Investigations on the biological effects of smoke-water and smoke-derived compounds in agriculture and horticulture

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**Submitted in fulfilment of the academic
requirements for the degree of
Doctor of Philosophy**

**Research Centre for Plant Growth and Development
School of Life Sciences
University of KwaZulu-Natal, Pietermaritzburg**

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FACULTY OF SCIENCE AND AGRICULTURE
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We hereby declare that we acted as Supervisors for this PhD student:

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Student Number: 210551088

Thesis Title: Investigations on the biological effects of smoke-water and smoke-derived compounds in agriculture and horticulture

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

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DECLARATION 2 – PUBLICATIONS

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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 (Published)

POŠTA M, LIGHT ME, PAPENFUS HB, VAN STADEN J, AND KOHOUT L (2013). Structure–activity relationships of analogs of 3,4,5-trimethylfuran-2(5H)-one with germination inhibitory activities. *Journal of Plant Physiology 170*, 1235-1242.

Dr Pošta (collaborator) synthesised karrikinolide and the analogues of trimethyl-butenolide that were used in this study. Dr Pošta also co-wrote the manuscript.

Dr Light supervised the germination assays and the analysis of data. Dr Light co-wrote the manuscript.

Mr Papenfus conducted the germination assays and prepared the photographs. Mr Papenfus also analyzed the data, prepared the graphs under the supervision of Dr Light and co-wrote the manuscript.

Prof. Van Staden (supervisor) partly funded the research and edited the manuscript.

Prof. Kohout (collaborator) funded the synthesis of all the compounds used in the study and edited the manuscript.

Publication 2 (Published)

PAPENFUS HB, KUMARI A, KULKARNI MG, FINNIE JF AND VAN STADEN J (2014). Smoke-water enhances *in vitro* pollen germination and tube elongation of three species of Amaryllidaceae. *South African Journal of Botany 90*, 87-92.

Mr Papenfus conceptualized the idea, conducted the experimental work in combination with Dr Kumari since the experiments were time dependent. Mr Papenfus also wrote the manuscript, analyzed the data and produced the graphs.

Dr Kumari contributed with the experimental work.

Dr Kulkarni supervised the analyses of data and edited the manuscript.

Prof. Finnie (co-supervisor) partly funded the project and edited the manuscript.

Prof. Van Staden (supervisor) partly funded the project and edited the manuscript.

Publication 3 (in press)

KUMARI A, PAPENFUS HB, KULKARNI MG, POŠTA M, FINNIE JF, AND VAN STADEN J (2014). Effect of smoke derivatives on *in vitro* pollen germination and pollen tube elongation of species from different plant families. *Plant Biology.*

Dr Kumari contributed with the experimental work and co-wrote the manuscript.

Mr Papenfus conceptualized the idea, conducted the experimental work in combination with Dr Kumari since experiments were time dependent. Mr Papenfus also co-wrote the manuscript, analyzed the data and produced the graphs.

Dr Kulkarni supervised the analyses of data and edited the manuscript.

Dr Pošta (collaborator) synthesised the karrikinolide and trimethylbutenolide compounds used in this study.

Prof. Finnie (co-supervisor) partly funded the project and edited the manuscript.

Prof. Van Staden (supervisor) partly funded the project and edited the manuscript.

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PAPENFUS HB, KULKARNI MG, POŠTA M, FINNIE JF AND VAN STADEN J (2014). Smoke-isolated trimethylbutenolide inhibits seed germination of different weed species by reducing amylase activity. *Weed Science.*

Mr Papenfus conceptualized the idea and conducted the experimental work. Mr Papenfus also analyzed the results, prepared the graphs and wrote the manuscript.

Dr Kulkarni supervised the analyses of results and the preparation of graphs.

Dr Pošta (collaborator) synthesised the karrikinolide and trimethylbutenolide compounds used in this study.

Prof. Finnie (co-supervisor) partly funded the project and edited the manuscript.

Prof. Van Staden (supervisor) partly funded the project and edited the manuscript.

Publication 5 (In preparation)

PAPENFUS HB, KULKARNI MG, KANNAN RRR, STIRK WA, BOTTINI R AND VAN STADEN J 2014. Interactions between a plant growth-promoting rhizobacterium (*Bacillus licheniformis*) and smoke-derived compounds and their effect on *Abelmoschus esculentus* (okra) growth.

Mr Papenfus conceptualized the idea and conducted the experimental work. Mr Papenfus also analyzed the results and wrote the manuscript.

Dr Kulkarni aided in harvesting and measuring the growth parameters. Dr Kulkarni also supervised the analyses of results and the preparation of graphs.

Dr Stirk aided in capturing growth parameters and edited the manuscript.

Dr Kannan aided in capturing growth parameters.

Prof Bottini (collaborator) partly funded the project and provided the rhizobacterium (*Bacillus licheniformis*) inoculum.

Prof. Van Staden (supervisor) partly funded the project and edited the manuscript.

Publication 6 (submitted)

PAPENFUS HB, FINNIE JF AND VAN STADEN J (2014). Plant-derived smoke increases the rate of germination and development of *Ansellia africana* seeds.

Mr Papenfus conceptualized the idea and conducted the experimental work. Mr Papenfus also analyzed the results, prepared the graphs and wrote the manuscript.

Prof. Finnie (co-supervisor) partly funded the project and edited the manuscript.

Prof. Van Staden (supervisor) partly funded the project and edited the manuscript.

Publication 7 (Published)

NAIR JJ, MUNRO OQ, POŠTA M, PAPENFUS HB, BEIER PJ AND VAN STADEN (2013). X-ray crystallographic structure determination of the smoke-derived karrikin KAR_3. *South African Journal of Botany 88*, 107-109.

Dr Nair supervised the project and co-wrote the manuscript.

Dr Munro (collaborator) determined the crystal structure of KAR_3.

Dr Pošta (collaborator) synthesised KAR_3.

Mr Papenfus tested the germination promoting activity of KAR_3 and produced the KAR_3 crystal. Mr Papenfus also co-wrote the manuscript.

Prof. Beier (collaborator) partly funded the project and provided the equipment for determining the X-ray crystallographic structure of KAR_3.

Prof. Van Staden partly funded the project and edited the manuscript.

Publication 8 (Published)

NAIR JJ, POŠTA M, PAPENFUS HB, MUNRO OQ, BEIER PJ AND VAN STADEN (2014). Synthesis, X-ray structure determination and germination studies on some smoke-derived karrikins. *South African Journal of Botany 91*, 53-57.

Dr Nair supervised the project and wrote the manuscript.

Dr Pošta (collaborator) synthesised the compounds used.

Mr Papenfus conducted the experimental work, analyzed the germination data and produced the graphs. Mr Papenfus also co-wrote the manuscript.

Dr Munro (collaborator) determined the crystal structure of the compounds.

Prof. Beier (collaborator) partly funded the project and provided the equipment for determining the X-ray crystallographic structure of the karrikins.

Prof. Van Staden partly funded the project and edited the manuscript.

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Declaration Publications FHDR 22/05/08 Approved

CONFERENCE CONTRIBUTIONS FROM THIS RESEARCH

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Papenfus HB, Pošta M, Light ME, Van Staden J and Kohout L. 2013. Synthesis of analogues of 3,4,5-trimethyl-2(5*H*)-furanone, 39'th congress of SAAB – South African Association of Botanists, Bergville.

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ABSTRACT

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Plant-derived smoke plays an important role in the germination of seeds of many plant species in their natural environments. Smoke and the germination stimulating compounds, karrikins, have been used to stimulate germination in over 1200 plant species from more than 80 different genera worldwide. Karrikinolide (KAR_1), the most commonly used karrikin, has been used to promote germination in the seed of a vast variety of plant species as it can be applied at a rate of 1 ppb (10^{-9} M). Recently another compound, namely trimethylbutenolide (TMB) was also isolated from plant derived smoke that significantly reduces the germination promoting activity of KAR_1. Although TMB has not been tested on a large number of plant species, it has great potential to be applied in agricultural and horticultural practices together with KAR_1.

Trimethylbutenolide has been reported to significantly reduce the stimulatory activity of KAR_1 at concentrations of 10^{-3} to 10^{-5} M. This difference in the activity ranges of TMB and KAR_1 points towards a mechanism through which the germination of seeds can be manipulated in natural systems. Trimethylbutenolide also occurs at much higher concentrations in plant derived smoke compared to KAR_1. It has been hypothesized that TMB will inhibit seed germination directly after a fire (smoke) so that germination of the seeds will only occur after sufficient rainfall has leached it from the seed. In order to utilize TMB in agricultural practices, a better understanding of the activity of this compound must be attained. The application of TMB in seed germination could also be enhanced if a more potent analogue of the compound can be identified. Therefore, 11 TMB analogues were prepared and tested using the lettuce seed germination bioassay. A concentration series (10^{-7} M to 10^{-3} M) of the analogues were tested alone, or in combination with KAR_1 (10^{-8} M). Only two compounds were found to reduce the germination promotory effect of 10^{-8} M KAR_1 in a similar manner as observed with TMB, with activity ranging from 10^{-5} M to 10^{-3} μM. Four compounds were found to have inhibitory activity at 10^{-4} and 10^{-3} M. The retention of activity by some of the analogues may be useful for designing novel compounds with improved activity. Furthermore, understanding the structure-activity

relationships of these compounds may be helpful in synthesising molecular probes that can be used to investigate the mechanism of action of these compounds in regulating seed germination.

Weeds pose a great problem to farmers worldwide, and controlling weeds demands a high input cost for herbicides and labour. Due to current environmental regulations, a limited number of herbicides are commercially available (with limited modes of action) to control weeds. The effects of smoke-water (SW), KAR$_1$ and TMB were tested on five major weed species in South Africa namely *Conyza albida*, *Hypochaeris radicata*, *Solanum mauritianum*, *Spilanthes decumbens* and *Talinum paniculatum*. Seeds of these weed species were subjected to 16 h light/8 h dark conditions or to constant dark conditions at constant 20, 25, 30°C and alternating 20/30°C. Smoke-water and KAR$_1$ significantly increased germination, while TMB significantly reduced the germination of these weed species compared to the respective controls. Furthermore, TMB treatment reduced the α-amylase activity of the tested weed seeds compared to the water control. These results indicate the possibility of manipulating the germination of certain weed seeds by SW, KAR$_1$ and TMB. Thus smoke and the smoke-isolated compounds could potentially be used in new weed management strategies.

The use of plant growth promoting rhizobacteria (PGPR) to improve crop yields is a well-established agricultural practice. Before SW, KAR$_1$ and TMB can be used in the field, the effect these compounds have on agriculturally beneficial bacteria such as the hormone-producing bacterium *Bacillus licheniformis* needs to be established. In the present study, the effects of a hormone-producing *Bacillus licheniformis* strain and SW, KAR$_1$ and TMB applied singularly and in combination on *Abelmoschus esculentus* (okra) growth in a pot trial were investigated. *Bacillus licheniformis*, SW 1:500 (v:v) and KAR$_1$ (10^{-7} M) applied singularly, significantly improved the shoot and root biomass and leaf area of okra. However, when applied in combination with *B. licheniformis* inoculum, there was a negative effect on plant growth. In contrast, the smoke inhibitor TMB (10^{-3} M) had a negative effect on plant growth that was overcome when combined with *B. licheniformis*. All treatments had no effect on chlorophyll, carotenoid, protein and sugar content in okra while α-amylase activity was slightly elevated in the roots with *B. licheniformis*, SW 1:500 (v:v) and KAR$_1$ (10^{-7} M) application. Estimation of the rhizobacteria populations at harvest showed

SW decreased the rhizosphere microbial population, KAR_1 stimulated rhizosphere microbial abundance and TMB reduced *B. licheniformis* establishment.

