 cd	44.6 ± 3.10 bc	4.8 ± 0.27 b	19.8 ± 1.28 b	57.7± 6.8 a	55.4 ± 5.2 ab	5.8 ± 0.60 a
SW 1:500	10.1 ± 0.19 a	80.1 ± 3.4 ab	11.7 ± 0.37 bc	53.1 ± 3.29 ab	5.7 ± 0.41 b	21.1 ± 1.03 b	59.1 ± 8.3 a	63.8 ± 1.9 a	6.5 ± 0.26 a
SW 1:250	8.8 ± 0.30 b	68.2 ± 2.8 b	10.2 ± 0.11 d	40.9 ± 1.89 c	4.6 ± 0.27 b	20.7 ± 0.80 b	49.0 ± 4.2 ab	59.8 ± 4.2 ab	6.4 ± 0.55 a
VCL 1:20	10.6 ± 0.23 a	76.7 ± 3.5 b	11.6 ± 0.28 bc	51.3 ± 3.61 ab	5.3 ± 0.43 b	24.6 ± 1.01 a	48.3 ± 5.0 abc	49.9 ± 3.9 bc	5.5 ± 0.50 a
VCL 1:10	10.0 ± 0.23 a	92.2 ± 5.9 a	12.1 ± 0.31 ab	49.2 ± 4.32 bc	5.0 ± 0.55 b	25.0 ± 0.73 a	52.6 ± 2.1 a	52.0 ± 5.4 abc	5.6 ± 0.68 a
VCL 1:5	10.3 ± 0.27 a	93.0 ± 6.9 a	13.0 ± 0.44 a	55.2 ± 4.23 ab	5.7 ± 0.49 b	25.0 ± 1.21 a	59.3 ± 5.1 a	54.1 ± 4.3 ab	5.1 ± 0.40 a

Mean values ± standard error (n = 10) in the same column with different letter(s) are significantly different (*P* = 0.05) based on Duncan's multiple range test.
*Treatment: SW = Smoke-water; VCL = Vermicompost leachate.

Table 6.2: Effect of soil drenching of different concentrations of vermicompost leachate (VCL) and smoke-water (SW) on the growth parameters of produced off-shoots in „Williams' bananas

*Treatment	Leaves		Shoots			Roots			
	Number	Area (cm^2)	Length (cm)	Fresh weight (g)	Dry weight (g)	Number	Length (cm)	Fresh weight (g)	Dry weight (g)
Control	7.3 ± 0.1 a	21.3 ± 0.7 a	6.6 ± 0.2 b	9.3 ± 0.6 ab	1.1 ± 0.33 a	10.3 ± 0.6 a	23.5 ± 0.5 c	5.0 ± 0.4 b	0.6 ± 0.17 a
SW 1:1000	7.2 ± 0.1 ab	23.3 ± 1.7 a	6.9 ± 0.4 b	9.4 ± 0.8 ab	0.8 ± 0.12 a	10.0 ± 0.4 a	32 ± 1.9 a	6.4 ± 0.5 ab	0.7 ± 0.07 a
SW 1:500	6.6 ± 0.3 abc	21.3 ± 3.1 a	7.0 ± 0.5 b	8.2 ± 1.3 ab	0.7 ± 0.21 a	10.8 ± 0.6 a	27 ± 2.8 abc	5.4 ± 0.9 b	0.6 ± 0.18 a
SW 1:250	6.5 ± 0.2 abc	24.2 ± 2.6 a	7.3 ± 0.5 b	9.4 ± 1.3 ab	0.9 ± 0.24 a	11.3 ± 0.7 a	26.8 ± 2.1 abc	9.4 ± 1.6 a	1.0 ± 0.30 a
VCL 1:20	6.1 ± 0.3 c	22.0 ± 2.2 a	7.3 ± 0.3 b	8.6 ± 1.2 ab	0.7 ± 0.12 a	9.8 ± 0.7 a	25 ± 2.3 bc	5.6 ± 1.4 b	0.7 ± 0.19 a
VCL 1:10	6.2 ± 0.2 c	21.3 ± 2.4 a	7.3 ± 0.4 b	7.2 ± 1.0 b	0.6 ± 0.09 a	9.9 ± 0.7 a	22.6 ± 2.0 c	4.3 ± 0.7 b	0.5 ± 0.09 a
VCL 1:5	6.3 ± 0.3 bc	27.7 ± 2.6 a	8.8 ± 0.5 a	11.3 ± 1.3 a	1.0 ± 0.15 a	10.8 ± 0.7 a	30.8 ± 2.4 ab	6.6 ± 0.9 ab	0.7 ± 0.12 a

Mean values ± standard error in the same column with different letter(s) are significantly different (P = 0.05) based on Duncan's multiple range test.
*Treatment: SW = Smoke-water; VCL = Vermicompost leachate.

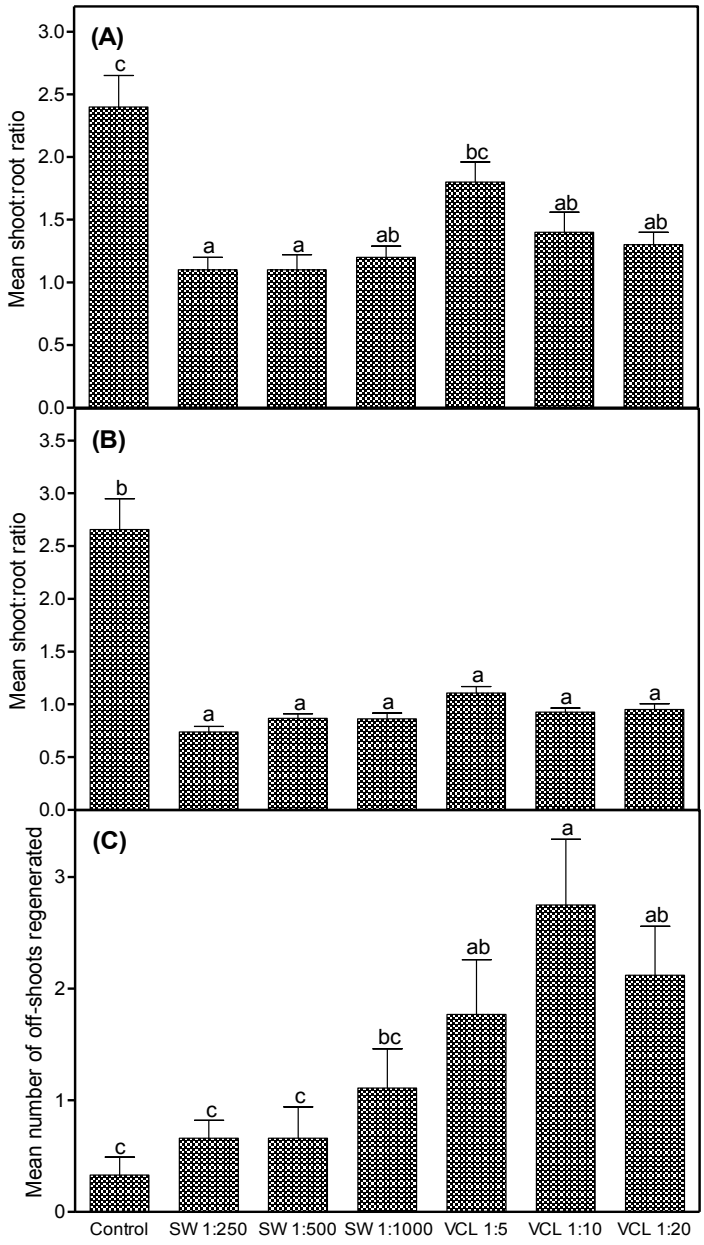

Figure 6.2: Effect of different concentrations of smoke-water and vermicompost leachate on the vegetative growth of „Williams' bananas (A) Mean shoot:root ratio of regenerated off-shoot plants; (B) Mean shoot:root ratio of parent plants; and (C) Mean number of off-shoots regenerated from the parent plant. In each graph, bars with different letter(s) are significantly different (P = 0.05) according to Duncan's multiple range test.

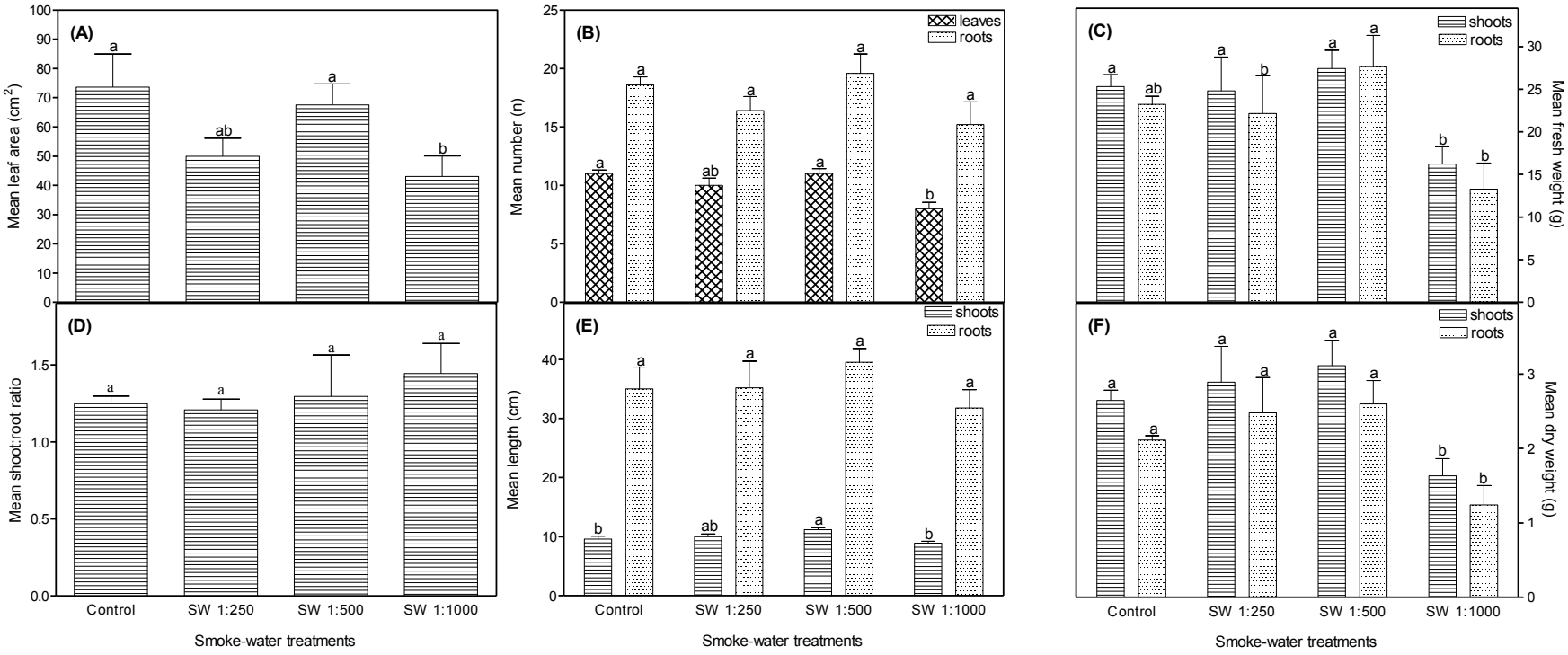

Figure 6.3: Effects of foliar application of different concentrations of smoke-water on the vegetative growth of 'Williams' bananas (A) Mean leaf area; (B) Mean number of leaves and roots; (C) Mean fresh weight of shoots and roots; (D) Mean shoot:root ratio; (E) Mean length of shoot and root; and (F) Mean dry weight of shoots and roots. In each graph, similar pattern bars with different letter(s) are significantly different ($P = 0.05$) according to Duncan's multiple range test.

Several studies have demonstrated that smoke, aqueous smoke extracts and smoke-derived butenolide (syn: karrikinolide) are useful and have a wide range of potential applications in the agricultural and ecological sectors **(Verschaeve et al., 2006; Light et al., 2009)**. In several plant species, the positive influence of smoke on seed germination **(Van Staden et al., 2000)**, rooting **(Taylor and Van Staden, 1998)**, secondary metabolite accumulation **(Zhou et al., 2011)**, yield and general seedling vigour **(Sparg et al., 2005; Van Staden et al., 2006; Kulkarni et al., 2008)** have been demonstrated. Similarly, several advantages of the use of vermicompost products in agricultural practices are reported **(García-Gómez et al., 2008; Wang et al., 2010; levinsh, 2011)**. Most of these positive influences have been attributed to the evidence of the presence of some plant growth promoters in smoke extract **(Chiwocha et al., 2009)** and vermicompost leachate **(Tomati et al., 1988; Suthar, 2010)**. Moreover, **Arthur et al. (2001)** observed that leachate from well-decomposed compost contain cytokinin-like substances derived from the hydrolysis of cytokinin glucosides by the enzyme *β*-glucosidase produced by microbes. The growth promoting effects observed by the test solution in this study could therefore, be attributed to the contents of the solutions.

In this study, the influence of the method of application of the SW was apparent; soil drenching was more effective than foliar application. Studies have shown better absorption of different compounds using soil drenching compared to foliar application **(Kumar and Poehling, 2006; Sarkar et al., 2007; Francis et al., 2009)**. **Parkunan et al. (2011)** postulated that soil drenching application is associated with continuous uptake by plants, over a relatively longer period of time compared to foliar application. Moreover, SW solutions of similar concentration are mostly applied using different volumes for soil drenching versus foliar spray. This delivers different amounts of smoke solutions to the test plants. As a result of these interacting factors, it is difficult to strictly compare the two application methods at similar concentration.