The effects of SW, KAR_1 and TMB were investigated on the germination and growth of orchid seeds using tissue culture techniques. Orchid seeds are classified as micro seeds as these seeds have limited to no seed reserves and contain undifferentiated embryos. The effect of smoke on orchid seed germination has not been evaluated. The effect of SW, KAR_1 and TMB were investigated on the endangered epiphytic orchid, *Ansellia africana*, which is indigenous to Africa. Infusing SW (1:250 v:v) into half strength MS medium significantly increased the germination rate index (GRI) and the development rate index (DRI) of the *A. africana* seeds. Infusing karrikinolide into the growing medium had no significant effect on the germination or development of the seeds. The TMB treatment, however, significantly reduced the GRI and DRI of the *A. africana* seeds. The results indicate that there are aspects of smoke that still needs to be explored, many of which could have significant implications in plant breeding, agriculture and horticulture.

Since large amounts of smoke are generated and released into the air during wildfires, it is possible that angiosperm pollen germination and pollen tube elongation may be affected by plant-derived smoke even when the plants are some distance from the fire. The effect of smoke on pollen germination and pollen tube elongation for three species of the Amaryllidaceae that occur naturally in areas prone to winter fire in South Africa were assessed as a pilot study. Pollen from seven other plant species of different families were also assessed after the initial effects were recorded. *In vitro* pollen germination and pollen tube growth of the 10 plant species were assessed by preparing hanging drop slides with different concentrations of SW, KAR_1 and TMB combined with Brewbaker and Kwack's medium and a sucrose and boric acid medium. These slides were incubated for 1 h at 25°C. Pollen germination and pollen tube lengths were recorded by capturing images with a compound microscope aided by a digital camera. Low concentrations of SW (1:1000 and 1:2000 v:v) significantly increased pollen germination and pollen tube length in the three Amaryllidaceae species as well as in *Aloe maculata*, *Kniphofia uvaria*, *Lachenalia aloides* and *Tulbaghia simmleri* when applied alone or in combination with either Brewbaker and Kwack's medium or sucrose and boric acid medium. Karrikinolide (10^{-6} and 10^{-7} M) treatment significantly improved pollen tube growth in

A. maculata, *K. uvaria*, *L. aloides* and *Nematanthus crassifolius* compared to the controls. Brewbaker and Kwack's or SB medium containing TMB (10^{-3} M) produced significantly longer pollen tubes in *A. maculata*, *K. uvaria* and *N. crassifolius*. Consequently, smoke from grassland fires may have favourable implications for the reproductive process of flowering plants. These results indicate that plant-derived smoke and the smoke-isolated compounds may stimulate pollen growth in a wide range of plant species.

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LIST OF ABBREVIATIONS

ABA	abscisic acid
ANB	aniline blue lactophenol
ANOVA	analysis of variance
BWK	Brewbaker and Kwack
CFU	colony forming units
Chl	chlorophyll
DNS	3, 5-dinitrosalicylic acid
DW	dry weight
FDA	fluorescein diacetate
FW	fresh weight
GA	gibberellin
IAA	indole-3-acetic acid
JA	jasmonic acid
KAR	karrikinolide
LB	Luria broth
MS	Murashige and Skoog
NO	nitric oxide
PGPR	plant growth promoting rhizobacteria
PGR	plant growth regulators
ppm	parts per million
SB	sucrose and boric acid
SE	standard error
S-KAR$_1$	sulphur-analogue of karrikinolide-1
SVI	seedling vigour index
SW	smoke-water
TMB	trimethylbutenolide
TTC	2,3,5-triphenyl tetrazolium chloride
UV-B	ultraviolet light-B

CHAPTER 1 – INTRODUCTION

1.1 PLANT-DERIVED SMOKE AND THE BIOACTIVE COMPOUNDS ISOLATED FROM SMOKE

During the last two decades the role of smoke on seed germination and overall plant growth has received considerable attention. The isolation of the germination promoter karrikinolide (KAR_1) and the germination inhibitor trimethylbutenolide (TMB) revealed a new class of plant growth regulator, the activity of which is still being elucidated by researchers all over the world. With the addition of each study on smoke and the compounds isolated from smoke, the potential uses of these compounds become more apparent, creating new avenues of research.

1.2 AIMS AND OBJECTIVES

1.2.1 Aims

- To determine the structure-activity relationships of TMB.
- To investigate the possible applications of smoke-water (SW), KAR_1 and TMB in agricultural practices.
- To investigate the possible roles of plant-derived smoke, KAR_1 and TMB in horticultural practices.

1.2.2 Objectives

- Test the germination inhibitory activities of 11 TMB analogues using the lettuce seed germination bioassay.
- Explore the use of KAR_1 and TMB in weed seed germination as possible weed control agents.

- To investigate the interaction(s) between a plant growth promoting rhizobacterium (*Bacillus licheniformis*) and smoke-derived compounds (SW, KAR_1 and TMB) on plant growth.
- Investigate the possible roles of SW, KAR_1 and TMB on orchid seed germination.
- Investigate the possible roles of SW, KAR_1 and TMB on pollen germination of various plant families.

1.3 GENERAL OVERVIEW

The effects of SW and KAR_1 on seed germination and plant growth have been well documented **(LIGHT *et al.* 2009; KULKARNI *et al.* 2011)**. Although some studies mention that SW and KAR_1 have many applications in agriculture and horticulture, little research has been conducted on this topic. For this reason various laboratory and field based experiments were conducted to highlight the uses of smoke and the smoke-derived compounds. Together these experiments create new avenues of research each with opportunities that can be exploited in the future.

Chapter 2 provides a comprehensive literature review on the topic of smoke and the bioactive compounds isolated from smoke. As a short introduction, chemical seed dormancy is discussed with special reference to germination inhibitors that have been isolated from plant material. Possible areas of agricultural application as well as research areas in which smoke may play a significant role are discussed.

Chapter 3 presents results of structure-activity relationships of 11 analogues of TMB, a germination inhibitor isolated from plant derived smoke. Two analogues of TMB are identified with inhibitory activity equivalent to that of the original inhibitor. This work was made possible by the collaborative effort of Dr M. Pošta who synthesised the analogues of TMB.

Chapter 4 presents results on the application of SW, the germination promoter (KAR_1) and the germination inhibitor (TMB) in weed control. This chapter shows that SW and KAR_1 can be used to stimulate germination in the five weed species investigated while TMB inhibits the germination of the weed species.

Chapter 5 presents data on the second agricultural application proposed in which the interaction between SW, KAR_1 and TMB with an agriculturally beneficial bacterium is investigated. The results indicate that hormonal cross-talk occurs between the smoke treatments and the bacterium. The addition of the beneficial bacterium overcame the inhibitory effects of TMB treatment.

Chapter 6 presents results on the effects of SW, KAR_1 and TMB on *in vitro* orchid seed germination. The results indicate that SW increases orchid seed germination as well as improves subsequent development of the orchid seeds. Infusing TMB into the growth medium significantly reduced the germination and growth of the orchid seeds.

Chapter 7 presents results on the effects of SW, KAR_1 and TMB on *in vitro* pollen germination and pollen tube elongation on ten different plant species from different plant families. The results indicated that SW, KAR_1 and TMB have significant stimulatory effects on pollen germination and pollen tube growth.

Chapter 8 provides general conclusions on the results presented in this study which is followed by **Chapter 9** (References).

CHAPTER 2 – LITERATURE REVIEW

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2.1 SEED DORMANCY AND GERMINATION CUES

2.1.1 Dormancy mechanisms in seeds

The germination of a seed can simply be defined as the removal of dormancy blocks. Although the above mentioned statement describes the process, it has several limitations as dormancy comes in different forms and in most cases can only be broken by specific environmental conditions or stimuli **(BASKIN and BASKIN 1998)**. Dormancy in seeds can be divided into two main groups namely endogenous dormancy and exogenous dormancy **(NIKOLAEVA 1977)**. According to **NIKOLAEVA (1977)** endogenous dormancy can be further subdivided into physiological, morphological and morphophysiological dormancy which is caused by physiological inhibiting mechanisms and underdeveloped embryos. Exogenous dormancy is categorized by aspects of the structure of the seeds (seed coat, endosperm and fruit wall) that restrict germination and can be subdivided into physical, chemical and mechanical dormancy types **(NIKOLAEVA 1977; BASKIN and BASKIN 1998)**. To break these kinds of dormancy mechanisms the specific chemical, physical, physiological or structural block must be removed **(BASKIN and BASKIN 1998)**. The removal of these dormancy blocks can vary depending on the type of dormancy present and can range from scarification (heat, chemical or physical breakdown of the seed coat), warm or cold stratification, physiological development during appropriate conditions, and lastly the addition of chemicals and nutrients or the leaching of chemical inhibitors **(BASKIN and BASKIN 1998)**. Despite the differences among the dormancy mechanisms, they all have one thing in common and that is to ensure that the subsequent seedling will be able to compete for resources and that the germination and growing conditions are as favourable as possible. For the purposes of this study, the focus will be on chemical dormancy.

2.1.2 Chemical dormancy in seeds

According to **EVENARI (1949)**, chemical inhibitors of germination can be defined as "substances produced by plants or substances of related structure not found in plants which inhibit or delay the germination of seeds of the same or other species". These inhibitors of germination are often produced in the pericarp of the seeds **(NIKOLAEVA 1977; BASKIN and BASKIN 1998)** but have also been isolated from the embryo, endosperm, seed coats and in the dispersal structures of seeds **(BEWLEY and BLACK 1982**, **1994)**. Chemical dormancy can be broken by either removing the seed part containing the inhibitors or by leaching out the chemical inhibitors **(NIKOLAEVA 1977; BASKIN and BASKIN 1998)**. Chemical dormancy also includes compounds that have been translocated into the seeds **(BASKIN and BASKIN 1998)**, however, most of the inhibitors mentioned in the literature are from a plant origin (Table 2.1).

The compounds with reported seed germination inhibitory activity are summarized in Table 2.1. The compounds with potent germination inhibitory activity all contain an isolated formyl (CHO) group. According to **EVENARI (1949)** and **MAYER and EVENARI (1952)**, the nature of this formyl group determines the effectiveness of the germination inhibitor rather than the inductive and electromeric influences. The germination inhibitory activity of many of the compounds listed in Table 2.1 were compared to coumarin which was regarded as one of the most potent seed germination inhibitors **(MAYER and EVENARI 1952; HENDERSHOT _et al._ 1962; KATO _et al._ 1978; FRIEDMAN _et al._ 1982)**. A large number of the germination inhibitors reported in Table 2.1 were from the lactone group which all included the characteristic CHO-group. It was evident from the literature that this group has the highest number of strong germination inhibitors. Although it was reported that ethylene and hydrogen cyanide have germination inhibitory activity (Table 2.1), it was also shown that they have germination stimulatory activity **(ABELES and LONSKI 1969; EGLEY and DALE 1970; TAYLORSON and HENDRICKS 1973; KEÇPCZYŃSKI and KEÇPCZYŃSKA 1997; MATILLA 2000; FLEMATTI _et al._ 2011)**. The discrepancy between the studies could be attributed to the concentration at which the compounds were administered.

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Table 2. 1 Chemical compounds derived from plant material with germination inhibitory activity

Chemical group	Compound name	Material isolated from	Effective concentration	Chemical structure	Reference
Aldehyde	Acetaldehyde	Variety of seeds	Inhibits the germination of grains at 0.1 mg.mL^{-1}		**(EVENARI 1949; BRADOW and CONNICK 1988)**
	Benzaldehyde	Break down products of amygdalin, which are common in Prunanceae and Pomaceae seeds	Complete inhibition of grains at a dilution of 1:3000		**(EVENARI 1949)** **(REYNOLDS 1978)**

	Cinnamalaldehyde	Cinnamon bark	Inhibits 50% of Great Lakes lettuce seeds at a concentration of 0.38 mM		**(EVENARI 1949; REYNOLDS 1978)**
Aldehyde	Salicylaldehyde	Characteristic aroma component of buckwheat	Complete inhibition of grains at a dilution of 1:500-1:1000		**(EVENARI 1949; REYNOLDS 1978; JANEŠ and KREFT 2008)**
Alkaloid	Cocaine, physostigmine, caffeine, chinine, cinchonine, cinchonidine, tropa acid, strychnine, berberine and codeine	Plant material from various plant species e.g. *Coffea arabica*	Caffeine completely inhibits wheat seed germination at a dilution of 1:200	Caffeine	**(EVENARI 1949; FRIEDMAN and WALLER 1983)**
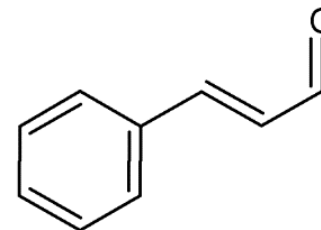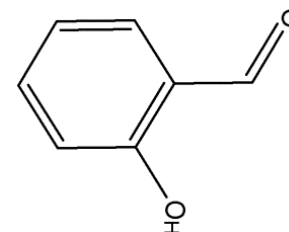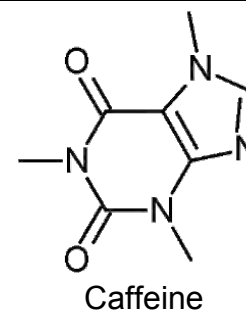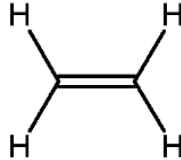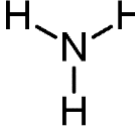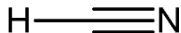
Alkene	Ethylene	Released by many fruits and plants	Ethylene produced by three apples in a 6 L container inhibited the germination of various seeds	**(EVENARI 1949)**
Ammonia	Ammonia	Liberated by enzymes during the digestion of nitrogenous compounds in seeds	Delays the germination of *Zea mays* at a concentration of 1:20000	**(EVENARI 1949)**
Hydrogen cyanide	Hydrogen cyanide	Metabolic products of amygdalin, which are common in Prunanceae and Pomaceae seeds	0.1% inhibits the germination of tomato seeds. 0.024% delay germination of *Lepidium* seeds (inhibition only evident if seeds are kept in presence of hydrogen cyanide)	**(EVENARI 1949)**

Lactone				
Anemonin	Fruit of the Ranunculaceae	Inhibits the germination of *Avena* spp. seeds at a dilution of 1:10000		**(EVENARI 1949)**
Coumarin	Various plant species e.g. *Trigonella arabica*	Completely inhibits the germination of 'Grand Rapids' lettuce seeds at 75 ppm		**(NUTILE 1945; EVENARI 1949; MAYER and EVENARI 1952; LERNER *et al.* 1959)**
Deacetylcryptocarya-lactone	*Cryptocarya moschata* seeds	Inhibits the germination of *Abutilon theophrasti* seeds at a concentration of 0.004 M		**(SPENCER *et al.* 1984)**
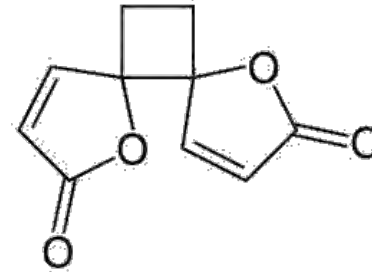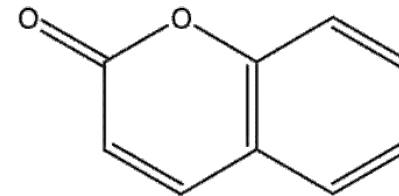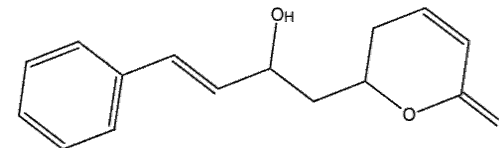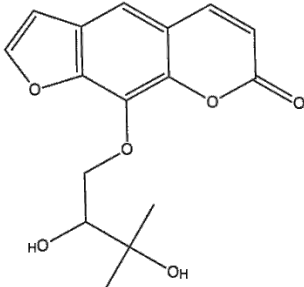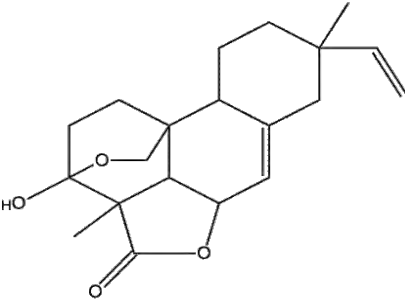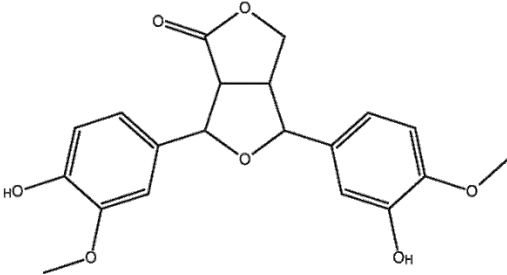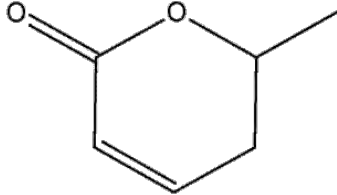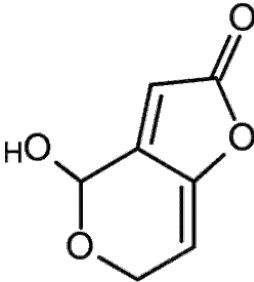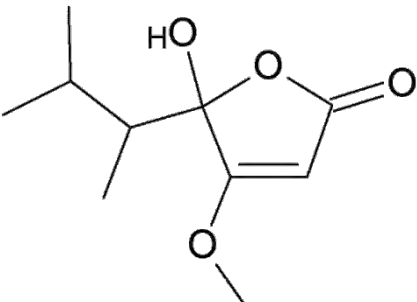
Heraclenol	*Petroselinium crispum*	Inhibits lettuce seed germination at 1000 ppm		**(KATO *et al.* 1978)**
Momilactone	*Oryza sativa*	Inhibits lettuce seed germination at 100-1000 ppm		**(KATO *et al.* 1977)**
Monoepoxylignanolide	*Aegilops ovata* husks	Inhibits lettuce seed germination at 0.5 mg.mL^{-1}		**(LAVIE *et al.* 1974)**

(Lactone)

Lactone	Parasorbic acid	Fruit of *Sorbus aucuparia*	Inhibits the germination of *Lepidium* spp. seeds at a dilution of 1:1000		**(EVENARI 1949)**
	Patulin	Metabolic product of fungi like *Penicillium expansum* from fermenting fruits like apples	Completely inhibits lettuce seed germination at a concentration of 0.2 mg.mL^{-1}		**(BERRIE *et al.* 1967)**
	Penicillic acid	Metabolic product of fungi like *Penicillium cyclopium* and *Penicillium canescens*	Inhibits 80% of corn seeds when applied at a concentration of 2 mg.ml^{-1}		**(BERRIE *et al.* 1967; KEROMNES and THOUVENOT 1985)**

Lactone	2-pentene-1:4-olid	Source not mentioned	Inhibits 50% of lettuce seeds at 1.19^{-3} M (equivalent to 42% of the activtiy of coumarin)	**(MAYER and EVENARI 1952)**
	Ramulosin	Metabolic product of the fungus *Pestalotia ramulosa*	Completely inhibits the germination of wheat and oats at 10 ppm	**(HENDERSHOT et al. 1962)**
	Xanthotoxin (furanocoumarins)	Fruit from Bishop's weed	Completely inhibits the germination of *Anastatics hierochuntica* at 10^{-4} M	**(FRIEDMAN et al. 1982)**
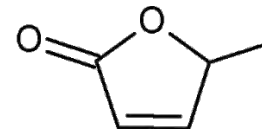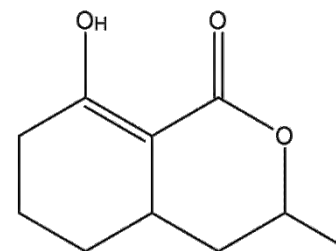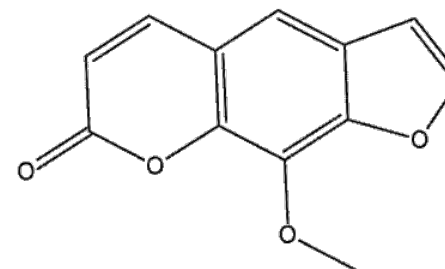
Mustard oil	Allyl-isothiocyanate	Common in all the plant organs of *Brassica sinapsis*	0.7% mustard oil (containing 92-95% allyl-isothiocyanate) inhibited germination of *B. sinapsis* seeds	**(EVENARI 1949)**
Organic acid	Citric acid	Common in fruit juices and plant sap	Inhibits germination at a 1:100 dilution	**(EVENARI 1949)**
	Malic acid	Common in fruit juices and plant sap	Inhibits seed germination at 1:200 dilution	**(EVENARI 1949)**
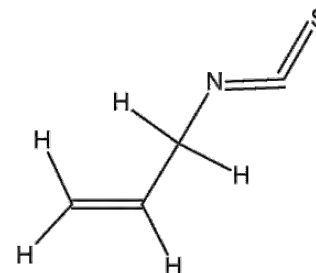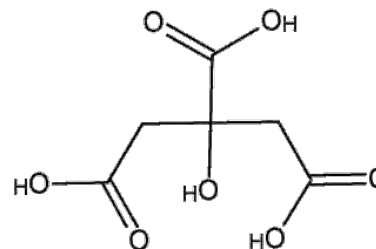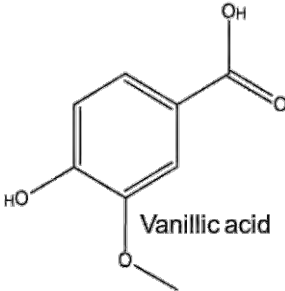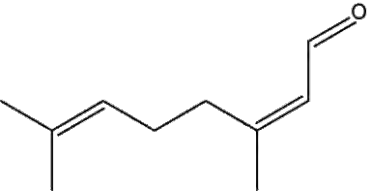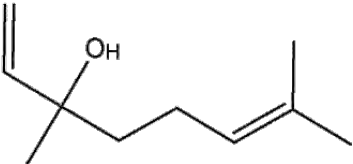
Organic acid	Verulic acid, abscisic acid and vanillic acid	Most plants	Inhibits 50% of lettuce seeds at a concentration of 2.17 mM	Vanillic acid	**(AKKERMAN and VELDSTRA 1947 as in FRIEDMAN and WALLER 1983; WILLIAMS *et al.* 1973; GATFORD *et al.* 2002)**
Terpenoid	Citral	Occurs in lemon grass oil	Completely inhibits seed germination at a dilution of 1:500		**(EVENARI 1949)**
	Linalool	Occurs in essential oils of lemon grass and several others	Inhibits 62% of seeds at a dilution of 1:500		**(EVENARI 1949)**