Due to its high susceptibility to toppling, well developed root systems contribute significantly to the stability of banana plants **(Blomme et al., 2008)**. As evidenced in this

study, roots generated from SW and vermicompost leachate were longer with an higher fresh and dry weight. This is an indication that smoke-water and vermicompost leachate would enhance the stability of the „Williams' banana when transferred to the field. Another parameter closely related to the effect of rooting is the shoot/root ratio; a vital measure to predict the seedling survival potentials during transplanting. A high ratio implies that the roots are not as abundant with the seedling liable to incidents of water stress after planting, either in drought areas or under conditions of high evaporative demand **(Bernier et al., 1995)**. All the concentrations of SW and vermicompost leachate (with the exception of 1:5) exhibited lower shoot/root ratios which were significantly (P = 0.05) different from the control plants **(Figure 6.2A)**. Hence, the favourable effect of these treatments on shoot/root ratios suggests the ability of the treated banana plants to tolerate drought and withstand transplanting shock.

In addition to the presence of micro-elements, vermicompost leachate contains components such as humic and fulvic acids that promote growth in several species. Humic acids are molecules that regulate many processes of plant development as well as enhancing macro- and micro-nutrients absorption **(Gutiérrez-Miceli et al., 2008)**. Studies have indicated that humic acid increased the number of roots thereby stimulating nutrient uptake and plant development **(Alvarez and Grigera, 2005)**. The presence of these compounds probably explains the relatively longer shoot length as well as higher leaf number and area in vermicompost leachate-treated „Williams' bananas compared to either the control or SW treatments. In a study by **Padmavathiamma et al. (2008)**, the authors demonstrated that vermicompost-amended acid-agriculture-soil significantly improved the yield, biometric character and quality of bananas. Similarly, treatment of *Brassica rapa* (pak choi: chinese cabbage) with vermicompost tea resulted in an increase in above-ground fresh and dry weights, leaf area and extractable mineral element concentration **(Pant et al., 2009)**. The authors attributed the favourable response to an enhanced mineral nutrient (particularly nitrogen) uptake by plant tissue.

For most of the vegetative parameters, the soil drenching with smoke-water at 1:500 dilution gave the best results. The effectiveness of similar smoke-water concentration which mainly stimulated more rooting in other plant species is well documented **(Van Staden et al., 2006; Kulkarni et al., 2008; Kulkarni et al., 2010)**). Success resulting from SW application depends on the concentration of aqueous smoke extract which varies among plant species **(Brown and Van Staden, 1997; Sparg et al., 2005)**. In this study, concentrations higher or lower than 1:500 dilution may be too strong or weak, respectively for plant growth. This probably explains the reduced vegetative parameters observed in the „Williams' bananas. The effects of organic fertilizers are genotype dependent, for example, different genotypes of sweet corn responded differently to organic fertilizer treatments **(Lazcano et al., 2011)**. In addition, effectiveness of vermicompost leachate depends on factors such as concentration and plant species **(Tomati et al., 1988)**. In terms of leaf number and area as well as shoot length, current findings indicated that higher concentrations (1:5 and 1:10) were more effective than the lower one (1:20). Similarly, an undiluted concentration of vermicompost leachate was recommended for optimum growth response during the cultivation of sorghum **(Gutiérrez-Miceli et al., 2008)**.

6.4 Concluding remarks

Despite the reported benefits, the effect of SW and vermicompost leachate on plant species has been associated with major shortcomings. For instance, with SW, it has been observed that no two batches contain exactly the same balance or concentration of compounds **(Light et al., 2009)**. The application of the pure butenolide (syn: karrikinolide) compound would eliminate most of these problems associated with the use of SW **(Light et al., 2009)**. Disparity in parameters such as chemical, physical and biological composition amongst different vermicompost samples has also been reported **(Campitelli and Ceppi, 2008)**. Hence, **Campitelli and Ceppi (2008)** recommended the need to devise tools for appropriate quality characterisation of vermicompost. In addition, vermicompost leachate must be used with care due to the tendency of contamination with pathogens and presence of phytotoxic compounds **(Gutiérrez-**

Miceli et al., 2008). Based on available literature, these growth promoting compounds do not cause any toxic or mutagenic effects. For instance, SW as well as some smoke-derived compounds did not exhibit any mutagenic activity **(Verschaeve et al., 2006; Trinh et al., 2010)**. Furthermore, SW-treated onion bulbs showed no genotoxic effect and were pronounced safe for consumption **(Kulkarni et al., 2010)**. In the current experiment, the positive effect on rooting is beneficial for acclimatization and establishment of tissue-cultured banana plantlets in nurseries and subsequent transfer to the field. Moreover, the high cost of inorganic fertilizers as well as environmental safety concerns makes the use of SW and vermicompost leachate potential alternatives for improving the growth and cultivation of bananas. Hence, soil drenching application of these compounds is a sustainable agricultural practice which is a cheaper and more environment-friendly means to increase „Williams' banana production. Moreover, the application method which is very easy can be practiced by small-scale banana farmers.

Chapter 7: Physiological responses and endogenous cytokinin profiles in relation to roscovitine and an inhibitor of cytokinin oxidase/dehydrogenase (INCYDE) treatments

7.1 Introduction

Cytokinins (CKs), specifically the aromatic types are N^6-substituted purine derivatives. They constitute an essential class of plant growth regulators (PGRs) that together with auxins act at low concentration and regulate various physiological and developmental processes in plants **(Letham and Palni, 1983; Schmülling, 2004)**. The absence or presence of hydroxyl groups as well as their stereoisomeric position are common features of CKs which account for their diverse activity **(Strnad, 1997; Mok and Mok, 2001; Sakakibara, 2006)**. Cytokinin homeostasis is an essential factor that determines the physiological activities, particularly the growth and development of plants **(Kamínek et al., 1997)**. In plant cells, CKs act as normal adenylate compounds which exist in mixtures of free bases, *N*-glucosides, *O*-glucosides, nucleosides as well as the mono-, di-, and tri-nucleotides **(Letham and Palni, 1983; Bajguz and Piotrowska, 2009)**. These compounds can be interconverted via reactions catalyzed by purine metabolizing enzymes such as glucosyltransferase, adenosine nucleosidase, and xylosyltransferase **(Chen, 1997)**. Based on their physiological metabolic pathways, CK inter-conversion, hydroxylation, conjugation and degradation remain important regulatory mechanisms in plant tissues **(Strnad et al., 1997)**. Cytokinin homeostasis is generally maintained by the regulation of both CK biosynthesis and catabolism which are influenced by two main enzymes: isopentenyltransferase (IPT) and cytokinin oxidase/dehydrogenase (CKX), respectively **(Frébort et al., 2011)**. In addition, CK homeostasis is regulated by several internal and external factors such as PGRs and inorganic nitrogen sources, which have been postulated to connect the nutrient signals and morphogenetic responses **(Auer, 1997; Sakakibara, 2006)**.

In plant tissue culture (PTC), CKs are often used to stimulate the growth and development of plants *in vitro* **(Zalabák et al., 2012)**. The exogenously applied CKs interact with the endogenous level thereby affecting the balance in the cultured explants

(Blakesley, 1991; Blagoeva et al., 2004a). The uptake and metabolism of the applied CKs determine the resultant growth and development of the plant **(Kamínek et al., 1997; Haberer and Kieber, 2002)**. Cytokinins may be reversibly or irreversibly conjugated with sugars, and amino acids **(Bajguz and Piotrowska, 2009)**. Often, the exogenous CK are converted into various forms of metabolites as a regulatory mechanism in order to maintain the CK homeostasis **(Letham and Palni, 1983)**. As suggested by the authors, these metabolites could be of a less active (conjugates), translocation or a storage form which are readily available in the event of depletion of the free CKs pool or the detoxification products (which are mostly unavailable to the plant tissue). In other words, since free CK bases remain the most active form, the various inter-conversion of the CK bases is essential for the regulation of CK levels and activities **(Bajguz and Piotrowska, 2009; Frébort et al., 2011)**.

The level of active CKs is reduced in plants mainly by side chain cleavage into simpler units by CKX. The enzyme is also useful for the maintenance of an optimal level of CKs required for growth and/or resetting a CK signalling system to a basal level **(Werner et al., 2001; Frébort et al., 2011)**. In addition, the free CKs can undergo conjugation with glucose to produce either reversible forms such as *O*-glucosides or irreversible forms such as *N*-glucosides **(Mok and Mok, 2001)**. The ability of the glycosides to interact with the 3, 7 and 9 position of the purine ring probably make them the most common and abundant CK conjugates both in lower **(Ördög et al., 2004)** and higher plants **(Van Staden and Crouch, 1996; Auer, 1997; Bairu et al., 2011b)**. Although CK 7*N*- and 9*N*-glucosides are considered as non-active and detoxification products in plants, some authors have observed a contrary effect to this widely-accepted assumption **(Letham and Palni, 1983; Upfold and Van Staden, 1990)**.

In PTC, the presence of large amounts of 7*N*- and 9*N*-glucosylation products is a common event in several micropropagated species **(Werbrouck et al., 1995; Blagoeva et al., 2004b; Dwivedi et al., 2010)**. Consequently, there is a decrease in the levels and availability of other CK forms especially the free bases which have more beneficial effects in plant tissues **(Brzobohatý et al., 1994)**. Often, these „cause and effect'

phenomena are responsible for the commonly observed physiological disorders in PTC. For example, there was a wide variation in the composition of CK metabolites of normal and necrotic shoots of *Harpagophytum procumbens* **(Bairu et al., 2011b)**, normal and hyperhydric shoots of *Aloe polyphylla* **(Ivanova et al., 2006)** as well as normal and variegated leaves of micropropagated bananas **(Zaffari et al., 1998)**. The abundance of *N*-glucosides was also implicated in the inhibition of rooting and subsequent acclimatization failure observed in *Spathiphyllum floribundum* **(Werbrouck et al., 1995; 1996)**. Among the commonly used CKs, BA has been associated with rapid formation of *N*-glucosides due to its structural limitation (absence of hydroxyl groups). The accumulation of BA *N*-glucosides particularly in the basal part of the shoots can have severe practical consequences in PTC **(Bairu et al., 2007; Valero-Aracama et al., 2010)**.

The prospect of suppressing these detrimental metabolites could enhance CK activity as well as modulate the plant responses positively. Along this line, a number of potential *N*-glucosylation inhibitors such as papaverine, methylxanthines, theophylline and olomoucine have been tested **(Letham et al., 1977; Tao et al., 1991; Blagoeva et al., 2004b)**. Recently, roscovitine (6-benzylamino-2-(R)-[1-(hydroxymethyl) propylamino]-9-isopropylpurine), was identified as the most potent inhibitor of *N*-glucosylation **(Blagoeva et al., 2003; 2004b; Dwivedi et al., 2010)**. Roscovitine, a 2,6,9-tri-substituted purine is a well-known inhibitor of the cyclin-dependent kinase (CDK), enzymes involved in the cell cycle progression that play a vital role in intracellular control of cell division **(De Azevedo et al., 1997; Meijer et al., 1997)**. Roscovitine and similar compounds such as bohemine and olomoucine have also been found to be effective inhibitors of CDKs **(Havlíček et al., 1997; Binarová et al., 1998; Spíchal et al., 2007)**.

Cytokinin oxidase/dehydrogenase is responsible for most of the CK catabolism and inactivation of the CKs in a single enzymatic step **(Schmülling et al., 2003)**, the presence of an inhibitor compound(s) could circumvent the activity of the enzyme. Recent research endeavours resulted in the discovery of a number of potent CKX

inhibitors **(Zatloukal et al., 2008)**. One such compound was INCYDE (2-chloro-6-(3-methoxyphenyl)aminopurine) which demonstrated CK activity in classical CK bioassays and was highly effectively in the inhibition of *Arabidopsis* CKX.

Evidence from the limited studies indicates the great potential of roscovitine in PTC **(Blagoeva et al., 2003; Stoynova-Bakalova and Petrov, 2009; Dwivedi et al., 2010)**. The effectiveness of INCYDE however, has not been studied in-depth as the compound was only discovered recently **(Zatloukal et al., 2008)**. In the field of PTC and biotechnology, bananas are important species because of their huge economic contribution to food security. Although PTC has contributed significantly to improvement in the quality of planting materials and the increase in production globally, banana remains one of the most highly prioritized research crops. The presence of inherent physiological problems including the detrimental effect associated with the commonly used CK (for e.g. BA) necessitates further studies on the role of CK in the improvement of PTC protocols. Besides, stringent studies on the endogenous CKs and factors affecting their concentration would contribute to better understanding of the banana hormone physiology *in vitro*. In the current Chapter, the role of roscovitine and INCYDE (with BA or *m*T) on the physiology (growth, photosynthetic pigment and phenolic contents) and CK metabolism of micropropagated „Williams' bananas was investigated.

7.2 Materials and methods

7.2.1 Explant culture and growth conditions

Aseptically maintained *in vitro* „Williams' banana plantlets, with medium composition and growth condition as described in **Section 3.2.2** were used for the experiment. However, the explants were obtained from medium supplemented with either 30 µM *m*T or BA. Roscovitine and INCYDE were prepared as described by **Havlíček et al. (1997)** and **Zatloukal et al. (2008)**, respectively. The treatments were BA + INCYDE, BA + roscovitine, BA + INCYDE + roscovitine, *m*T + INCYDE, *m*T + roscovitine and *m*T + INCYDE + roscovitine while 30 µM *m*T and BA were included as controls. Based on previous studies **(Zatloukal et al., 2008; Dwivedi et al., 2010)**, roscovitine and INCYDE

were tested at 5 and 100 µM, respectively. Shoot-tip explants (10 mm) were cut in half longitudinally and inoculated in screw-cap culture jars (110 x 60 mm, 300 ml volume) containing 36 ml of growth medium. After 42 d, plantlets were harvested (separated into aerial and underground sections) and growth parameters such as shoot number and plant height were measured.

7.2.2 Quantification of photosynthetic pigment and phytochemical content

After 42 days incubation, fresh leaf samples were extracted using acetone (0.2 g/ml) and the amount of chlorophyll a, chlorophyll b and carotenoid content were quantified using the method of **Lichtenthaler (1987)** as outlined in **Section 4.2.2**. For the phytochemical analysis, oven-dried plant materials were extracted with 50% methanol (MeOH) at a ratio of 0.1 g per 10 ml. The mixture was sonicated in an ice-bath for 20 min and filtered using Whatman filter paper. Total phenolics, flavonoids and proanthocyanidins were determined as outlined in **Sections 3.2.6.1, 3.2.6.2** and **3.2.6.3**, respectively.