Terpenoid	1-hydroxyepiacorone (Sesquiterpenoid)	*Acori rhizoma* plant material	Completely inhibits lettuce seeds at 100 ug.mL^{-1}		**(NAWAMAKI and KUROYANAGI 1996)**
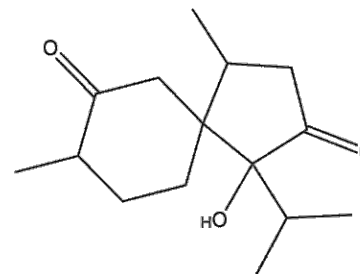
Essential oils were also mentioned in the literature for their inhibitory activity on seed germination but were omitted from the table since these oils are made up of a mixture of compounds and not one active principle. In some studies several compounds from the same chemical group inhibited the germination of seeds, however, only the most potent compounds were illustrated. All the chemical structures were drawn using ACD/ChemSketch software (Version 12.01).

2.1.3 Germination cues associated with fire

Shrub species from fire-prone ecosystems show diverse responses to fire. The regeneration of various plant species in the post-fire environment is evident in shrublands around the world **(KRUGER 1977; KEELEY and ZEDLER 1978)**. It is well-known that fire plays an active role in shaping the vegetation of ecosystems. During the last two decades the role of fire in seed germination attracted considerable attention. However, the specific aspect of fire that is responsible for breaking seed dormancy has been highly disputed. Many different aspects of fire influence the germination of fire-dependent seeds such as heat (heat shock) **(JEFFERY *et al.* 1988)**, light **(KEELEY and FOTHERINGHAM 2000)**, the reduction in foliage (competition for light, space, water and nutrients) **(KEELEY and FOTHERINGHAM 2000)**, increases in nutrient levels **(DEBANO and CONRAD 1978)**, sterilization of soil **(FLETCHER 1910 AS IN KEELEY AND FOTHERINGHAM, 2000; SABIITI and WEIN 1987; WICKLOW 1988)**, a reduction of herbivores **(QUINN 1994)** and smoke **(DE LANGE and BOUCHER 1990)**. Today it is well known that these aspects of fire and also, the post fire environment, play significant roles in stimulating the germination of seeds. In many instances, fire dependent seeds often require a combination of two or more of the above mentioned aspects of fire to germinate successfully **(KEELEY and FOTHERINGHAM 2000)**.

2.2 GERMINATION STIMULATORY ACTIVITY OF PLANT-DERIVED SMOKE

2.2.1 Production of smoke-water

It was only in 1990 that the role of smoke in stimulating the germination of seeds was discovered **(DE LANGE and BOUCHER 1990)**. **DE LANGE and BOUCHER (1990)** observed that the seeds of *Audouinia capitata* (a threatened fynbos species of South Africa) only germinated after the vegetation had burnt. **DE LANGE and BOUCHER (1990)** showed that the seeds of *A. capitata* did not only germinate in response to a fire event but also when treated with cold plant-derived smoke or smoke-water [(SW) that was prepared by bubbling plant-derived smoke through distilled water]. This clearly indicated that plant-derived smoke contained compounds that stimulated

seed germination in *A. capitata*. Shortly after the discovery of smoke-stimulated seed germination, several studies reported that many more fynbos species were also responsive to plant-derived smoke **(BROWN 1993a; BROWN and BOTHA 2004)**.

Since the discovery of the germination stimulatory activity of SW, many researchers set out to establish the activity range of plant-derived SW **(LIGHT and VAN STADEN 2004)**. It was therefore necessary to standardize the crude SW extracts. Although many different methods were developed to produce SW, the basic principles are the same. Dry plant material (and in some instances a mixture of moist and dry plant material) is burned for a certain time and the resultant smoke steeped through a specific volume of distilled water for a particular length of time. Several variations of this method have been reported **(DE LANGE and BOUCHER 1990; BROWN 1993a**, **b; KEELEY 1993; BAXTER *et al.* 1994; ROCHE *et al.* 1994; BAXTER *et al.* 1995; DIXON and ROCHE 1995; DIXON *et al.* 1995; JÄGER *et al.* 1996)**. Irrespective of the plant material burned, the resultant SW stimulated the germination of smoke-sensitive seeds. It was later reported that the active principle was mainly formed when the plant material was heated to a temperature between 160 and 200°C **(JÄGER *et al.* 1996)**. According to **JÄGER *et al.* (1996)**, heating the plant material above 200°C resulted in loss of activity as a result of possible volatilization of the active principles. The same study also reported that heating agar and cellulose to the desired temperature also produced the germination stimulatory compounds. The active principle could therefore be produced from burning any plant material **(JÄGER *et al.* 1996)**.

2.2.2 Effects of plant-derived smoke-water on germination

Since the initial discovery by **DE LANGE and BOUCHER (1990)**, many studies reported on the germination stimulatory activity of SW. Smoke-stimulated seed germination experiments were mainly conducted on plant species from the Cape Floristic region of South Africa **(BROWN 1993a**, **b; BROWN *et al.* 1994; PIERCE *et al.* 1995; ROCHE *et al.* 1997b; BROWN *et al.* 2003)**, the Southwest Botanical Province of Western Australia **(ROCHE *et al.* 1994; DIXON *et al.* 1995; ROCHE *et al.* 1997a)** and the Californian Floristic Province **(KEELEY and FOTHERINGHAM 1998)**. Surprisingly, SW also stimulated seed germination in plant species that are

not from fire-prone environments **(DREWES *et al.* 1995; DOHERTY and COHN 2000; ADKINS and PETERS 2001)**. The potential of smoke in agriculture, horticulture and ecological restoration is very promising as it has a broad activity range, stimulating germination of seeds from indigenous plants as well as weeds and commercially important crops **(KULKARNI *et al.* 2011)**.

2.2.3 The discovery of the active principle in plant-derived smoke

The germination promotory activity of SW unlocked a new avenue of research and instigated the search for the active principles responsible for the germination stimulatory activity. The isolation of the active principle was, however, very problematic since plant-derived smoke contains thousands of chemical compounds **(MAGA 1988)**. The isolation of the germination stimulating compound in SW was further complicated by its relatively low concentration compared to other compounds present in smoke **(LIGHT *et al.* 2009)**. Fourteen years after the initial discovery that smoke-water stimulated seed germination, two independent research groups isolated the active principle in close succession to each other from burning cellulose paper **(FLEMATTI *et al.* 2004)** and from burning fynbos material *Passerina vulgaris* and *Themeda triandra* leaf material **(VAN STADEN *et al.* 2004)**. The compound responsible for stimulating seed germination was confirmed as the butenolide 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one (Fig. 2.1) and was later named karrikinolide-1 (KAR$_1$, for numbering purposes in **Chapter 2**, this compound will also be referred to as **1**). Five additional active karrikin analogues with closely related structures consisting of a butenolide moiety fused to a pyran ring with various methyl substitutions have since been identified and synthesised i.e. KAR$_2$ – KAR$_6$ **(FLEMATTI *et al.* 2007)**.
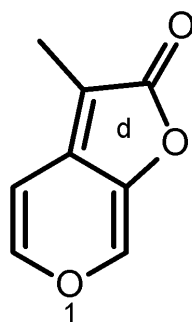
Figure 2. 1 The chemical structure of karrikinolide (**1**) 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one], the main germination stimulating compound isolated from plant-derived smoke **(FLEMATTI *et al.* 2004; VAN STADEN *et al.* 2004)**. The chemical structure was drawn using ACD/ChemSketch software (Version 12.01).

2.3 EFFECTS OF KARRIKINOLIDE ON SEED GERMINATION AND OVERALL PLANT GROWTH

2.3.1 Characteristics of karrikinolide

The germination stimulatory activity of the synthesised butenolide 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one was shown to be equivalent to that of the crude SW extract at similar concentration levels **(FLEMATTI *et al.* 2004)**. Since butenolide refers to a type of compound, the compounds responsible for the germination stimulatory activity were named karrikins. The name was derived from the Australian Nyungar Aboriginal word "karrick" which translates in English to smoke **(COMMANDER *et al.* 2008)**. Since 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one was isolated first and was considered the main germination stimulating compound, it is commonly referred to as karrikinolide-1 or KAR_1 **(COMMANDER *et al.* 2008)**. The isolated KAR_1 had a very broad activity range, stimulating the germination of Grand Rapid lettuce seeds from 10^{-4} M down to a concentration of 10^{-9} M **(FLEMATTI *et al.* 2004; VAN STADEN *et al.* 2004)**. Even before the isolation of KAR_1, it was already established that the active principle in plant-derived smoke was water soluble, thermostable and lasted a long time in solution **(BALDWIN *et al.* 1994; VAN STADEN *et al.* 2000)**. It was later also established by **VERSCHAEVE *et al.* (2006)** that KAR_1 exhibited no toxicity or genotoxicity at the levels (10^{-4} – 10^{-9} M) at which the germination stimulatory activity was established. These characteristics make it possible to use

KAR$_1$ in research and agricultural practices as a germination stimulant **(LIGHT *et al.* 2009)**.

2.3.2 Formation of karrikinolide (Maillard reaction)

Before the isolation of KAR$_1$ was achieved, **BALDWIN *et al.* (1994)** showed that the germination promoting activity of SW can be achieved by burning cellulose. It was later reported that KAR$_1$ is synthesised through a Maillard reaction during the burning of plant material **(LIGHT *et al.* 2005)**. The Maillard reaction is the chemical reaction that takes place between amino acids and reducing sugars when heated to temperatures between 140-165°C and gives browned foods their flavour **(HODGE 1953)**. The water soluble extracts obtained by heating proteins or amino acids with sugars at 180°C for 30 min produced a similar germination response in 'Grand Rapids' lettuce seeds **(LIGHT *et al.* 2005)**. According to **LIGHT *et al.* (2005)** the compounds formed between the sugars D-xylose or D-ribose and the amino acids arginine, asparagine, aspartic acid, glycine, serine, tyrosine or valine produced solutions with the greatest germination stimulatory activity. From these studies it seemed probable that the burning of any type of organic plant material could be used to produce karrikins. Most humans therefore consume considerable amounts of karrikins every day with foods like toast, coffee, cooked meats and vegetables.