7.2.3 Extraction, purification and quantification of endogenous cytokinins

At the end of the culture cycle (42 days), plantlets from each treatment were washed and separated into aerial and underground sections. Plant materials were immediately frozen in liquid nitrogen, freeze-dried and lyophilized. Prepared samples were extracted in 1 ml of Bieleski buffer (60% MeOH, 25% $CHCl_3$, 10% HCOOH and 5% H_2O) together with a cocktail of 23 deuterium-labeled (Olchemim Ltd, Olomouc, Czech Republic) CK internal standards at 1 pmol per sample, to check recovery during purification and to validate the determination **(Novák et al., 2008)**. Purification of the samples was achieved by using a combination of cation (SCX-cartridge), anion [DEAE-Sephadex-C18-cartridge] exchangers and immunoaffinity chromatography (IAC) based on wide-range specific monoclonal antibodies against CKs **(Novák et al., 2003)**. The resultant eluates from the IAC columns were evaporated to dryness and dissolved in 20 µl of the mobile phase used for quantitative analysis.

Samples were analyzed using an ultra-performance liquid chromatography (UPLC) (Acquity UPLC™; Waters, Milford, MA, USA) coupled to a Quattro *micro*™ API (Waters, Milford, MA, USA) triple quadrupole mass spectrometer equipped with an electrospray interface [ESI(+)] and photodiode array detector (Waters PDA 2996). Thereafter, the purified samples were injected onto a C18 reversed-phase column (Waters Acquity UPLC BEH C18; 1.7 µm; 2.1 x 50 mm). Elution was performed using a methanolic gradient consisting of 100% MeOH (A) and 15 mM formic acid (B) adjusted to pH 4.0 with ammonium. With a flow rate of 0.25 ml/min and column temperature of 40 °C, the following protocol was applied: 0 min 10% A + 90% B - 8 min linear gradient; 50% A + 50% B then column equilibration.

Endogenous CK quantification was obtained by multiple reaction monitoring (MRM) of $[M + H]^+$ and the appropriate product ion. For selective MRM experiments, optimal conditions (dwell time, cone voltage, and collision energy in the collision cell) corresponding to exact diagnostic transition were optimized for each CK **(Novák et al., 2008)**. Quantification was performed with Masslynx software using a standard isotope dilution method. The ratio of endogenous CK to appropriate labelled standard was determined and subsequently used to quantify the level of endogenous CKs in the original banana plantlet extract, based on the known quantity of added internal standard **(Novák et al., 2003)**.

7.2.4 Data analyses

Endogenous CK contents were quantified and expressed as mean ± standard deviation from three technical replicates. The growth, phytochemical and photosynthetic pigment contents data were measured and subjected to one-way analysis of variance (ANOVA) using SPSS software package for Windows (SPSS®, version 10.0 Chicago, USA). Where there was statistical significance (P = 0.05), the mean values were further separated using the Duncan's multiple range test.

7.3 Results and discussion

7.3.1 Effect of roscovitine and INCYDE on the growth of 'Williams' bananas

Regeneration rate was more than 50% in all the treatments. The 93% response in BA + roscovitine-treated cultures was the highest rate observed **(Figure 7.1A)**. Highest shoot number was obtained in plantlets treated with *m*T + INCYDE while BA + roscovitine + INCYDE treatment had the lowest number of shoots **(Figure 7.1B)**. The addition of INCYDE to BA-supplemented medium reduced the shoot length in the regenerants. Fresh and dry weights were highest in BA + roscovitine-treated and lowest in BA + INCYDE-treated plantlets **(Figure 7.1D and E)**. The inhibitory effect of *m*T or BA + INCDYE on the fresh and dry weights was partially restored with the addition of roscovitine to the medium. Furthermore, banana plantlet regenerated from BA + roscovitine had a significantly (P = 0.05) higher dry matter compared to other BA supplemented cultures. In addition, when compared to their respective control (*m*T or BA) plantlets, the addition of roscovitine and/or INCYDE had no significant (P = 0.05) stimulatory effect on other growth parameters. It was also observed that beside BA + roscovitine-treated banana plantlets with a few roots, all other treatments inhibited root formation (data not shown).

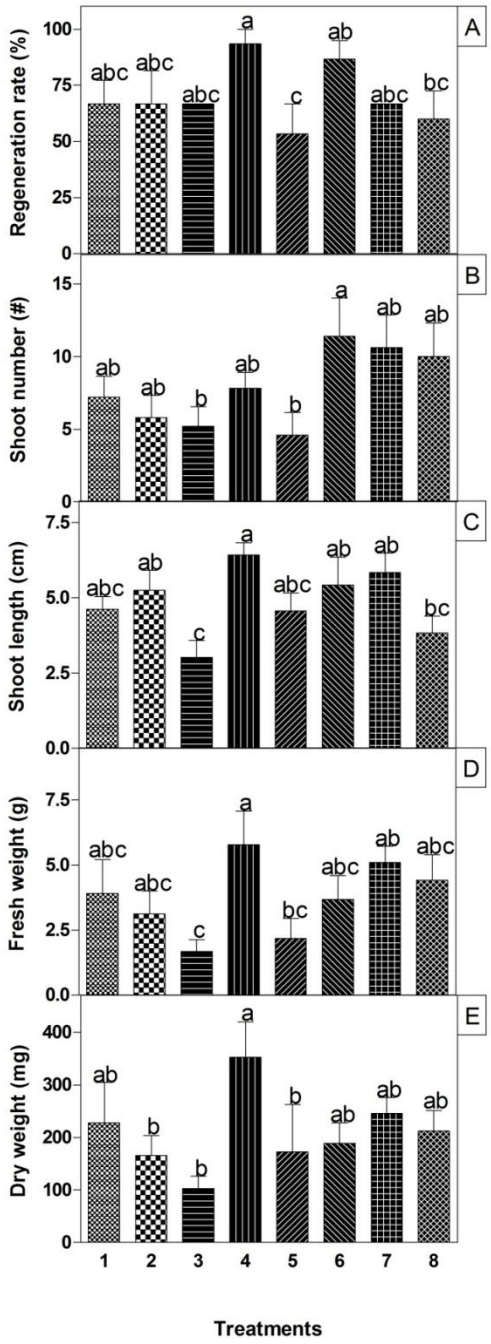

Figure 7.1: Effect of roscovitine and INCYDE on growth of the micropropagated „Williams' bananas. A = regeneration rate; B = shoot number; C = shoot length; D = fresh weight and E = dry weight. 1 = *meta*-topolin; 2 = benzyladenine; 3 = benzyladenine + INCYDE; 4 = benzyladenine + roscovitine; 5 = benzyladenine + INCYDE + roscovitine; 6 = *meta*-topolin + INCYDE; 7 = *meta*-topolin + roscovitine; 8 = *meta*-topolin + INCYDE + roscovitine. In each graph, bars with different letter(s) are significantly different (P = 0.05) based on Duncan's multiple range test.

A better understanding of the structure-activity relationship of CKs and their effect on plant physiology remains vital for improving the growth and development of plants *in vitro* **(Chen, 1997; Sakakibara, 2006)**. From a commercial point of view, the proliferation rate of any plant species especially the economically important ones such as bananas should not be compromised. Thus, the possibility of any stimulatory or inhibitory effect of roscovitine and INCYDE on the growth parameters of the banana plantlets was evaluated. Roscovitine is a well-known potent and selective inhibitor of animal CDKs where it effectively blocks cells at G1/S and G2/M transitions **(Meijer et al., 1997)**. Although, a similar observation was observed in *Petunia* hybrid culture **(Tréhin et al., 1998)**, studies on the effect of roscovitine on the plant cell cycle is limited **(Planchais et al., 2000)**. In plant cells, roscovitine has been demonstrated as one of the potential synchronizing agents and used to characterize the requirements of different CDK activities for cell cycle progression **(Planchais et al., 1997; Binarová et al., 1998; Tréhin et al., 1998; Blagoeva et al., 2003)**. The reversibility and specificity of roscovitine activities makes it a suitable chemical inhibitor for synchronization in the plant cell cycle **(Planchais et al., 2000)**. In the current study, the interaction of roscovitine with the CKs seems to play some vital role in the cell cycle as *m*T or BA with roscovitine treatments had a slightly higher shoot multiplication rate compared to the controls (*m*T or BA alone). **Pasternak et al. (2000)** postulated that CK play a regulatory role in the first cells of protoplast-derived cells of alfalfa at the G2/M border which may be related to the post-translational regulation of the CdcMsA/B kinase. Although INCYDE retained high CK activity in three classical CK bioassays **(Zatloukal et al., 2008)**, the interaction of the compound with BA resulted in a slight reduction in the regeneration and proliferation of the explants. The opposite effect observed in the presence of *m*T is probably due to the presence of the hydroxyl group in *m*T. In addition, the increased total endogenous CK levels in *m*T + roscovitine and/or INCYDE-treated plantlets **(Table 7.1 and 7.2)** may have partially contributed to the better proliferation in these treatments.

Table 7.1: Effect of roscovitine and INCYDE with aromatic cytokinins on endogenous cytokinins content (pmol/g FW) in „Williams' banana aerial parts

Cytokinin type	Cytokinin treatment and resultant endogenous cytokinin content (pmol/g FW)								
	*m*T	BA	BA+INCYDE	BA+Roscovitine	BA+INCYDE+ Roscovitine	*m*T+INCYDE	*m*T+Roscovitine	*m*T+INCYDE+Roscovitive	
tZ	14 ± 0.7	10 ± 2.0	19 ± 7.1	2 ± 0.0	<LOD	<LOD	<LOD	<LOD	
tZOG	8 ± 1.3	4 ± 0.6	10 ± 0.9	3 ± 1.4	7 ± 1.1	2 ± 1.1	<LOD	2 ± 1.0	
tZR	2 ± 1.1	0.2 ± 0.2	3 ± 0.6	0.2 ± 0.0	3 ± 2.8	<LOD	<LOD	<LOD	
tZROG	7 ± 0.5	3 ± 0.6	10 ± 0.8	7 ± 1.1	10 ± 2.0	4 ± 1.2	11 ± 2.3	9 ± 1.8	
tZ9G	<LOD	4 ± 4.9	7 ± 0.0	<LOD	<LOD	72 ± 0.0	<LOD	<LOD	
tZR5'MP	14 ±	<LOD	19 ± 4.0	<LOD	<LOD	<LOD	<LOD	<LOD	
Total tZ	**44 ± 3.6**	**21 ± 8.3**	**68 ± 13.4**	**12 ± 2.5**	**20 ± 5.9**	**79 ± 2.4**	**11 ± 2.3**	**11 ± 2.8**	
cZ	6 ± 0.5	1 ± 0.2	4 ± 1.9	1 ± 0.6	2 ± 0.0	<LOD	<LOD	<LOD	
cZOG	11 ± 0.4	5 ± 0.4	16 ± 0.4	11 ± 2.1	13 ± 3.4	4 ± 0.3	10 ± 2.7	9 ± 0.3	
cZR	97 ± 15.2	22 ± 3.7	128 ± 25.2	68 ± 4.2	59 ± 20.7	21 ± 5.7	62 ± 1.2	12 ± 3.3	
cZROG	40 ± 1.3	13 ± 2.0	59 ± 5.0	46 ± 7.2	77 ± 6.1	11 ± 0.4	42 ± 7.7	9 ± 2.7	
cZ9G	601 ± 386.1	1048 ± 841.9	528 ± 273.8	1116 ± 103.0	798 ± 485.5	474 ± 390.2	827 ± 253.6	1759 ± 1434.4	
cZR5´MP	78 ± 28.0	47 ± 12.2	235 ± 49.9	209 ± 121.3	218 ± 12.0	61 ± 32.9	165 ± 42.0	23 ± 10.4	
Total cZ	**833 ± 431.5**	**1137 ± 860.5**	**970 ± 356.1**	**1450 ± 238.5**	**1168 ± 527.7**	**571 ± 429.5**	**1106 ± 307.2**	**1812 ± 1451.1**	
DHZ	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	
DHZOG	2 ± 0.8	1 ± 0.3	3 ± 0.1	2 ± 1.3	3 ± 0.1	1 ± 0.1	5 ± 0.1	2 ± 0.8	
DHZR	2 ± 0.2	1 ± 1.1	2 ± 1.1	0 ± 0.1	2 ± 1.3	<LOD	<LOD	1 ± 0.0	
DHZROG	3 ± 1.1	1 ± 0.1	3 ± 1.8	3 ± 0.9	6 ± 1.3	1 ± 0.2	5 ± 0.2	2 ± 0.7	
DHZ9G	3 ± 0.0	8 ± 5.1	41 ± 0.0	7 ± 0.0	27 ± 25.2	<LOD	3 ± 0.0	2 ± 0.0	
DHZR5´MP	54 ± 0.0	20 ± 0.0	14 ± 14.8	2 ± 0.0	35 ± 0.0	11 ± 0.0	<LOD	30 ± 0.0	
Total DHZ	**64 ± 2.0**	**30 ± 6.6**	**64 ± 17.7**	**14 ± 2.3**	**74 ± 27.9**	**13 ± 0.3**	**12 ± 0.3**	**36 ± 1.4**	
iP	0.3 ± 0.0	0.5 ± 0.2	1 ± 0.3	<LOD	<LOD	<LOD	<LOD	<LOD	
iPR	9 ± 0.2	7 ± 1.1	66 ± 8.2	24 ± 1.0	22 ± 3.5	3 ± 0.9	42 ± 2.0	<LOD	
iP9G	26 ± 5.3	248 ± 16.7	72 ± 3.9	145 ± 23.8	194 ± 85.9	27 ± 8.2	110 ± 15.0	47 ± 0.6	
iPR5´MP	12 ± 0.9	11 ± 2.1	96 ± 9.7	42 ± 9.1	39 ± 7.7	<LOD	66 ± 5.8	<LOD	
Total iP	**47 ± 6.4**	**266 ± 20.0**	**235 ± 22.0**	**212 ± 33.9**	**255 ± 97.0**	**30 ± 9.1**	**218 ± 22.9**	**47 ± 0.6**	
BA	18 ± 1.2	8712 ± 900.0	11846 ± 341.2	6755 ± 4.2	10637 ± 9279.7	967 ± 57.5	92 ± 32.7	214 ± 17.1	

BAR	1	±	0.1	286	±	35.5	425	±	35.5	435	±	41.6	2353	±	196.1	9	±	1.0	0.5	±	0.5	1	±	0.3
BA9G	18	±	3.4	8730	±	352.3	31342	±	810.2	30693	±	1973.8	72254	±	16570.7	3096	±	2.4	56	±	8.2	125	±	29.6
BAR5´MP	9	±	0.5	926	±	16.2	1017	±	64.8	979	±	214.9	5258	±	756.2	16	±	1.6	<LOD			<LOD		
Total BA	**46**	**±**	**5.1**	**18654**	**±**	**1304.0**	**44630**	**±**	**1251.7**	**38862**	**±**	**2234.5**	**90501**	**±**	**26802.7**	**4088**	**±**	**62.5**	**148**	**±**	**41.4**	**340**	**±**	**47.0**
mT	902	±	60.3	33	±	8.8	53	±	0.7	41	±	0.2	182	±	221.0	36579	±	1745.6	5183	±	89.6	22894	±	1293.5
mTOG	294	±	10.8	49	±	11.3	120	±	22.5	80	±	24.7	699	±	222.1	31151	±	2236.7	2249	±	396.4	30589	±	115.8
mTR	39	±	0.7	1	±	0.1	2	±	0.7	1	±	0.4	5	±	0.2	803	±	52.3	28	±	3.3	294	±	39.0
mTROG	32	±	8.1	4	±	0.7	2	±	0.9	5	±	4.7	7	±	3.4	7804	±	332.7	250	±	26.9	5850	±	127.4
mT9G	63	±	7.6	3	±	1.5	17	±	15.1	11	±	0.6	19	±	5.3	3087	±	3.6	224	±	1.9	4316	±	547.0
mTR5´MP	108	±	1.5	27	±	25.3	3	±	0.3	<LOD			<LOD			2136	±	628.3	64	±	32.7	804	±	7.7
Total mT	**1439**	**±**	**88.9**	**117**	**±**	**47.7**	**197**	**±**	**40.2**	**138**	**±**	**30.7**	**912**	**±**	**452.2**	**81560**	**±**	**4999.2**	**7997**	**±**	**550.9**	**64748**	**±**	**2130.4**
oT	<LOD			50	±	7.8	80	±	10.3	15	±	0.6	191	±	153.7	2	±	0.3	0.2	±	0.0	1	±	0.2
oTOG	<LOD			26	±	3.0	51	±	0.1	23	±	9.5	619	±	117.8	24	±	0.0	<LOD			<LOD		
oTR	1	±	0.0	1	±	0.6	1	±	0.6	1	±	0.1	11	±	0.6	<LOD			<LOD			1	±	0.1
oTROG	<LOD			<LOD			<LOD			<LOD			<LOD			<LOD			<LOD			<LOD		
oT9G	<LOD			63	±	1.1	329	±	0.7	142	±	19.8	1216	±	213.3	9	±	7.0	16	±	0.0	14	±	0.0
oTR5´MP	<LOD			<LOD			<LOD			<LOD			<LOD			<LOD			<LOD			<LOD		
Total oT	**1**	**±**	**0.0**	**141**	**±**	**12.5**	**461**	**±**	**11.7**	**180**	**±**	**30.0**	**2036**	**±**	**485.5**	**35**	**±**	**7.4**	**16**	**±**	**0.0**	**16**	**±**	**0.3**
pT	<LOD			<LOD			<LOD			<LOD			<LOD			<LOD			<LOD			<LOD		
pTOG	2	±	0.0	1	±	0.9	6	±	0.3	3	±	3.0	10	±	2.9	4	±	2.1	5	±	2.7	4	±	3.0
pTR	<LOD			<LOD			<LOD			<LOD			<LOD			<LOD			<LOD			<LOD		
pTROG	<LOD			<LOD			<LOD			<LOD			<LOD			<LOD			<LOD			<LOD		
pTR5´MP	<LOD			<LOD			<LOD			<LOD			<LOD			<LOD			<LOD			<LOD		
Total pT	**2**	**±**	**0.0**	**1**	**±**	**0.9**	**6**	**±**	**0.3**	**3**	**±**	**3.0**	**10**	**±**	**2.9**	**4**	**±**	**2.1**	**5**	**±**	**2.7**	**4**	**±**	**3.0**
K	43	±	16.1	18	±	11.1	39	±	13.4	11	±	1.8	11	±	0.0	52	±	10.1	27	±	8.3	40	±	14.7
KR	0.2	±	0.0	0.1	±	0.1	0.1	±	0.0	0.1	±	0.0	1	±	0.3	<LOD			<LOD			<LOD		
K9G	1	±	0.7	2	±	0.1	6	±	0.1	7	±	1.9	10	±	2.8	<LOD			<LOD			<LOD		
KR5´MP	<LOD			1	±	0.0	2	±	0.0	1	±	0.0	<LOD			<LOD			<LOD			<LOD		
Total K	**44**	**±**	**16.8**	**21**	**±**	**11.2**	**47**	**±**	**13.5**	**18**	**±**	**3.7**	**22**	**±**	**3.1**	**52**	**±**	**10.1**	**27**	**±**	**8.3**	**40**	**±**	**14.7**
Total CK	**2519**	**±**	**554.4**	**20388**	**±**	**2271.7**	**46677**	**±**	**1727**	**40887**	**±**	**2579.1**	**94998**	**±**	**28404.9**	**86432**	**±**	**5522.4**	**9541**	**±**	**936.0**	**67053**	**±**	**3651.4**

Values are reported as mean ± standard deviation (n = 3); <LOD = below the limit of detection. Besides topolins, all the CKs abbreviations were based on **Kamínek et al. (2000)**

Table 7.2: Effect of roscovitine and INCYDE with aromatic cytokinins on endogenous cytokinins content (pmol/g FW) in „Williams' bananas underground parts

Cytokinin types	Cytokinin treatment and resultant endogenous cytokinin content (pmol/g FW)							
	*m*T	BA	BA+INCYDE	BA+Roscovitine	BA+INCYDE+Roscovitine	*m*T+INCYDE	*m*T+Roscovitine	*m*T+INCYDE+Roscovitive
tZ	0.04 ± 0.03	2 ± 0.8	0 ± 0.3	2 ± 1.4	1 ± 0.3	1 ± 0.2	4 ± 0.2	1 ± 0.0
tZOG	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
tZR	<LOD	2 ± 0.1	1 ± 0.5	3 ± 1.1	2 ± 0.4	1 ± 0.2	3 ± 0.2	1 ± 0.0
tZROG	0.1 ± 0.01	1 ± 0.1	0 ± 0.2	1 ± 0.2	0.4 ± 0.01	0 ± 0.1	0 ± 0.1	2 ± 0.2
tZ9G	4 ± 0.4	13 ± 2.8	4 ± 1.4	7 ± 3.1	4 ± 0.2	3 ± 0.2	4 ± 2.9	2 ± 0.7
tZR5'MP	0.33 ± 0.2	6 ± 0.7	2 ± 1.0	31 ± 20.9	13 ± 8.1	4 ± 2.2	1 ± 1.1	20 ± 1.4
Total tZ	**5 ± 0.6**	**24 ± 4.5**	**8 ± 3.3**	**44 ± 26.6**	**19 ± 9.1**	**9 ± 2.9**	**13 ± 4.4**	**26 ± 2.3**
cZ	2 ± 0.9	7 ± 2.9	2 ± 0.2	4 ± 1.3	4 ± 0.2	21 ± 7.9	41 ± 6.6	2 ± 1.7
cZOG	1 ± 0.1	<LOD	0 ± 0.1	<LOD	<LOD	1 ± 0.3	2 ± 0.5	<LOD
cZR	14 ± 1.1	9 ± 2.4	4 ± 1.7	58 ± 17.3	56 ± 18.4	110 ± 3.3	288 ± 20.1	63 ± 7.0
cZROG	5 ± 0.3	2 ± 0.3	2 ± 1.0	59 ± 5.2	46 ± 12.7	41 ± 2.1	137 ± 17.4	20 ± 2.4
cZ9G	97 ± 33.6	199 ± 5.6	75 ± 21.8	175 ± 25.0	179 ± 42.7	217 ± 29.8	81 ± 6.8	21 ± 5.5
cZR5´MP	63 ± 1.8	45 ± 19.9	54 ± 17.2	490 ± 108.1	253 ± 40.5	394 ± 57.4	640 ± 126.2	194 ± 10.3
Total cZ	**180 ± 37.8**	**262 ± 31.0**	**138 ± 42.0**	**785 ± 156.8**	**538 ± 114.6**	**784 ± 100.8**	**1189 ± 177.6**	**300 ± 26.9**
DHZ	<LOD	1 ± 0.3	0 ± 0.1	0 ± 0.2	0 ± 0.01	0 ± 0.1	1 ± 0.1	0.2 ± 0.03
DHZOG	0 ± 0.0	1 ± 0.2	0 ± 0.2	1 ± 0.5	1 ± 0.4	0 ± 0.1	0 ± 0.3	1 ± 0.3
DHZR	2 ± 0.2	2 ± 0.2	1 ± 0.2	2 ± 0.7	1 ± 0.5	17 ± 0.8	29 ± 0.8	7 ± 0.3
DHZROG	0 ± 0.1	1 ± 0.5	0 ± 0.1	2 ± 1.0	1 ± 0.4	5 ± 0.3	19 ± 3.5	3 ± 0.0
DHZ9G	1 ± 0.2	8 ± 1.3	2 ± 0.4	4 ± 0.8	4 ± 0.3	3 ± 0.2	11 ± 1.0	1 ± 0.2
DHZR5´MP	12 ± 0.8	7 ± 0.5	6 ± 1.1	110 ± 40.2	26 ± 16.6	114 ± 63.9	94 ± 1.8	57 ± 5.7
Total DHZ	**16 ± 1.4**	**19 ± 3.0**	**9 ± 2.2**	**120 ± 43.3**	**33 ± 18.3**	**139 ± 65.5**	**155 ± 7.4**	**69 ± 6.6**
iP	<LOD	12 ± 4.3	4 ± 1.3	8 ± 2.9	7 ± 0.8	5 ± 1.6	8 ± 3.7	4 ± 1.2
iPR	7 ± 2.1	19 ± 7.6	23 ± 9.5	54 ± 9.7	58 ± 6.3	10 ± 4.5	76 ± 4.7	6 ± 2.0
iP9G	49 ± 4.6	211 ± 10.0	109 ± 28.9	30 ± 7.6	42 ± 12.0	27 ± 5.0	218 ± 90.2	5 ± 2.7
iPR5´MP	36 ± 9.6	110 ± 11.1	56 ± 12.7	226 ± 57.5	132 ± 40.3	53 ± 18.2	131 ± 16.1	14 ± 4.0
Total iP	**91 ± 16.3**	**352 ± 33.0**	**193 ± 52.3**	**319 ± 77.7**	**240 ± 59.4**	**96 ± 29.3**	**434 ± 114.7**	**30 ± 9.9**
BA	218 ± 79.9	133696 ± 34010.0	58646 ± 23349.5	108767 ± 40570.4	90962 ± 31271.6	3811 ± 313.5	610 ± 236.6	2482 ± 1088.7
BAR	1 ± 0.6	855 ± 54.8	941 ± 218.7	2538 ± 77.4	3656 ± 700.2	10 ± 2.5	9 ± 2.6	14 ± 2.1