2.3.3 Germination stimulatory activity of KAR$_1$

After the initial discovery of KAR$_1$, numerous studies were conducted to determine the activity range of KAR$_1$. Since isolating KAR$_1$ from plant-derived smoke yielded low amounts of the compound, KAR$_1$ is generally synthesised according to one of the methods described by **FLEMATTI *et al.* (2007)**, **NAGASE *et al.* (2008)** and **SUN *et al.* (2008)** for use in scientific studies. Since the initial discovery of KAR$_1$, it has been used to stimulate seed germination in a wide variety of plant species **(**reviewed in **CHIWOCHA *et al.* 2009; LIGHT *et al.* 2009)**. Many studies on the effects of smoke and the smoke-derived compounds are still conducted at research institutes all over the world. According to a recent survey by **JEFFERSON *et al.* (2014)** the seeds of 1355 plant species from 120 different plant families have been treated with

either aerosol smoke, SW or KAR_1 solutions in various experiments. According to **DIXON et al. (2009)** the seeds of more than 1200 species in more than 80 different genera worldwide are stimulated by either smoke or KAR_1. Interestingly, only 80% of these species are from fire prone areas. This suggests that even though a plant may not be in a fire prone environment, its ancestors may have adapted to an environment where fire was once common. This could explain why cultivated plant species such as lettuce [*Lactuca sativa* **(DREWES et al. 1995; LIGHT et al. 2002)**], celery [*Apium graveolens* **(THOMAS and VAN STADEN 1995)**], red rice [*Oryza sativa* **(DOHERTY and COHN 2000)**] and wild oats [*Avena sterilis* spp. *ludoviciana* **(ADKINS and PETERS 2001)**] respond to smoke. Since SW and KAR_1 stimulate the germination of crops, it has potential to be used as a tool in agriculture.

The ability of SW to overcome the light requirement of lettuce achenes **(DREWES et al. 1995)** (hereafter referred to as seeds) played a fundamental part in the isolation of KAR_1. Lettuce seeds, especially the 'Grand Rapids' cultivar, were used in numerous studies to investigate aspects pertaining to seed germination **(LIGHT 2006)**. The seeds are ideal for assessing the germination stimulatory activity of extracts and compounds since they are light sensitive and therefore do not germinate in the dark at optimal germination temperatures. This cultivar of lettuce seeds also germinated within 24 h which made it possible to test many test solutions in a short time frame **(LIGHT 2006)**. 'Grand Rapids' lettuce seeds were used in assessing the germination stimulatory activity of the fractions isolated from plant-derived smoke **(DREWES et al. 1995)** and also in the subsequent confirmation of the germination stimulatory activity of the isolated KAR_1 **(FLEMATTI et al. 2004; VAN STADEN et al. 2004)**.

2.3.4 Effects of SW and KAR_1 on the growth and development of plants

Apart from stimulating germination, other beneficial effects of smoke treatments include better seedling growth and vigour, increased root growth, improved resistance to salinity, temperature and drought stress, increased flowering and improved crop yield as well as promoting growth in heavy metal contaminated soils (reviewed in **LIGHT et al. 2009; KULKARNI et al. 2011)**. Smoke-water solutions have been shown to increase the growth of tomato roots in an *in vitro* system

(TAYLOR and VAN STADEN 1998). **VAN STADEN et al. (2006)** reported that KAR_1 (10^{-7} M) applied as a post-germination treatment produced tomato seedlings with roots ten times longer than that of the control and three times longer in beans and okra seedlings compared to their respective controls. Seedling vigour index (SVI) is a mathematical formula, frequently used to compare the overall growth differences between plants. The SVI takes several growth parameters into consideration and is calculated as follow: SVI = [stem thickness (mm)/seedling length (mm) + root fresh weight (g)/shoot fresh weight (g)] × [shoot fresh weight (g) + root fresh weight (g)]. The overall mass and vigour indexes of tomato, bean, okra and maize seedlings were significantly higher with SW (1:500 v:v) and KAR_1 treatments **(VAN STADEN et al. 2006)**. According to **SPARG et al. (2006)** the vigour of maize seedlings was noticeably greater when the seeds were treated with either aerosol smoke or SW (1:500; v:v) solution. It was reported that aerosol smoke and SW significantly increased the SVI of three medicinal plants **(SPARG et al. 2005)**. Treating maize seedlings with a similar treatment regime produced maize seedlings with significantly higher seedling vigour indexes. Similarly SW also increased the seedling vigour index of papaya **(CHUMPOOKAM et al. 2012)**. Smoke-water and KAR_1 have also been used to increase the seedling vigour indexes of rice **(KULKARNI et al. 2006a)**, tomato **(JAIN and VAN STADEN 2007)**, melon **(MAVI et al. 2010)** and *Eragrostis tef* **(GHEBREHIWOT et al. 2008)**.

Yield increases have also been reported for tomatoes **(KULKARNI et al. 2008)** and onions **(KULKARNI et al. 2010)** in response to SW and KAR_1 treatments. Karrikinolide treatments increased the growth parameters of tomato and onion plants; however, SW treatments produced significantly higher yields. This indicates that there might be other compounds in smoke that stimulate the growth of plants alone or synergistically. It could also be that the group of karrikin compounds function individually or as a unit to produce the significant growth promoting effects. Several of these studies suggested that KAR_1 exhibits both cytokinin-like and auxin-like activities **(JAIN et al. 2008; LIGHT et al. 2010)**. The karrikins are now regarded as a new group of naturally occurring plant growth regulators **(CHIWOCHA et al. 2009)**.

2.4 ISOLATION AND ACTIVITY OF THE GERMINATION INHIBITING COMPOUND FROM PLANT-DERIVED SMOKE

2.4.1 The isolation of trimethylbutenolide

Several studies reported that smoke responsive plant species responded the best to SW solutions administered at concentrations ranging from 1:500 – 1:1000 (v:v). However, a negative effect was observed when SW was applied at relatively high concentrations **(DREWES *et al.* 1995; LLOYD *et al.* 2000; ADKINS and PETERS 2001; DAWS *et al.* 2007)**. During the isolation of KAR_1, the compounds in SW were separated using high pressure liquid chromatography. After initial screening for germination stimulatory activity, the fraction containing KAR_1 was isolated **(VAN STADEN *et al.* 2004)**. It was only after testing the other separated fractions for germination stimulating activity that **LIGHT *et al.* (2010)** discovered a fraction that reduced the germination of 'Grand Rapids' lettuce seeds. This observation led to the isolation of another butenolide, 3,4,5-trimethylfuran-2(5*H*)-one, hereafter referred to as trimethylbutenolide [TMB (Fig. 2.2)]. For numbering purposes in **Chapter 2** this compound will also be referred to as **2**. Trimethylbutenolide has been shown to inhibit the germination of light sensitive lettuce seeds (Fig. 2.3) **(LIGHT *et al.* 2010)**. One of the remarkable features is that TMB shares a common but-2-enolide ring with KAR_1, indicated by the letter d (Figs. 2.1 and 2.2) **(LIGHT *et al.* 2010)**.
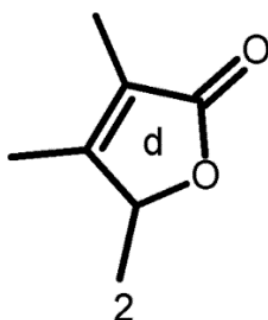
Figure 2. 2 The chemical structure of trimethylbutenolide (**2**) 3,4,5-trimethylfuran-2(5*H*)-one, the germination inhibiting compound isolated from plant-derived smoke **(LIGHT *et al.* 2010)**.
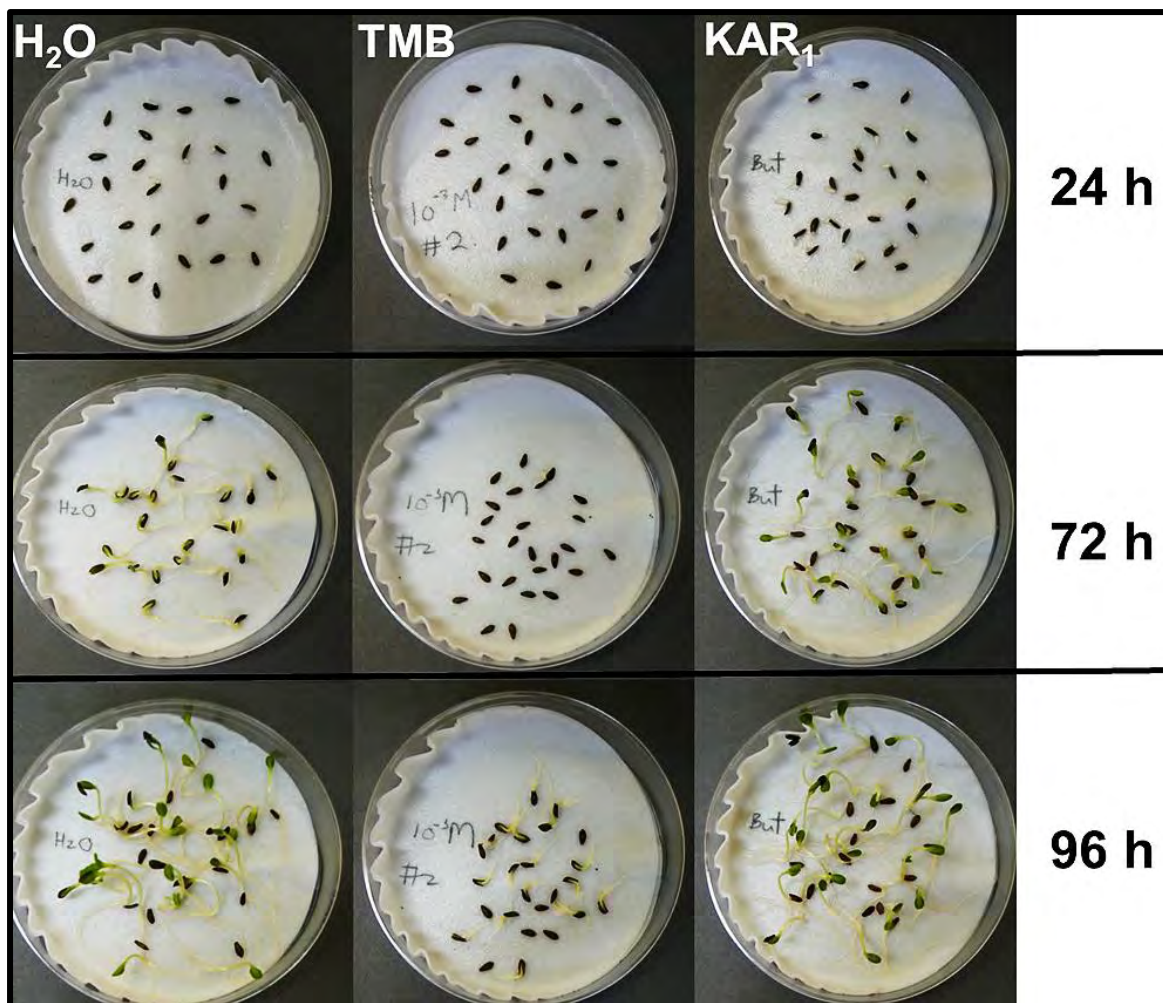
Figure 2. 3 The antagonistic effect of the germination inhibitor, trimethylbutenolide, on the germination of 'Grand Rapids' lettuce seeds. The first, second and third rows show germination after 24 h, 48 h and 72 h. The column on the left was the water control, the column in the middle was the TMB (10^{-3} M) treatment and the column on the right was the KAR$_1$ (10^{-8} M) positive control. The TMB-treated seeds were rinsed after 72 h **(**reproduced as described by **LIGHT *et al.* 2010)**.
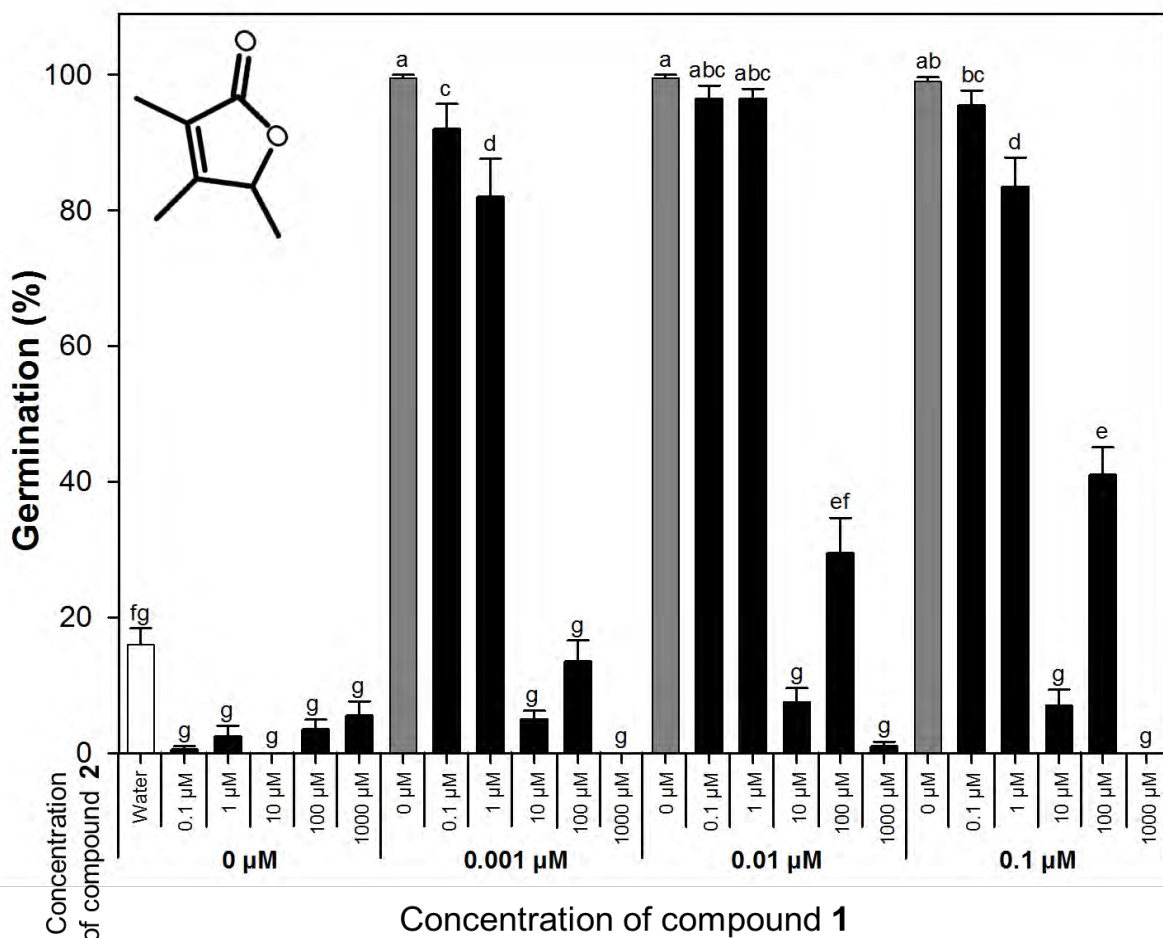
Figure 2. 4 Germination inhibitory activity of trimethylbutenolide on 'Grand Rapids' lettuce seeds in the dark at 25°C for 24 h. Trimethylbutenolide (**2**) was applied on its own (0.1 – 1000 µM) or in combination with KAR$_1$ [**1** (0.001 – 0.1 µM)]. Distilled water served as the control. Bars indicate the means of the results from two experiments ± SE. Bars with similar letters are not significantly different from each other ($P < 0.05$) according to Duncan's multiple range test (reproduced as described by **LIGHT et al. 2010)**.