BA9G	64 ± 46.4	231971 ± 17203.1	107425 ± 37160.8	192241 ± 50161.4	186642 ± 17584.5	2958 ± 421.9	360 ± 29.8	837 ± 232.5
BAR5´MP	1 ± 0.4	6904 ± 963.2	1734 ± 861.0	3288 ± 2657.5	5327 ± 2536.5	40 ± 8.6	7 ± 3.1	30 ± 0.5
Total BA	**284 ± 127.3**	**373427 ± 52231.1**	**168746 ± 61590.0**	**306834 ± 93466.6**	**286587 ± 52092.7**	**6819 ± 746.5**	**986 ± 272.1**	**3363 ± 1323.8**
mT	19816 ± 1715.2	666 ± 41.3	341 ± 179.4	2062 ± 1272.7	1013 ± 24.4	58057 ± 3077.0	59127 ± 539.7	38593 ± 1402.8
mTOG	3928 ± 873.4	3909 ± 670.3	1501 ± 469.5	968 ± 538.5	748 ± 279.1	66078 ± 9963.9	145144 ± 19824.4	60043 ± 5169.3
mTR	167 ± 65.8	42 ± 9.2	38 ± 10.2	37 ± 8.7	46 ± 7.4	15866 ± 2822.0	10996 ± 40.9	27138 ± 465.7
mTROG	652 ± 71.7	116 ± 54.5	39 ± 22.3	79 ± 39.7	61 ± 3.7	17305 ± 1527.3	6556 ± 824.8	38652 ± 2464.6
mT9G	5020 ± 849.9	1689 ± 137.8	952 ± 330.6	619 ± 374.5	513 ± 168.0	244469 ± 22819.4	425229 ± 78859.3	68110 ± 11401.7
mTR5´MP	332 ± 150.4	48 ± 31.2	77 ± 36.8	180 ± 105.0	86 ± 58.6	16054 ± 481.1	1597 ± 228.0	13269 ± 4864.2
Total mT	**29916 ± 3726.4**	**6469 ± 944.3**	**2947 ± 1048.9**	**3945 ± 2338.9**	**2467 ± 541.2**	**417830 ± 40690.9**	**648649 ± 100317.0**	**245805 ± 25768.2**
oT	6 ± 0.7	8181 ± 1549.0	2900 ± 596.2	6496 ± 723.5	7168 ± 1313.6	40 ± 19.6	7 ± 1.3	58 ± 3.0
oTOG	2 ± 0.9	5034 ± 715.8	1633 ± 958.0	209 ± 40.5	401 ± 229.9	15 ± 3.7	29 ± 14.5	23 ± 0.0
oTR	0 ± 0.2	89 ± 31.1	42 ± 15.6	83 ± 26.4	124 ± 22.9	<LOD	<LOD	<LOD
oTROG	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
oT9G	1 ± 0.3	19766 ± 2077.5	8532 ± 2629.2	7166 ± 1669.3	10506 ± 3139.8	115 ± 64.2	103 ± 14.8	38 ± 7.4
oTR5´MP	35 ± 37.8	49 ± 27.9	86 ± 70.5	116 ± 98.9	105 ± 92.3	<LOD	<LOD	<LOD
Total oT	**44 ± 39.9**	**33118 ± 4401.2**	**13193 ± 4269.5**	**14071 ± 2558.6**	**18305 ± 4798.4**	**170 ± 87.4**	**139 ± 30.5**	**119 ± 10.4**
pT	1 ± 0.1	4 ± 1.3	1 ± 0.3	5 ± 0.3	3 ± 0.6	0 ± 0.2	1 ± 0.5	1 ± 0.3
pTOG	<LOD	17 ± 5.8	7 ± 0.4	9 ± 0.7	6 ± 2.4	<LOD	<LOD	<LOD
pTR	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
pTROG	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
pTR5´MP	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Total pT	**1 ± 0.1**	**21 ± 7.1**	**8 ± 0.7**	**14 ± 1.0**	**10 ± 3.1**	**0 ± 0.2**	**1 ± 0.5**	**1 ± 0.3**
K	58 ± 50.7	217 ± 129.1	85 ± 27.4	1151 ± 250.9	450 ± 238.9	394 ± 121.1	93 ± 2.5	1641 ± 2.2
KR	<LOD	3 ± 0.8	1 ± 0.2	4 ± 0.8	3 ± 1.6	2 ± 0.3	4 ± 0.7	3 ± 0.9
K9G	2 ± 0.1	831 ± 6.1	339 ± 51.5	486 ± 143.9	449 ± 95.5	35 ± 7.0	11 ± 1.5	47 ± 0.2
KR5´MP	<LOD	5 ± 0.7	2 ± 1.2	35 ± 32.0	12 ± 7.4	5 ± 0.8	<LOD	21 ± 1.3
Total K	**60 ± 50.7**	**1057 ± 136.8**	**427 ± 80.3**	**1676 ± 427.6**	**914 ± 343.4**	**435 ± 129.2**	**108 ± 4.7**	**1712 ± 4.5**
Total CK	**30597 ± 4000.6**	**414749 ± 57791.9**	**185667 ± 67089.1**	**327808 ± 99097.1**	**309113 ± 57980.2**	**426282 ± 41852.6**	**651674 ± 100929.1**	**251425 ± 27153.0**

Values are reported as mean ± standard deviation (n = 3); <LOD = below the limit of detection. Besides topolins, all the CKs abbreviations were based on **Kamínek et al. (2000)**

7.3.2 Effect of roscovitine and INCYDE on endogenous cytokinin content

Generally, aromatic CKs were the major CK-type occurring in both aerial **(Figure 7.2A)** and underground **(Figure 7.2B)** parts. With the exception of the aerial part in which *m*T and *m*T + roscovitine treatments had (mainly cZ and its derivatives) 39% and 14%, respectively the isoprenoid CK constituent in all the treatment was generally low (< 1%). **Figure 7.3A and B** depict the relative abundance (%) and pattern of the distribution and/or transportation of the isoprenoid and aromatic CKs within the plantlets in each treatment. The regenerated banana plantlets had more than 50% of the isoprenoid CK contents in the aerial region with the exception of *m*T + roscovitine and *m*T + INCYDE treatments. Conversely, the control (*m*T and BA) as well as *m*T + roscovitine and BA + roscovitine plantlets had at least 90% of their aromatic CK contents restricted to the underground region.

A total of 47 different CKs (aromatic and isoprenoid) were detected at varying concentrations across all the treatments in the aerial **(Table 7.1)** and underground **(Table 7.2)** parts. All the detected CKs were grouped into 9 CK-types namely: *t*Z, *c*Z, DHZ, iP, BA, *m*T, *o*T, *p*T and K. In *m*T containing treatments, *m*T-type was the most abundant CK and it was in the magnitude of *m*T + INCYDE > *m*T + INCYDE + roscovitine > *m*T + roscovitine > *m*T in the aerial part while it was *m*T + roscovitine > *m*T + INCYDE > *m*T > *m*T + INCYDE + roscovitine in the underground parts. On the other hand with BA-treated plantlets, BA-type CKs were the most abundant and total CK was in the order of BA + INCYDE + roscovitine > BA + INCYDE > BA + roscovitine > BA (aerial part) and BA > BA + roscovitine > BA + INCYDE + roscovitine > BA + INCYDE (underground part). There was a high concentration of *o*T and its derivatives, especially in the underground region of BA-treated plantlets **(Table 7.2)**. The total *o*T level in BA-treated plantlets was approximately 750% higher than in *m*T-treated ones. Furthermore, *o*T9G constituted the bulk (≥ 50%) of the total *o*T-type CK content. However, the addition of roscovitine and/or INCYDE to BA supplemented medium reduced the total *o*T content by more than 50%.

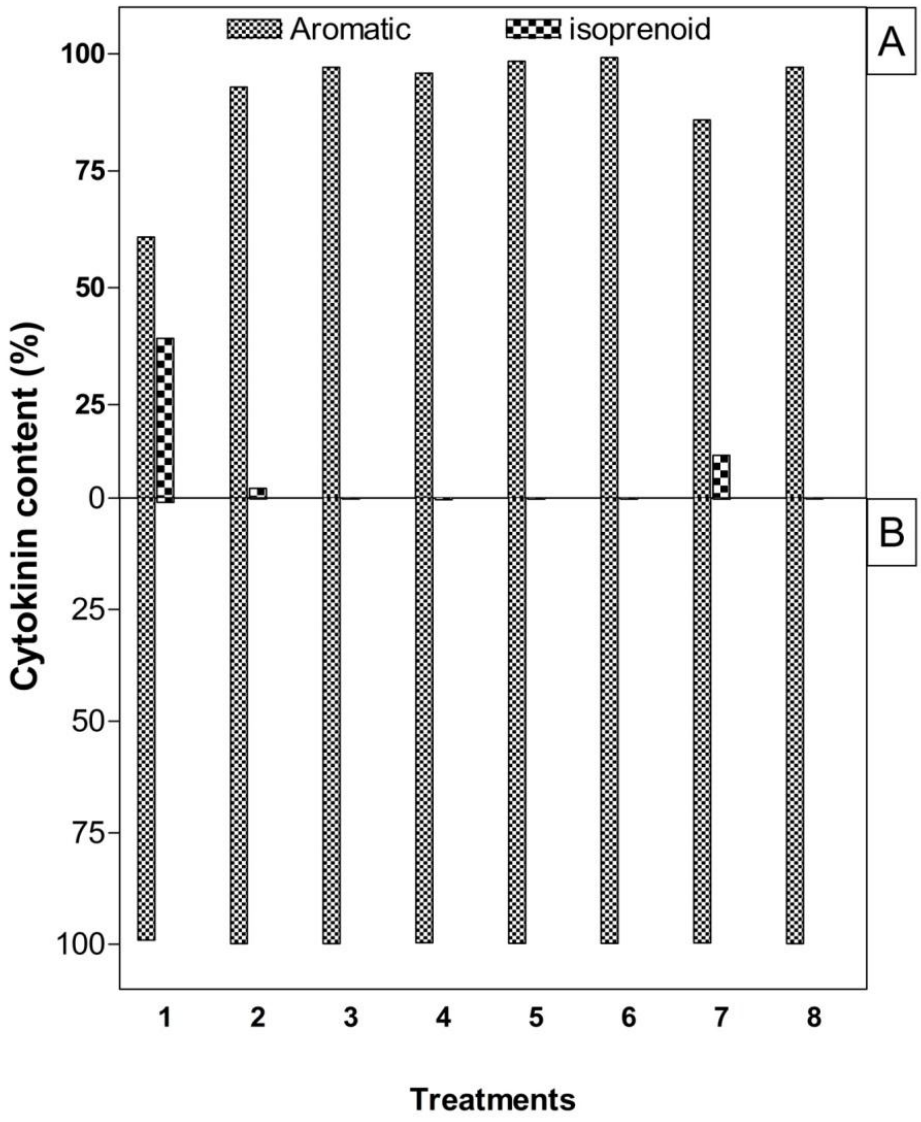

Figure 7.2: Total isoprenoid and aromatic cytokinin content (%) in aerial (A) and underground (B) parts of the micropropagated „Williams' bananas. 1 = *meta*-topolin; 2 = benzyladenine; 3 = benzyladenine + INCYDE; 4 = benzyladenine + roscovitine; 5 = benzyladenine + INCYDE + roscovitine; 6 = *meta*-topolin + INCYDE; 7 = *meta*-topolin + roscovitine; 8 = *meta*-topolin + INCYDE + roscovitine.

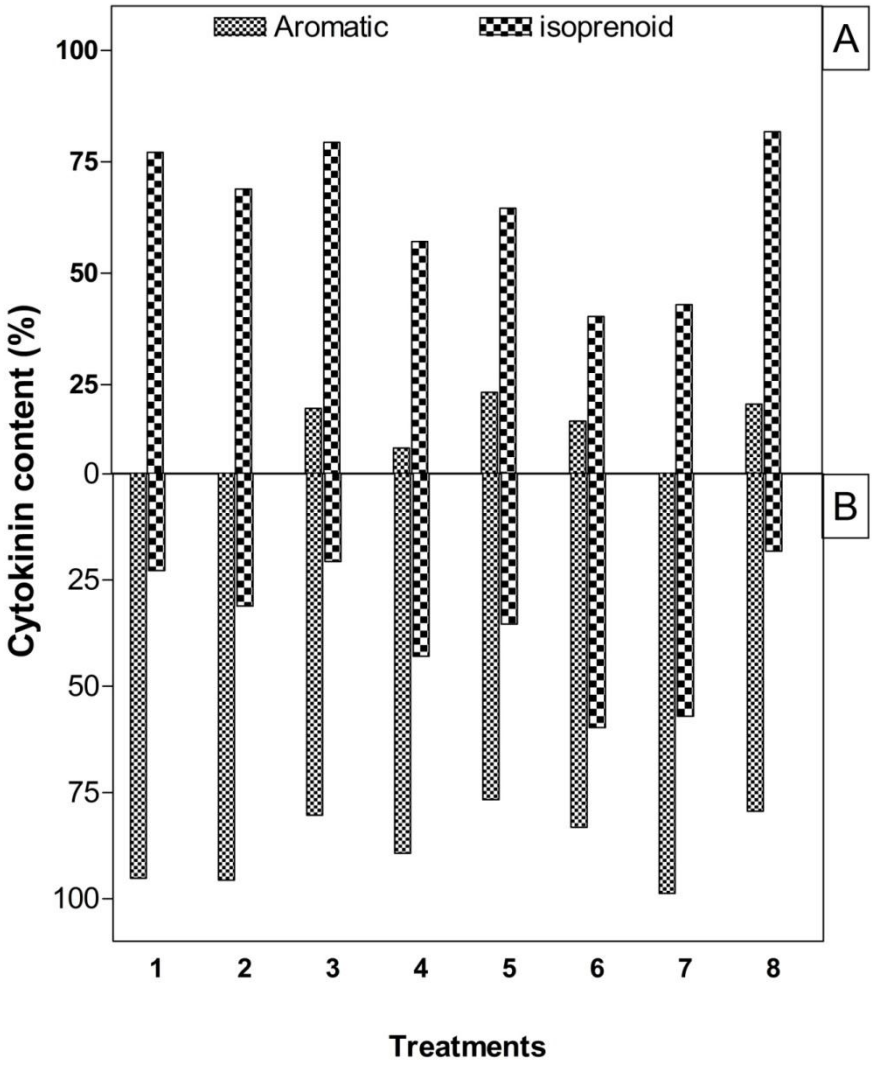

Figure 7.3: Relative abundance (%) and distribution pattern of the isoprenoid and aromatic cytokinins in the micropropagated „Williams' bananas. A = aerial and B = underground regions. 1 = *meta*-topolin; 2 = benzyladenine; 3 = benzyladenine + INCYDE; 4 = benzyladenine + roscovitine; 5 = benzyladenine + INCYDE + roscovitine; 6 = *meta*-topolin + INCYDE; 7 = *meta*-topolin + roscovitine; 8 = *meta*-topolin + INCYDE + roscovitine.

The importance of exogenous application of CKs for *in vitro* shoot development cannot be overemphasized, particularly in the micropropagation of bananas **(Bairu et al., 2008)**. The response of explants observed in terms of the growth and development *in vitro* are regulated by the interaction and balance between the applied PGRs (type and concentration) and the endogenously produced ones **(George, 1993; Krikorian, 1995)**. Factors such as the exogenous application of CKs are known to affect the biochemical pathways that regulate endogenous CK levels **(Krikorian, 1995; Blagoeva et al., 2004a)**. Since aromatic type CKs were used in the current study, as expected, the aromatic CKs were several fold more abundant compared to the isoprenoid CKs which are generally more common in plant tissue. When compared to the lower levels that ranged from approximately 0.2-39% for the total isoprenoid CK pool in the regenerants, the aromatic CKs were more abundant with the highest level (99.8%) observed in BA-treated plantlets **(Figure 7.2)**. **Ivanova et al. (2006)** reported a similar scenario whereby the application of exogenous BA to the media resulted in an alteration from isoprenoid to aromatic CKs in the newly-formed *Aloe polyphylla* shoots compared to those grown on CK-free and Z-supplemented media. As only aromatic CKs were used in the current study, the detection of isoprenoid CKs indicates the occurrence of the *de novo* CK synthesis pathway in the regenerated banana *in vitro*. However, the high concentration of the exogenously applied aromatic CK probably suppressed the isoprenoid CK synthesis pathway in the cultured explants. In addition, CKX have been shown to exhibit preferences for the isoprenoid CKs while the aromatic CKs are more resistant **(Zalabák et al., 2012)**. Interestingly, the current findings show that roscovitine and/or INCYDE when combined with *m*T or BA (with the exception *m*T + INCYDE) possibly prevented the breakdown of isoprenoid CK during the *in vitro* growth of „Williams' bananas **(Table 7.1 and 7.2)**.