2.4.2 The inhibitory activity of trimethylbutenolide on seed germination

The inhibitory activity of TMB on seed germination was tested in combination with three different concentrations of KAR$_1$ (0.001 – 0.1 µM) which stimulated the germination of lettuce seeds under normal conditions **(LIGHT et al. 2010)**. From Figure 2.4 it is clear that TMB significantly reduced the germination stimulatory activity of the three concentrations of KAR$_1$ (0.001 – 0.1 µM) at concentrations ranging from 10 – 1000 µM with a clear reduction in activity at concentrations above

10 µM. It is of great interest that the inhibitory activity of TMB was more potent when administered at 10 µM compared to the higher concentration of 100 µM. A similar result was obtained when 10 µM TMB was combined with all three concentrations of KAR_1 (0.001 – 0.1 µM), irrespective of the increased stimulatory activity of KAR_1 (Fig. 2.4). This observation emphasizes the role of the smoke-isolated butenolides as plant growth regulators as they seem to be more active at a specific (optimal) concentration. Trimethylbutenolide does not only function as a specific antagonist to the stimulatory activity of KAR_1 as it also significantly reduced the germination promotory activity of karrikinolide-3 (KAR_3; 10 nM), a related karrikinolide compound, and S-KAR_1 at 10 nM (synthesised sulphur analogue of KAR_1) **(NAIR et al. 2014)**. The above mentioned dose response relationship was also apparent when 10 µM TMB was combined with 10 nM KAR_3 and S-KAR_1 (Fig. 2.5) **(NAIR et al. 2014)**.
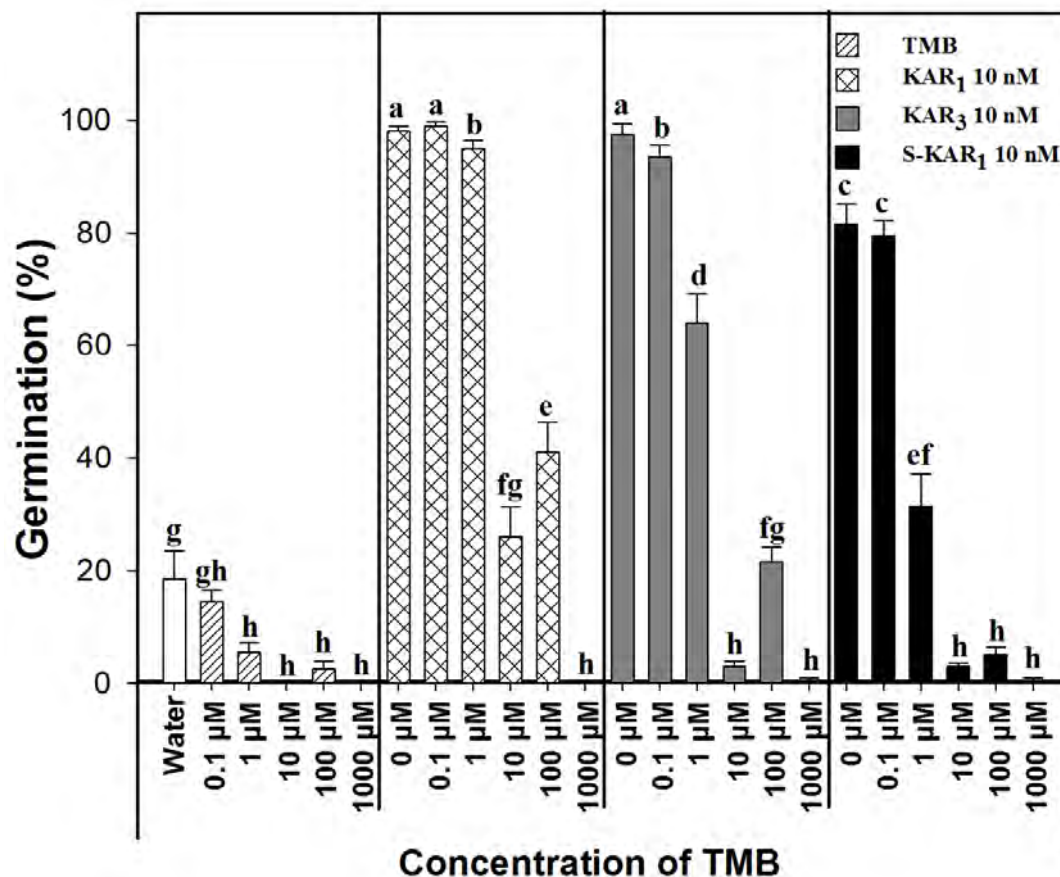
Figure 2. 5 Germination inhibitory activity of trimethylbutenolide (0.1 – 1000 µM) when combined with 10 nM KAR_1, KAR_3 and S-KAR_1 (sulphur analogue of KAR_1). Distilled water served as the control. Bars indicate the means of the results from two experiments ± SE. Bars with similar letters are not significantly different from each other ($P < 0.05$) according to Duncan's multiple range test **(NAIR et al. 2014)**.

2.4.3 Similarities between TMB and natural seed germination inhibitors

The effective inhibitory concentration of TMB (10 – 1000 µM) is much higher **(LIGHT et al. 2010)** than the activity range reported for KAR_1 **(FLEMATTI et al. 2004; VAN STADEN et al. 2004)** and KAR_3 **(FLEMATTI et al. 2007)** with effective concentrations from 0.001 – 1 µM. However, when TMB was applied within its activity range (10 – 1000 µM), it reduced the germination promotory activity of 10 nM KAR_1, KAR_3 and S-KAR_1 (Fig. 2.5) **(NAIR et al. 2014)**. Coumarin (Table 2.1) is regarded as one of the most potent inhibitors of germination **(EVENARI 1949; MAYER and EVENARI 1952)**. It was reported that the germination of certain seeds was inhibited by coumarin at concentrations ranging between 1 – 1000 ppm (6.84 µM – 6842.63 µM) **(NUTILE 1945; EVENARI 1949)**. A study conducted by **NUTILE (1945)** showed that coumarin could effectively reduce the germination of 'Grand Rapids' lettuce seeds at concentrations of 10 ppm (68.43 µM). Trimethylbutenolide could thus also be regarded as a potent inhibitor of seed germination as it inhibited the germination of 'Grand Rapids' lettuce seeds when applied at similar concentrations (Fig. 2.4).

Since the effective concentration of TMB is much higher than that of the germination promoting compounds, it seems apparent that TMB functions as a chemical block as part of a chemical dormancy mechanism in natural systems. This is further supported by the similarities between the chemical structure of TMB and the chemical structures of the known inhibitors synthesised by plants such as anemonin, 1-hydroxyepiacorone and 2-pentene-1:4-olid (Table 2.1). Compounds that are structurally similar to TMB or at least have the furanone ring structure included as part of their molecular structures include patulin, penicillic acid, monoepoxy-liganolide, 1-hydroxyepiacorone, anemonin, heraclenol, momilactone, xanthotoxin and 2-pentene-1:4-olid. Trimethylbutenolide also shares the characteristic CHO-group that have been associated with the germination inhibitory activity of some of the plant-synthesised inhibitors listed in Table 2.1 **(EVENARI 1949)**.

2.4.4 Proposed role of TMB in a natural system

There are several similarities between TMB and anemonin (a potent germination inhibitor), which basically consists of two furanone ring structures bound together by a cyclobutane bridge. It is conspicuous that these inhibitors can be produced in plants (seeds) and also by the Maillard reaction during fires. This commonality between the above mentioned inhibitors (see **Section 2.4.3**) and TMB is an interesting phenomenon in terms of the evolutionary derivation of seed germination inhibitors. It is possible that at some point during the evolution of angiosperms, the butenolide molecules produced by fires became signals for unfavourable environments, and thereby blocking the germination of seeds. However, these environments would quickly turn into very favourable ones, as soon as sufficient precipitation leached the inhibitors from the seeds, allowing the seeds to germinate in the now moist nutrient rich environment. This is supported by the results illustrated in Figure 2.3 in which TMB-treated seeds germinated within 24 h after being rinsed with distilled water **(LIGHT *et al.* 2010)**.

After the karrikins (germination promoters and inhibitors) saturate the soil and soil seed banks after a fire event, the seeds responsive to the smoke-derived compounds would supposedly remain dormant. This can be attributed to the higher amounts of TMB in plant-derived smoke compared to KAR_1 **(GHEBREHIWOT *et al.* 2013)**. According to **GHEBREHIWOT *et al.* (2013)** the soil from a post-fire environment contain 380 ± 32 $nmol.g^{-1}$ TMB and only 3.15 ± 0.45 $nmol.g^{-1}$ KAR_1 **(GHEBREHIWOT *et al.* 2013)**. Trimethylbutenolide must first be leached from the seeds after a sufficient amount of precipitation. The lowered amount of TMB bound to the seeds will allow the karrikins to stimulate germination in the now favourable environment. As was found with KAR_1, it is possible that several different forms of TMB exist in the smoke of plant-derived smoke. The inhibitory activity reported by **GHEBREHIWOT *et al.* (2013)** might therefore be from a group of TMB compounds rather than one inhibitory compound.

2.5 POTENTIAL OF SMOKE TECHNOLOGY IN AGRICULTURE

2.5.1 The problem of weeds in South Africa

Weeds can be divided into two groups, those that were recently introduced into the country and are trying to establish (minor invasive plant species) and those that have established and are reproducing at a rapid rate (major invasive plant species) **(MARAIS *et al.* 2004)**. In South Africa, 117 plants have been identified as major invasive plant species and 84 as emerging invasive plant species **(NEL *et al.* 2004)**. Many of the major invasive species are being actively monitored and included in management practices. These control programs include labour intensive practices and are time consuming. While focusing on eradicating the major invasive species, the emerging invasive species are neglected, which creates opportunities for emerging weeds to become major invaders **(NEL *et al.* 2004)**. Bugweed (*Solanum mauritianum*) is one of the weeds that is targeted for eradication in South Africa **(MARAIS *et al.* 2004)**. According to **MARAIS *et al.* (2004)** the bugweed infestation covers 89 491 ha (hectares equivalent 100% cover) and would only be eradicated within 23 years using the current management strategies. After the removal of this weed, however, large numbers of bugweed seedlings establish in the mother plant's place which requires follow up treatments **(BROMILOW 2010)**. New management strategies are, therefore, required to facilitate the control of weeds like bugweed that encompasses not only larger plants, but also the seed that will produce a new infestation in the next season.

2.5.2 Problems associated with the use of herbicides

Plant diseases, pests and weeds reduce the global food production potential by a third each year, of which, weeds alone may cause crop losses of more than 75% **(BROMILOW 2010)**. Herbicides play an integral part in managing weeds in agricultural practices. However, the removal of weeds by means of herbicides has several negative connotations; herbicides increase the input costs to farmers, and many weed species have adapted to herbicide treatment and are now herbicide tolerant **(BROMILOW 2010; BUSI *et al.* 2013)**. Herbicide tolerance in weeds is brought about by the continual application of a specific herbicide or class of

herbicides with a similar mode of action to the weed species **(JASIENIUK *et al.* 1996)**. The plants that survive the herbicide treatment will convey the tolerance traits to their progeny, thereby increasing both the frequency of the resistance alleles and also the number of resistant plants **(JASIENIUK *et al.* 1996)**. A recent international survey reported that 221 weed species have evolved resistance to 152 different herbicides and 22 of the 25 modes of action **(HEAP 2014)**. The number of herbicides that are still deemed effective in controlling certain resistant weed species are therefore declining rapidly, which holds adverse consequences for future weed control. Furthermore, the increasing demand to produce organically grown crops compels farmers to reduce their use of agrochemicals **(DAYAN *et al.* 2009; HELGA 2009)**.

2.5.3 Controlling weeds by manipulating the germination of seeds in the soil seed bank

Herbicides kill plants by altering biochemical processes such as photosynthesis, respiration, oxidative phosphorylation, RNA synthesis, protein synthesis and lipid synthesis **(ASHTON and CRAFTS 1981)**. This is often accomplished by inhibiting specific enzymes associated with these processes **(BUSI *et al.* 2013)**. According to **BROMILOW (2010)** the seeds produced by weeds often remain unaffected by herbicide treatment. These seeds will remain in the soil seed bank and produce a new weed infestation in the next growing seasons **(BROMILOW 2010)**. An effective weed management strategy focuses not only on removing established plants but also on reducing the number of germinating weeds while crops are still emerging, creating weed-free periods **(ZIMDAHL 1988; WYSE 1992)**. Such weed-free periods may allow the crop to establish and compete effectively with any subsequent emerging weeds **(WYSE 1992; GALLANDT 2006)**. In an attempt to reduce the weed seeds in the soil seed bank and reduce the emerging of weeds, **DYER (1995)** suggested that an integrated method of depleting weed seeds from the soil seed bank will enhance weed management in agriculture. The weed seeds in the soil seed bank can be managed by several physical and chemical triggers, which stimulate the seeds to germinate **(DYER 1995)**.

2.5.4 Agriculturally beneficial bacteria and their role in crop production

There is a global trend towards organic farming in order to increase crop productivity to meet the demands of an expanding population while simultaneously improving water use efficiency and mitigating environmental pollution and high energy inputs associated with synthetic fertilizers, pesticides and herbicides. Conventional farming is also adopting low input, environmentally friendly practices **(GOMIERO *et al.* 2011; DA COSTA *et al.* 2013)**. Areas of research for sustainable and environmentally friendly agricultural practices include the introduction of plant growth promoting rhizobacteria (PGPR) and the application of natural plant biostimulants.

Soil microbes are essential for maintaining soil nutrient levels that sustain plant growth **(MIRANSARI 2013)**. Plant growth promoting rhizobacteria are a diverse group of rhizosphere colonizing bacteria that enhance plant growth, suppress root disease and protect plants from environmental stresses such as drought, salinity and heavy metals **(MAYAK *et al.* 2004)**. There are multiple mechanisms by which these PGPR influence plant growth. These include the synthesis of plant growth regulators such as cytokinins, auxins, gibberellins and nitric oxide (NO) that promote growth and stress-related plant growth regulators such as abscisic acid and jasmonic acid. It also includes the biosynthesis of antimicrobial compounds and induction of enhanced plant defence mechanisms, nitrogen fixation, iron sequestration by siderophores, phosphate solubilization and production of 1-amino-cyclopropane-1-carboxylic acid deaminase **(MAYAK *et al.* 2004; DA COSTA *et al.* 2013; BHARTI *et al.* 2014; CASSÁN *et al.* 2014)**. Plant growth promoting rhizobacteria have been used for many decades to improve crop yields **(MIRANSARI 2013)** and in disease management **(NEGI *et al.* 2011)**. Many PGPR inoculums are available commercially where they are applied with either an organic or inorganic carrier to improve the survival of the bacteria and to maintain a threshold PGPR population to ensure positive effects on plants. The most common genera include the symbiotic *Rhizobium* and the free living *Azospirillum*, *Pseudomonas* and *Bacillus* strains **(BASHAN *et al.* 2014)**.

Before smoke biotechnology can be used on a large-scale, a number of questions need to be addressed including the impact it has on the soil microbial population **(LIGHT *et al.* 2009)** and in particular, on PGPR. The interaction between bacteria

and SW, KAR$_1$ and TMB treatment needs to be examined for agricultural practices. A negative response to these treatments may restrict the use of smoke-technology in agricultural practices.

2.6 *IN VITRO* GERMINATION OF ORCHID SEEDS

2.6.1 Orchid seed germination

Orchid seeds are classified as micro seeds as they have limited to no endosperm (reserves like starch, proteins and lipids) to support germination and early growth **(BASKIN and BASKIN 1998)**. After the orchid pod (capsule) dehisces, millions of dust-like seeds are distributed by wind **(BASKIN and BASKIN 1998)**. Once a seed lands on a suitable substrate, it will rely on a symbiotic fungus (often *Rhizoctonia* spp.) to supply it with various compounds, nutrients and water **(ARDITTI 1979; CHANG 2006)**. Only a couple of seeds from the millions of seeds released from an orchid pod will germinate and grow into a mature plant in a natural environment **(KNUDSON 1922)**. For this reason commercial growers of orchids germinate orchid seeds on nutrient rich agar which circumvents the need for a mutualistic fungus **(CHUGH *et al.* 2009)**. Tissue culture techniques revolutionized the production of new varieties of orchids since thousands of orchids can be produced from the seeds of one orchid pod **(KNUDSON 1922)**. Large numbers of plants can be cultivated from orchid seeds grown on a suitable growth medium, containing all the hormones, macronutrients, micronutrients, minerals, sucrose and vitamins required to sustain the germination and growth of the orchid seeds without the need for a symbiotic fungus **(ARDITTI 1967; CHUGH *et al.* 2009)**. According to **ARDITTI *et al.* (1990)** the most important factors to stimulate the germination of orchid seeds are either sugar or a symbiotic fungus.