Cytokinin uptake, metabolism and transport within the explants are vital parameters for the growth and development in PTC **(Auer, 1997)**. In the current study, the wide disparity between the aerial and underground parts is probably a function of the CK transport mechanism. In the eight treatments, the concentration of total CK detected in the underground parts was higher than in the aerial parts. Plantlets regenerated from

*m*T + roscovitine had the highest total CK content (661215 pmol/g FW) with the underground parts having approximately a 68-fold more than the aerial parts. A general trend observed was that the addition of roscovitine and/or INCYDE with *m*T improved the total CK pool in both aerial and underground parts of the regenerants. A similar pattern was observed in the aerial parts when BA was combined with roscovitine and INCYDE; however, both compounds reduced the total CK content in the underground sections as well as the sum total in the plantlets. It is noteworthy to highlight that the decrease in total CK pool was mainly due to the reduction in the quantity of 9-glucosides which are generally detrimental to plant growth. This reduction in 9-glucosides levels could be attributed to the presence of either roscovitine or INCYDE when acting individually with BA.

Often, most of the physiological activity of Z as a free base has been attributed to *t*Z while the *cis* isomer is regarded as an inactive or weakly active form of CK **(Kamínek et al., 1987; Haberer and Kieber, 2002)**. Although a precise role for *c*Z-type CKs remains to be fully elucidated, recent studies have been identifying their potential functions in plants **(Dwivedi et al., 2010; Gajdošová et al., 2011)**. In accordance with previous findings **(Blagoeva et al., 2004b; Dwivedi et al., 2010)** where olomoucine and its analogues such as roscovitine enhanced the *c*Z levels *in vitro*, roscovitine stimulated the production of more *c*Z in both aerial and underground regions of the regenerated plantlets compared to the controls **(Table 7.1 and 7.2)**. Although, *c*Z was less abundant in the underground region, the concentrations were several folds higher than the *t*Z in the plantlets. In addition to the potential role of cZ and/or its derivatives in regulation of physiological processes, **Gajdošová et al. (2011)** postulated that these compounds may be relevant under growth-limiting conditions connected to developmental processes or external signals.

In BA-supplemented treatments, 9-glucosides account for approximately 60% of total CK content **(Figure 7.4A and B)**. In terms of their distribution, an estimated 80% were detected in the underground part of the plantlets **(Figure 7.5B)**. The quantity and

distribution of CK derivatives are important information which may explain some observed physiological disorders in PTC **(Aremu et al., 2012)**.

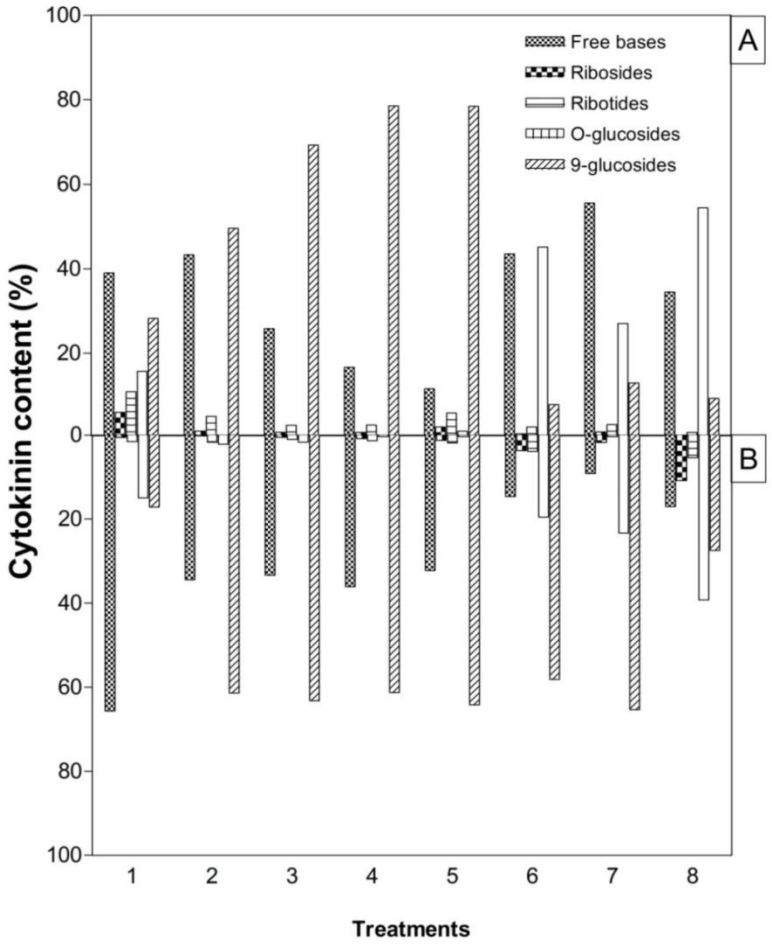

Figure 7.4: Total cytokinin pool (%) of the different cytokinin forms in aerial (A) and underground (B) parts of the micropropagated „Williams' bananas. 1 = *meta*-topolin; 2 = benzyladenine; 3 = benzyladenine + INCYDE; 4 = benzyladenine + roscovitine; 5 = benzyladenine + INCYDE + roscovitine; 6 = *meta*-topolin + INCYDE; 7 = *meta*-topolin + roscovitine; 8 = *meta*-topolin + INCYDE + roscovitine.

It is generally known that most 9*N*-glucosides, particularly BA9G are formed and stored in the basal region of plant and transport difficulty has been implicated in problems such as acclimatization failure **(Werbrouck et al., 1995)**, shoot-tip necrosis **(Bairu et al., 2011b)** and high toxicity **(Amoo et al., 2011)**. In the current study, when compared to BA alone, the presence of roscovitine and/or INCYDE with BA produced a substantial

quantity of the 9-glucosides in the aerial parts **(Figure 7.5A and B)**. The enhanced transportation of 9-glucosides could be a regulatory mechanism to alleviate the detrimental effect in the underground region of the plantlets.

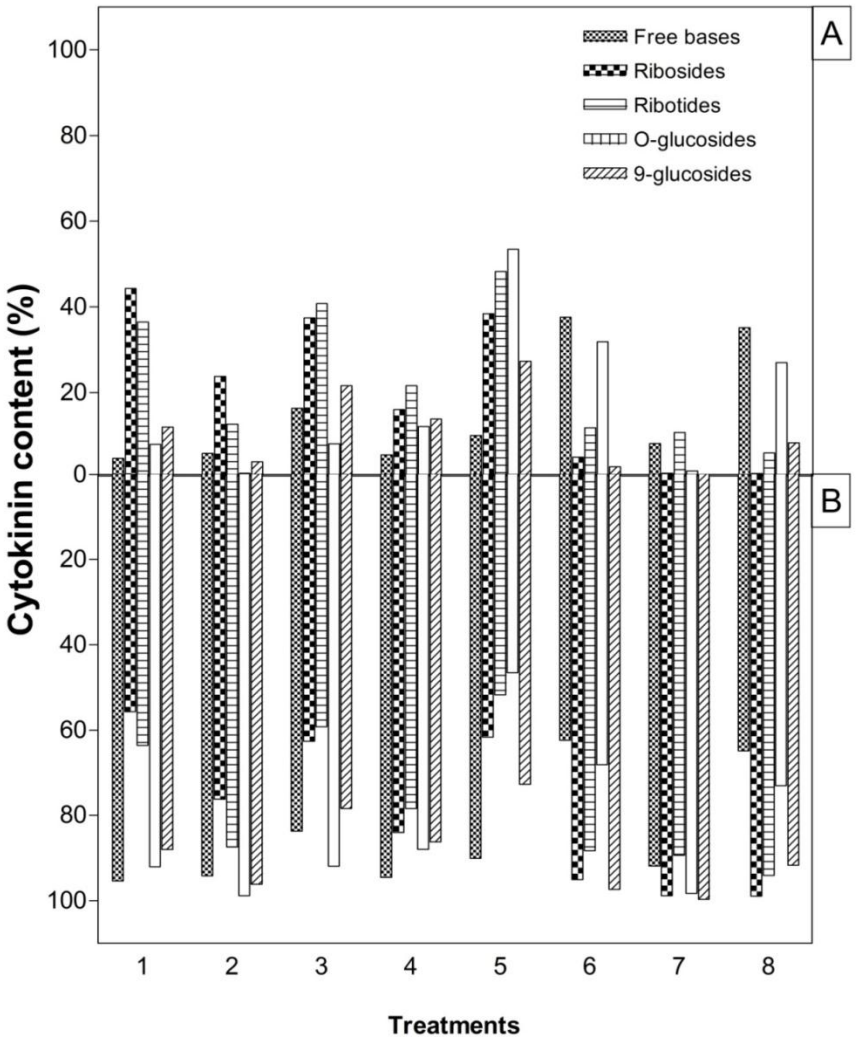

Figure 7.5: Relative abundance (%) and distribution pattern of the different cytokinin forms in the micropropagated „Williams' bananas. A = aerial and B = underground regions. 1 = *meta*-topolin; 2 = benzyladenine; 3 = benzyladenine + INCYDE; 4 = benzyladenine + roscovitine; 5 = benzyladenine + INCYDE + roscovitine; 6 = *meta*-topolin + INCYDE; 7 = *meta*-topolin + roscovitine; 8 = *meta*-topolin + INCYDE + roscovitine.

7.3.3 Effect of roscovitine and INCYDE on photosynthetic and phenolic contents

The combination of *m*T with roscovitine significantly increased the total phenolic and flavonoid levels in the plantlets but there was a drastic reduction in the amount of proanthocyanidins compared to the use of *m*T alone **(Table 7.3)**. When incorporated with *m*T, INCYDE or its combination with roscovitine did not have a notable effect on the phenolic contents detected. Plantlets treated with BA and INCYDE as well as BA + INCYDE + roscovitine had a significantly lower amount of total phenolics and flavonoids.

Table 7.3: Effect of roscovitine and INCYDE on the phenolic content of micropropagated „Williams' bananas

#Treatment	[1]Total phenolics (mg GAE /g DW)	[2] Total flavonoids (mg CE /g DW)	[3]Proanthocyanidins (µg CCE /g DW)
Control *m*T	58.4±2.23 d	16.3±0.13 f	534.8±6.10 cd
Control BA	118.3±5.83 a	34.3±0.24 a	612.2±13.00 ab
BA + INCYDE	91.7±3.28 bc	23.5±0.11 c	476.3±8.37 e
BA + Roscovitine	110.7±7.80 a	30.2±0.38 b	640.7±7.59 a
BA + INCYDE + Roscovitine	97.7±6.50 b	21.8±0.26 d	581.2±15.64 bc
*m*T + INCYDE	67.9±2.17 d	16.3±0.16 f	463.9±8.54 e
*m*T + Roscovitine	83.8±2.01 c	21.0±0.23 e	405.4±42.15 f
*m*T + INCYDE + Roscovitine	59.4±0.54 d	13.0±0.04 g	500.6±8.86 de

#Treatment: *m*T = *meta*-Topolin (30 µM); BA = Benzyladenine (30 µM); INCYDE = 100 µM; Roscovitine = 5 µM

[1]GAE = Gallic acid equivalents; [2]CE = Catechin equivalents; [3]CCE = Cyanidin chloride equivalents

Mean values ± standard error (n = 5) in the same column with different letter(s) are significantly different (*P* = 0.05) based on Duncan's multiple range test.

Table 7.4 shows the effect of combining roscovitine and INCYDE (alone or together) with either *m*T or BA on the photosynthetic pigment content of micropropagated „Williams' bananas. Generally, BA-treated plantlets had a higher pigment content compared to the *m*T treatments. Furthermore, the addition of roscovitine or INCYDE to BA media resulted in the production of more pigment than the use of BA alone. The combination of BA, roscovitine and INCYDE was detrimental to the production of photosynthetic pigments. Conversely, treatment with *m*T alone had an higher pigment

content compared to the presence of roscovitine or INCYDE. There was an increase in total chlorophyll content in *m*T + roscovitine + INCYDE-treated plantlets compared to the addition of *m*T + roscovitine or INCYDE treatments. The same combination (*m*T + roscovitine + INCYDE) had the highest chlorophyll/carotenoid ratio among all the treatments.

Even though the precise role of phenolic compounds in the processes of differentiation and morphogenesis are not fully understood **(Schnablová et al., 2006)**, their importance in plant growth remain undisputed **(Buer et al., 2010; De Klerk et al., 2011)**. It is well known that PGRs (e.g. CKs) influence levels of secondary metabolites in *in vitro* plantlets **(Ramachandra Rao and Ravishankar, 2002; Coste et al., 2011)**. In this study, the interaction of CKs with roscovitine and/or INCYDE exhibited diverse responses in terms of the amount of secondary metabolites quantified **(Table 7.3)**. Overall, the combination of roscovitine and/or INCYDE with BA had higher total phenolic and flavonoid contents than *m*T-treatments. Remarkably, there was a direct relationship between the total amount of endogenous CK and total phenolic content depending on the type of aromatic CK used. When *m*T was combined with roscovitine and/or INCYDE, there was an increase in total endogenous CKs **(Table 7.1 and 7.2)** which correlated with an increase in total phenolics compared to the use of *m*T alone **(Table 7.3)**. There was a reduction in the total endogenous CK content when BA was combined with roscovitine and/or INCYDE, with a decrease in the amount of phenolics compared to the BA-treated ones **(Table 7.3)**. Evidence from the study by **Schnablová et al. (2006)** demonstrated that overproduction of endogenous CKs caused a stress response in non-rooting *Pssu-ipt* transgenic tobacco grown *in vitro*. The authors observed the over-accumulation of phenolic compounds, synthesis of pathogenesis related proteins, and an increase in peroxidase activity. The use of these CK analogues could possibly play a vital role in the production of essential plant secondary metabolites as highlighted by **Planchais et al. (2000)** and **Blagoeva et al. (2003)**.

Table 7.4: Effect of roscovitine and INCYDE on the photosynthetic pigment contents of micropropagated „Williams' bananas

[#]Treatment	Chlorophyll a (µg/g FW)	Chlorophyll b (µg/g FW)	Carotenoid (µg/g FW)	Total chlorophyll (µg/g FW)	Chlorophyll a/b	Total chlorophyll /carotenoid
Control *m*T	257.8±27.78 abc	98.6±11.39 abc	80.9±8.84 ab	356.4±39.13 abc	2.6±0.03 abc	4.4±0.08 b
Control BA	328.0±42.40 abc	128.5±17.59 abc	95.6±10.83 ab	456.5±59.94 abc	2.6±0.04 ab	4.7±0.14 ab
BA + INCYDE	363.8±71.92 ab	137.3±23.56 ab	98.7±16.63 ab	501.0±95.38 ab	2.6±0.08 ab	5.0±0.13 a
BA + Roscovitine	389.5±83.03 a	157.2±32.14 a	117.2±21.39 a	546.7±115.16 a	2.5±0.03 bc	4.6±0.15 ab
BA + INCYDE + Roscovitine	298.5±45.86 abc	110.3±18.93 abc	87.9±12.01 ab	408.8±64.72 abc	2.7±0.06 a	4.6±0.14 ab
*m*T + INCYDE	199.3±37.16 c	73.3±12.80 c	58.5±8.42 b	272.5±49.94 c	2.7±0.04 a	4.6±0.26 ab
*m*T + Roscovitine	188.7±14.00 c	78.6±5.99 bc	61.2±3.58 b	267.3±19.89 c	2.4±0.04 c	4.4±0.21 b
*m*T + INCYDE + Roscovitine	217.0±35.33 bc	86.2±13.18 bc	59.7±9.60 b	303.3±48.36 bc	2.5±0.08 bc	5.1±0.08 a

[#]Treatment: *m*T = *meta*-Topolin (30 µM); BA = Benzyladenine (30 µM); INCYDE = 100 µM; Roscovitine = 5 µM
Mean values ± standard error (n = 5) in the same column with different letter(s) are significantly different (*P* = 0.05) based on Duncan's multiple range test.

Cytokinins are important for photosynthesis as they are known to influence the quantity of photosynthetic pigments among their numerous other functions. Furthermore, the protective action of CKs under stress conditions maintains the structure and function of the photosynthetic apparatus **(Chernyad'ev, 2009)**. In this study, the use of roscovitine and INCYDE in conjunction with the CKs did not cause any significant effect on the quantity of the photosynthetic pigments **(Table 7.4)**. Nevertheless, there was a slight increase in the amount of pigments when roscovitine and/or INCYDE were used together with BA and a minimum reduction in quantity in the presence of *m*T compared to the controls (BA or *m*T alone). Particularly for BA, higher concentrations are known to cause detrimental effects as demonstrated in carrot and *Arabidopsis* cultures **(Carimi et al., 2003)**. These authors observed that high concentrations of BA (27 µM) block cell proliferation and induce programmed cell death. Using a similar high concentration (30 µM) in this study, roscovitine or INCYDE alleviated the possible detrimental effects of BA on the ability of the *in vitro* plantlets to synthesize more photosynthetic pigments. Conversely, the use of *m*T with both roscovitine and INCYDE produced an opposite effect. The basis of the contrasting responsiveness of the CKs remains unclear suggesting the need for more stringent studies at a molecular level.

7.4 Concluding remarks

Often, the majority of available evidence of the molecular characterization and explanation of CK interactions are based on *Arabidopsis* mutants. However, the practical application of such findings on plants which are generally characterized by a more complex genome and physiology may exhibit different responses. From a practical perspective, current findings give valuable insights into the potential of roscovitine and INCYDE in micropropagation of „Williams' bananas and possibly in other plant species. Depending on the types of CK, it has been demonstrated that the endogenous CK levels can be easily regulated with the addition of roscovitine and/or INCYDE. Particularly, the use of these compounds could be useful for controlling some tissue culture-induced physiological disorders related to endogenous CKs. In addition, roscovitine (in the presence of *m*T) enhanced the levels of the total phenolics and

flavonoids in the micropropagated „Williams' bananas. Both CK analogues have also been identified to have a vital role in the regulation of the photosynthetic pigment and phytochemical content of micropropagated plants. The use of these compounds for the manipulation of plant physiology to improve the various aspects of growth and synthesis of secondary metabolites remains a challenging research topic. Furthermore, the presence of a significant quantity of *o*T in BA-treated cultures raises important questions such as their source and origin? Why are they not available in *m*T-treated cultures? Currently, the role of *o*T and *p*T in plant growth and development is not fully understood by researchers, more in-depth studies with the use of radio-labelled compounds could possibly elucidate their metabolism and functions in due course.

Chapter 8: Influence of high cytokinin concentration on the genetic stability of tissue-cultured 'Williams' bananas

8.1 Introduction

Micropropagation has become an integral part of the banana industry due to the numerous benefits derived from the use of tissue-cultured planting materials. As evidenced with other important medicinal, food and fruit crops **(Mithila et al., 2001; Mondal et al., 2004; Dobránszki and Teixeira da Silva, 2010; Rout et al., 2010; Moyo et al., 2011a)**, the application of micropropagation has been vital in meeting the increasing demand for banana production globally **(Vuylsteke et al., 1998; Strosse et al., 2004)**. Despite the enormous benefits associated with the micropropagation technique **(Petolino et al., 2003; Caponetti et al., 2005)**, some inherent problems remain a common occurrence during the process **(Kevers et al., 2004; Bairu et al., 2009c; Bairu et al., 2011a)**. Particularly, the incidence of somaclonal variation is common in many banana micropropagation protocols **(Reuveni and Israeli, 1990; Côte et al., 1993; Damasco et al., 1998; Bairu et al., 2006)**. Common characteristics of the somaclonal variants include the presences of dwarf off-types, variations in foliage as well as colour and morphology of pseudostems **(Damasco et al., 1998; Israeli et al., 1991; Smith and Hamill, 1993)**. Among the various factors implicated in the incidence of somaclonal variation (see **Section 2.3**), the effect of the type and concentration of plant growth regulators (PGRs) particularly the cytokinins (CKs) remains critical **(Nehra et al., 1992; Bairu et al., 2011a)**.

As emphasized by **Smith and Hamill (1993)** as well as **Bairu et al. (2011a)**, the early detection of the presence of somaclonal variants remains critical as it saves valuable time and minimizes the overall economic loss to the users of tissue-cultured planting materials. As highlighted in **Section 2.3.2**, comprehensive reviews focusing on the various techniques employed for detecting the genetic fidelity of micropropagated plants have been documented **(Agarwal et al., 2008; Bairu et al., 2011a; Neelakandan and Wang, 2012)**. No doubt, the use of different molecular tools has been firmly accepted

as the most reliable approach for early detection of genetic variation in regenerated plantlets. The use of different molecular markers for diversity and genetic stability analysis in several plant species such as sorghum **(Aruna et al., 2012)**, rice **(Araújo et al., 2001)**, aloe **(Rathore et al., 2011)** and many banana cultivars **(Ray et al., 2006; Venkatachalam et al., 2007b; Borse et al., 2011)**. Among the various molecular markers, the use of ISSR markers remains popular due to their relative simplicity, reliability, cost effectiveness and highly discriminative nature. In addition, there is no need for any prior primer sequence information and they have been widely-accepted in numerous fields such as genetic diversity, phylogenetic studies, ecology and evolutionary biology **(Bahulikar et al., 2004; Chandrika et al., 2008; 2010; Aruna et al., 2012)**.

Several studies on different banana cultivars focusing on the role of CKs and other tissue culture conditions are available **(Sahijram et al., 2003; Bairu et al., 2006; Ray et al., 2006; Oh et al., 2007; Venkatachalam et al., 2007b)**. Nevertheless, some salient and confounding factors were clearly prominent in the majority of the previous studies. For instance, the residual or carry-over effect of different CKs remained inexplicable **(Zaffari et al., 2000; Bairu et al., 2006)**. In the current experiment, the effect of a relatively high concentration (30 µM) of CK (*meta*-topolins and benzyladenine, BA) on „Williams' banana plantlets maintained over seven shoot multiplication cycles were evaluated using ISSR markers.

8.2 Materials and methods

8.2.1 Plant material

In vitro banana plantlets obtained as described in **Section 3.2.2** and maintained on modified MS medium with the same type of CKs (30 µM) were regularly subculture at 42 day intervals. Plantlets at the seventh subculture cycle were used for the current experiment. Two types of controls which included the initial plantlets used for culture initiation and plantlets maintained on a CK-free modified MS medium with the same number of subcultures as the CK-treated plantlets were used.

8.2.2 Isolation and quantification of plant genomic DNA

After a 42 day culture cycle, the leaves from the individual CK-treated and control plantlets were harvested and crushed in liquid nitrogen in a mortar and pestle. Similarly, rooted plantlets purchased from Du Roi Laboratory used for the culture initiation were harvested and stored at -70 °C. Using a DNA extraction kit (ZR Plant/Seed DNA MiniPrep™, California, USA), the genomic DNA of approximately 150 mg pulverized plant material was isolated following the manufacturer's instructions. Thereafter, the concentration and purity of the isolated genomic DNA was measured at 260 and 280 nm using a UV visible spectrophotometer (NanoDrop 2000, Thermo Scientific Inc., Wilmington, USA).

8.2.3 Amplification of isolated DNA using inter simple sequence repeat (ISSR) primers

Polymerase chain reactions (PCR) were performed for the isolated DNA products. Briefly, 50 ng of genomic DNA was added to 25 µl PCR Master Mix (2X) (Thermo Scientific, California, USA) consisting of 0.05 u/µl *Taq* DNA polymerase, reaction buffer, 4mM $MgCl_2$, 0.4 mM of each dNTP (dATP, dCTP, dGTP and dTTP). Thereafter, 0.5 µl of individual ISSR primers, University of British Columbia (UBC) (Table 8.1) was added and the nuclease-free water was used to adjust the final volume to 50 µl. Amplification was performed using a real-time PCR machine (Applied Biosystems Veriti™ Thermal Cycler, California, USA). The amplification reaction included an initial denaturation temperature of 95 ºC for 2 min (1 cycle), an elongation step that involved 40 cycles of 30 s denaturation at 95 °C, 30 s annealing at 45 °C and 1 min extension at 72 °C, followed by final extension for 15 min at 72 °C.

Table 8.1: Characteristics of the ISSR primers used for the current study

Primer code		Primer sequence	Tm max	Number of bands
1	UBC-808	AGAGAGAGAGAGAGAGC	57.19	6
2	UBC-817	CACACACACACACACAA	54.78	6
3	UBC-827	ACACACACACACACACG	57.17	5
4	UBC-835	AGAGAGAGAGAGAGAGYC	59.9	6
5	UBC-841	GAGAGAGAGAGAGAGAYC	59.9	6
6	UBC-844	CTCTCTCTCTCTCTCTRC	59.9	5
7	UBC-859	TGTGTGTGTGTGTGTGRC	59.9	5
8	UBC-860	TGTGTGTGTGTGTGTGRA	57.62	6
9	UBC-868	GAAGAAGAAGAAGAAGAA	50.79	8
10	UBC-880	GGAGAGGAGAGGAGA	56.17	6

UBC = University of British Columbia
Tm = Melting temperature

8.2.4 Amplified DNA product electrophoresis

After thermocycling, 5 µl of 6X DNA loading dye (10 mM Tris-HCl pH 7.6, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol and 60 mM EDTA) were mixed with 10 µl of PCR reaction product. The amplification products were analyzed by gel electrophoresis in 1% agarose (Hispanagar, Burgos, Spain) containing 25 µl per 100 ml nucleic acid gel stain (GRGreen, Gaithersburg, USA). For reference purposes, a DNA molecular weight marker (GeneRuler™1kb plus DNA, Fermentas, California, USA) was included on each gel. Using a Max Submarine Agarose Gel unit (Hoefer Scientific Instruments, San Francisco, California, USA), the agrose gel was run in 1X TAE at 7 V/cm for 8 h. Resultant images were visualized and photographed using a Gel Documentation system fitted with 8 b CCD camera and UV light (Syngene Gel Doc, Syngene, Synoptics Ltd, UK). The captured images were used for data analysis.

8.2.5 Band scoring and data analysis

In order to accredit the polymorphism to a given ISSR marker, locus absence of an amplified band in at least one individual CK sample was taken as a minimum criterion. Percentage band polymorphism (PBP) was calculated as the number of polymorphic

bands × 100/total number of bands obtained for each primer. Amplified DNA banding patterns generated by ISSR primers in the lanes were scored as (1) for the presence or (0) for the absence. All amplifications were repeated twice and only reproducible bands were scored. Matrix data were analyzed using the GenAlEx 6.4 (Genetic Analysis in Excel) **(Peakall and Smouse, 2006)**.

8.3 Results and discussion

In view of the importance of banana for food security, it remains one of the most highly prioritized research fruit/crops globally. For decades, the role of different factors which could contribute to the improvement of the micropropagation process of banana has received considerable attention **(Kodym and Zapata-Arias, 2001; Ray et al., 2006; Martin et al., 2007; Venkatachalam et al., 2007b; Bairu et al., 2008; Borse et al., 2011)**. Despite the vast number of studies especially, on the role of CKs on the genetic fidelity of micropropagated bananas, it is obvious that several other factors contributes to the overall outcome **(Sahijram et al., 2003)**. As a result, the need for more information on the safe number of subculture cycles, CK levels, genotype, ploidy level amongst others for the different banana cultivars has been emphasized **(Sahijram et al., 2003; Bairu et al., 2008; Borse et al., 2011)**.

In the current study, the 10 ISSR markers generated bands ranging from 35 to 49 as observed in the initial and *m*T-treated plantlets, respectively **(Table 8.2)**. The percentage of polymorphic bands increased from approximately 24% in the initial plantlets to 56% in *m*T regenerated plantlets. Clearly, the presence of the different CKs increased the degree of variation. The ability of different CK type and concentration to induce somaclonal variation in tissue-cultured banana has been observed by several researchers **(Bairu et al., 2006; Martin et al., 2006; Bairu et al., 2008)**. **Venkatachalam et al. (2007b)** used RAPD and ISSR markers to confirm the genetic stability of banana micropropagated in the presence of high CK levels. However, the well-known differences in responses of different banana cultivars under *in vitro* conditions may have contributed to the variation observed in this study.

Table 8.2: ISSR analysis of the genetic stability of „Williams' banana plantlets using different cytokinins

#Treatment	Polymorphic loci (%)	Bands					
		Total	Average	NBF	UB	NLCB - 25%	NLCB - 50%
Initial plantlets	23.73	35	3.5	35	0	0	0
Control	35.59	46	4.6	46	1	0	5
*m*T	55.93	49	4.9	49	1	0	6
*m*TR	37.29	41	4.1	41	1	0	3
Me*m*T	40.68	48	4.8	48	2	1	5
Me*m*TR	42.37	44	4.4	44	1	1	3
BA	40.68	45	4.5	45	0	1	4
Me*m*TTHP	37.29	48	3.7	48	1	1	5

NBF = Number of different bands with a frequency >= 5%
UB = Number of bands unique to each treatment
NLCB - 25% = Number of locally common bands present in 25% or fewer treatments
NLCB - 50% = Number of locally common bands present in 50% or fewer treatments
#Treatment: *m*T = *meta*-Topolin; *m*TR = *meta*-Topolin riboside; Me*m*T = *meta*-Methoxy topolin; Me*m*TR = *meta*-Methoxy topolin riboside; BA = Benzyladenine; Me*m*TTHP = *meta*-Methoxy topolin 9-tetrahydropyran-2-yl.

The increase in percentage of polymorphic bands in the control plantlets is an indication that other factors besides the presence of CK in the medium contributed to the observed variations. Often, it has been postulated that the incidence of somaclonal variation is multi-dimensional ranging from the effect of initial explant, genotype and culture conditions including culture duration and number of subcultures **(Sahijram et al., 2003; Venkatachalam et al., 2007b; Bairu et al., 2011a)**. Particularly, the effect of subculture has been studied in numerous cultivars **(Reuveni et al., 1993; Damasco et al., 1998; Bairu et al., 2006; Venkatachalam et al., 2007c; Borse et al., 2011)**. Findings from the majority of the studies indicate an increase in degree of variation with an increase in the number of subculture cycles. The rapid increase in variation during the later stages of the multiplication phases could be associated with extended/ accumulated duration in culture or the multiplication of the variants produced in preceding stages, or even both factors **(Damasco et al., 1998; Bairu et al., 2006)**. Apparently, the high instability in most variants could have contributed to the high occurrence of variants within the individual CK regenerants in this study.

8.4 Concluding remarks

Based on the current findings, it is evident that the effect of subculturing contributed significantly to the higher rate of variation in 'Williams' bananas *in vitro*. The presence of CK in the culture media apparently aggravate the stress on the explants as indicated in the relatively higher percentage polymorphic bands compared to the controls. Among the tested CKs, the use of *m*TR and Me*m*TTHP caused the least detrimental effect on the regenerants while *m*T-treated plantlets had the most polymorphic bands. The ability of the ISSR markers to detect variation in the regenerants at the early *in vitro* stage is further evidence of the relevance of the use of molecular markers in micropropagation. In terms of the basic aim of banana micropropagation which is the production of a large pool of uniform and healthy plantlet devoid of any genetic variations, it is probably better to reduce the subculturing cycles of the initial explants. In fact, regardless of the type of CK applied, the establishment of new culture lines after about five subculture cycles is recommended in order to minimize the rate of variability.

Chapter 9: General conclusions and recommendations

Bananas remain an important food crop and commercial banana industries have acknowledged the use of plant tissue culture (PTC) as a vital tool to attain increased productivity. The role of topolins in the micropropagation of „Williams' bananas was critically evaluated based on evidence of the increasing potential of topolins in various PTC protocols. The highest shoot proliferation (7.3±1.0) was obtained with a 30 µM *m*T-supplemented medium. In contrast to other CK treatments requiring higher concentrations, highest shoot number per explant rate was attained using 10 µM Me*m*T and Me*m*TTHP treatments. Similarly, 10 µM Me*m*TTHP had the least root inhibitory effect during the shoot proliferation phase. From a commercial perspective, the efficiency of the aforementioned topolins at relatively lower concentrations will inevitably reduce the overall input cost during the micropropagation process. During the rooting phase, topolin treatments (*m*T = 40%) produced more off-shoots than BA-treated (13%) ones. The additional shoots produced makes the use of topolins an attractive and profitable CK as it contributes to the overall total number of potential shoots. In terms of abnormality index, *m*TR-regenerated plantlets were of the best quality across all the CKs tested. Unfortunately, some topolins (Me*m*T and Me*m*TR) had higher toxic effects on the resultant shoots compared to BA. Similarly, molecular marker-based genetic fidelity assessment indicates that the use of *m*TR and Me*m*TTHP had the least detrimental effect on the regenerants while *m*T-treated plantlets had high variability. Although all the CKs demonstrated high polymorphism, it is evident that the effect of subculturing contributed significantly to the higher rate of variation in the micropropagated 'Williams' bananas *in vitro*. The presence of CK in the culture media apparently aggravated the stress on the explants as indicated in the relatively higher percentage polymorphic bands compared to the controls. Perhaps, the establishment of new culture lines after approximately five subculture cycles will minimize the rate of variability. Overall, the current findings from the general growth parameters provide further evidence on the increasing potential of the topolins in PTC.

Some physiological and anatomical parameters were generally better in some topolin-treated compared to BA-treated cultures. In all cases, the maximum photosynthetic pigment content was attained between 40-50 days. Although the control plantlets had the highest pigment content, 10 µM Me*m*TTHP had the best pigment stimulatory effect among the tested CKs. In addition, scanning electron microscopy (SEM) of the foliar surface revealed that the stomata density was highest in 10 µM Me*m*TTHP-treated and lowest in 10 µM Me*m*TR-treated plantlets. Generally, prolonging culture duration as well as increasing the CK concentrations reduced the pigment content. However, the drastic breakdown in chlorophyll pigments beyond 50 days was slightly inhibited by the presence of *m*T, *m*TR, Me*m*TTHP and BA compared to the control. The current findings are further evidence of the importance of the type and concentration of applied CK in PTC. The availability of such physiological data on the effect of applied CK is useful as it could be decisive to both tissue culturists and commercial industries on the choice of CK for a particular task. Along the same line, the levels of plant secondary metabolites such as phenolics and flavonoids were generally higher in most of the topolin-treated (*m*T, Me*m*TTHP) plantlets. The majority of the plant secondary metabolites are known to play vital roles for plant survival upon transfer to *ex vitro* conditions. Furthermore, as plant secondary metabolites have been implicated in the therapeutic activity of plants used as medicine, the use of topolins possibly offers an alternative approach to increase the valuable metabolite content in medicinal plants. Besides the potential of the topolins as elicitors, an increase in the levels of secondary metabolites in micropropagated plantlets has been hypothesized to enhance their survival under *ex vitro* conditions. However, more in-depth molecular-based studies resulting in a better understanding of the basic mechanisms responsible for the higher metabolite levels in the topolin-treated plantlets would be indispensable.

Although the CK- and auxin-like activity of SW and KAR_1 has been postulated and demonstrated by several researchers, both compounds were only important for root stimulation in „Williams' banana micropropagation. Their ability to increase the phytochemical content and photosynthesis was identified as additional benefits associated with the use of both compounds. The activity of the compounds at extremely

low concentrations under *in vitro* conditions highlights the great potential still to be derived from the application of these compounds in PTC. Therefore, it will be a worthwhile experience to critically elucidate the underlying mechanisms of action of these compounds. The possible synergetic or antagonistic effects of SW and KAR_1 in the presence of other PGRs, also offers a new niche for plant physiologists and tissue culturists to explore.

Additional experiments with the objective of improving acclimatization competence of micropropagated „Williams' banana using either SW or vermicompost leachate under greenhouse conditions demonstrated great potential. For instance, soil drenching with SW significantly increased the root length (1:1000 and 1:500 dilutions) as well as fresh and dry weights (1:1000; 1:500 and 1:250 dilutions) when compared to foliar application. Vermicompost leachate (1:10 and 1:5 dilutions) significantly enhanced the shoot length, root length, leaf area and dry weights. Vermicompost leachate (1:20; 1:10 and 1:5 dilutions) also significantly increased the number of off-shoots. The positive effect on rooting is beneficial for acclimatization and establishment of micropropagated banana plantlets in nurseries and subsequent transfer to the field. The production of more off-shoots by the vermicompost leachate will inevitably increase the expected crop yield. The current findings contribute to the global efforts by researchers to evaluate the competence of environmental-friendly organic fertilizers and smoke solutions to improve the growth, yield and quality of various crops. The ease of application of the compounds remains an additional benefit as it could be easily embraced and employed by peasant farmers for crop improvement. Moreover, the high cost of inorganic fertilizers as well as environmental safety concerns makes the use of SW and vermicompost leachate a better alternative approach for increasing banana production to meet the escalating demands.

The use of CK analogues (roscovitine and INCYDE) with either *m*T- or BA-requiring cultures provided some insights on the endogenous CK profiles and physiology of banana *in vitro*. It was shown that the use of the CK analogue improved the growth as well as the levels of the phytochemicals and photosynthetic pigments. The CK analogue

particularly, roscovitine significantly increased the levels of endogenous CK in the presence of *m*T. On the other hand, both compounds reduced the levels of endogenous CK in the underground parts of BA-requiring cultures. There was a marked reduction in the quantity of 9-glucosides. These toxic CK metabolites are known to be detrimental to growth and are implicated in various physiological disorders in tissue-cultured plantlets. In the presence of CK analogous such as roscovitine and INCYDE, the current findings suggest that BA remains useful in PTC as the commonly observed physiological problems could be easily minimized. From a practical perspective, the use of roscovitine and INCYDE in micropropagation could be crucial in the alleviation of commonly observed *in vitro*-induced physiological abnormalities. As observed by few other researchers, the presence of an extremely large pool of *o*T in BA-treated plantlets (about 750% more than in *m*T treatment) remained unexplained and a mystery. Further stringent studies involving the use of labelled BA would possibly provide some insight and elucidate the source or the biosynthetic pathway of these compounds.

As highlighted by **Bairu et al. (2008)**, the current findings are further substantiation on the potential of topolins in banana micropropagation. Although some questions still remain unanswered, vital physiological and fundamental insights on the role of topolins in micropropagated „Williams' banana has been established. These could be easily applied to other plant species or banana cultivars. The current findings are a further demonstration of the increasing importance of topolins in PTC especially, compared to BA. Of particular interest is the new derivative Me*m*TTHP which demonstrated several advantages in most of the experiments performed. Perhaps, Me*m*TTHP could be an alternative CK for the micropropagation of plant species known for physiological disorders such as rooting inhibition and poor acclimatization competency. Nevertheless, it should be noted that there are species that may respond better to CKs other than the topolins. To avoid reaching an incorrect conclusion, it is recommended that fresh cultures are started to investigate the role of topolins to avoid possible carry-over effects of preceding CKs and culture additives. Lastly, it is necessary to emphasize that topolins should not be taken at face value and must pass through the routine process of CK selection.

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APPENDIX 1: Protocol for modified MS basal medium for *Musa* spp. shoot-tip culture

Component of stock solution

Stock	Salt component	Mass/500 ml stock (g)	Mass/1000 ml	Volume stock (ml l^{-1}) final medium
A: Macro 1	KNO_3	47.50	95.0	20
	NH_4NO_3	41.25	82.5	
	$CaCl_2.2H_2O$	11.00	22.0	
B: Macro 2	$MgSO_4.7H_2O$	9.25	18.5	20
C: Macro 3	KH_2PO_4	4.25	8.5	20
D: Micro	$MnSO_4.H_2O$	8.45	16.9	1
	H_3BO_3	3.20	6.20	
	KI	0.41	0.83	
	$ZnSO_4.4H_2O$	4.30	8.60	
	$Na_2MoO_4.2H_2O$	0.125	0.25	
	$CuSO_4.5H_2O$	0.0125	0.025	
	$CoCl_2.6H_2O$	0.0125	0.025	
E: Fe-stock	$FeSO_4.7H_2O$	2.785	5.57	5
	$Na_2EDTA.2H_2O$	3.725	7.45	
F: Vitamins & amino acids	Glycine	1.0	2.0	1
	Thiamine.HCl	0.2	0.4	
	Pyridoxin.HCl	0.25	0.5	
	Nicotinic acid	0.25	0.5	
G: Anti-oxidant	Ascorbic acid	10.0	20.0	1

Other additives

Sugar: 30 g l^{-1}
Gelrite: 3 g l^{-1}
Plant growth regulators (cytokinins and auxins)

Vuylsteke, D. R. (1998). Shoot-tip Culture for the Propagation, Conservation, and Distribution of *Musa* Germplasm. Ibadan, Nigeria, International Institute of Tropical Agriculture.