2.6.2 *In vitro* germination of *Ansellia africana* orchid seeds

Leopard orchid or *Ansellia africana* Lindl., is an epiphytic orchid species indigenous to the tropical zones of Africa **(MARTINS 2009)**. *Ansellia africana* is a threatened species as it is often harvested for its medicinal value **(POOLEY 1998; GOLDING**

and **BANDEIRA 2002)**. Some studies reported that *A. africana* seeds germinate readily on half strength and full strength Murashige and Skoog (MS) media **(BYTEBIER *et al.* 1996; VASUDEVAN and VAN STADEN 2010)**. **VASUDEVAN and VAN STADEN (2010)** showed that pre-treating the dehisced *A. africana* seeds with 50% NaOCl solution significantly increased the germination of the seeds. **VASUDEVAN and VAN STADEN (2011)** later showed that introducing cytokinins into the growth media increased the overall growth of the plants. Different types of compounds have been used in an attempt to stimulate the germination of orchid seeds. These include nitrogen sources, vitamins, minerals and hormones **(ARDITTI 1967)**.

2.7 POLLEN GROWTH STUDIES

2.7.1 The role of fire in flowering

Fire has a great influence on vegetation ecology and the functioning of several different types of ecosystems. In fire-prone environments, fire-stimulated flowering is also a common phenomenon, especially in herbaceous plants **(GILL and GROVES 1981; RUNDEL 1981)**. For example, flowering of *Cyrtanthus ventricosus* (Amaryllidaceae), a fynbos geophyte commonly known as the 'fire-lily', is associated with fire **(OLIVIER and WERNER 1980; LE MAITRE and BROWN 1992)**. It has also been reported that fire plays a role in mass flowering of *Watsonia borbonica* (Iridaceae) which leads to abundant fruit set **(LE MAITRE 1984)** and seedling recruitment **(KRUGER 1978; KRUGER and BIGALKE 1984; LE MAITRE 1984)**. A similar observation was made of the fruit production of the grasstree *Xanthorrhoea preissii*, which produced more fruits in summer-burnt populations compared to autumn- and spring-burnt populations **(LAMONT *et al.* 2000)**.

Fire is a common phenomenon in most regions of South Africa with the majority of the flora being exposed to smoke from naturally occurring wildfires. As described in **Section 2.2**, fire plays an important role as a germination stimulant. It is possible that plant-derived smoke from burning vegetation may affect flowers and their pollen when they are present during or directly after the vegetation surrounding it has burnt. This theory was based on observations made during the winter months in South

Africa, when grassland fires are common. For example Aloes produce flowers during the winter months and are often exposed to both fire and smoke directly (Fig. 2.6). It is possible that the smoke from these fires may affect the reproductive success of plants at the pollen level. Despite numerous reports of fire-stimulated flowering, no study has been carried out on the effects of smoke on angiosperm pollen.
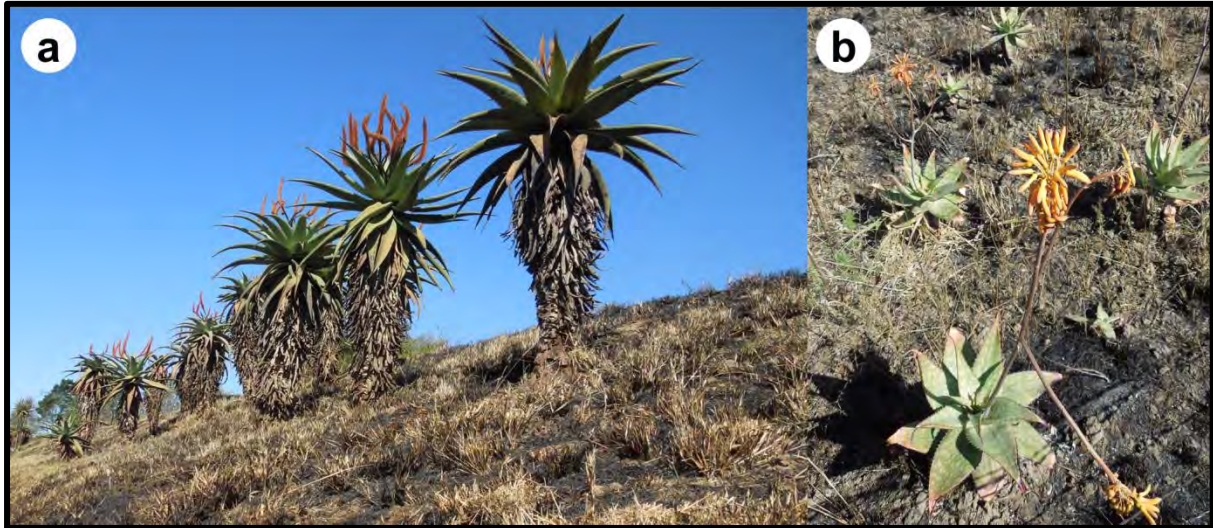
Figure 2. 6 Flowers of a) *Aloe ferox* and b) *Aloe maculata* exposed to fire and smoke. Photographs were taken one day after burning at a grassland near Pietermaritzburg.

2.7.2 *In vitro* pollen germination and pollen tube elongation

Pollen grains will often burst when exposed to water for extended periods of time. This made it very difficult to study pollen using *in vitro* techniques. *In vitro* pollen growth studies were revolutionized by a study conducted by **BREWBAKER AND KWACK (1963)** who showed that pollen grains can be germinated and grown *in vitro*, by supplementing the growth medium (which usually contained sucrose) with boric acid and calcium. Since this discovery was made, many pollen growth studies were conducted which emphasized the roles of sucrose, boric acid and calcium in different plant species. Most *in vitro* pollen growth studies were therefore focused on determining the specific growth requirements of pollen from a particular plant species **(BREWBAKER and KWACK 1963; KHATUN and FLOWERS 1995; SATO *et al.* 1998; BOLAT and PIRLAK 1999; FRANKLIN-TONG 1999; TUINSTRA and**

WEDEL 2000; WANG *et al.* 2004; KUMARI *et al.* 2009; LYRA *et al.* 2011; ABDELGADIR *et al.* 2012)**.

Several pollen growth studies have indicated that the addition of plant growth regulators such as auxins, brassinosteroids, cytokinins and gibberellic acid could induce pollen germination and pollen tube growth when applied to pollen *in vitro* **(SMITH 1942; BAMZAI and RANDHAWA 1967; HEWITT *et al.* 1985; VOYIATZIS and PARASKEVOPOULOU-PAROUSSI 2000; SINGH *et al.* 2002)**. Given the plant growth regulator-like germination inducing effects of smoke, smoke may also have an effect on pollen.

2.7.3 The effects of pollution on pollen

Abiotic factors such as temperature, light and UV-B radiation have been shown to affect the growth of pollen **(DHINGRA and VARGHESE 1985; WANG *et al.* 2006; ACAR and KAKANI 2010)**. Another abiotic factor that is generally neglected and which could potentially have an effect on pollen growth is the chemical substances that are present in the atmosphere. Pollutants such as anthropogenic compounds, heavy metals, pesticides and acid rain have been shown to affect pollen growth **(COX 1984; KAPPLER and KRISTEN 1987; WOLTERS and MARTENS 1987; ABBOTT *et al.* 1991; MUNZURO LU and G R 2000; TUNA *et al.* 2002; GÜR and TOPDEMIR 2005)**. According to these studies, the chemical substances that are released into the atmosphere can affect pollen growth negatively. Smoke is regarded as an environmental pollutant and is a common factor studied in fire ecology **(BAXTER and VAN STADEN 1994)** and could therefore either have beneficial or detrimental effects on pollen growth.

2.8 SUMMARY

During the last two decades major advances have been made in the field of smoke and karrikin research. Smoke and the compounds isolated from smoke play very important roles in the germination of seeds from many plant species. The smoke-derived compounds function as chemical signals in the chemical dormancy mechanisms of plant species which are part of an ecosystem where fire is common. Since these ecosystems have adapted to fire (smoke), the possibility exists that plant-derived smoke may affect not only seeds but also pollen.

The potential implications of smoke-technology in agriculture are immense, with many new uses being discovered on a regular basis. Many studies have been conducted to determine the activity and role of KAR_1. These studies indicated that KAR_1 affects seed germination and also the growth of seedlings from various commercially important crops. The role of SW and KAR_1 is also conspicuous in weed research, as it provides an alternative means for eradicating weeds in an environmentally friendly manner. However, it still needs to be determined what effect SW and KAR_1 can have on the microbial populations in the soil. In contrast to the advances that have been made in karrikin research, the activity and role of TMB in seed germination and different areas of plant growth have not been determined as yet. This study therefore focuses not only on the uses of KAR_1 but also on the activity and uses of TMB in weed control, interactions with agricultural bacteria, orchid seed germination and development and pollen growth.

CHAPTER 3 – STRUCTURE-ACTIVITY RELATIONSHIPS OF ANALOGUES OF 3,4,5-TRIMETHYLFURAN-2(5*H*)-ONE WITH GERMINATION INHIBITORY ACTIVITIES

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3.1 INTRODUCTION

The germination inhibitor trimethylbutenolide [TMB; 3,4,5-trimethylfuran-2(5*H*)-one (**2**)] was isolated from plant-derived smoke **(LIGHT *et al.* 2010)** (see **Section 2.4**). This compound not only inhibited seed germination **(LIGHT *et al.* 2010)**, but also significantly reduced the germination promotory activity of the highly active germination promoter, karrikinolide (**1** or KAR$_1$; 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one) another smoke-derived compound **(LIGHT *et al.* 2010)**. According to **GHEBREHIWOT *et al.* (2013)**, plant-derived smoke contains about 120 times more of compound **2** than **1**. It was reported by **LIGHT *et al.* (2010)** that TMB is effective at concentrations ranging between $10^{-5} – 10^{-3}$ M (Fig. 2.4) which is much higher than the effective concentration range reported for **1 (FLEMATTI *et al.* 2004; VAN STADEN *et al.* 2004)**. Since TMB is available at much higher concentrations in smoke and considering that it is only effective in reducing the germination promotory activity of **1** at relatively high concentrations, several interesting inferences can be made about the roles of TMB in seed dormancy (see **Section 2.4.4**).

Earlier studies have shown that seeds often contain germination inhibitors which were regularly isolated from the pericarp and seed dispersal appendages **(BASKIN and BASKIN 1998)**. These inhibitors are all derived from plant material which makes the discovery of TMB unique, since it is produced by burning plant material **(LIGHT *et al.* 2010)**. Many of the seed germination inhibitors that were isolated belong to the lactone chemical group (see Table 2.1 for a reference of the germination inhibitors previously isolated from seeds). Coumarin is regarded as one of the most potent germination inhibitors isolated from seeds **(EVENARI 1949; MAYER and EVENARI**

1952). Many of the compounds listed in Table 2.1 which are considered potent germination inhibitors share a characteristic CHO-group **(EVENARI 1949)** which is also present in the chemical structures of **1 (FLEMATTI *et al.* 2004; VAN STADEN *et al.* 2004)** and **2 (LIGHT *et al.* 2010)**. Coumarin and TMB are, however, structurally quite different (see **Chapter 2** Table 2.1 and Fig. 2.2) but have similar germination inhibitory activities when applied to 'Grand Rapids' lettuce seeds **(NUTILE 1945; EVENARI 1949; LIGHT *et al.* 2010)**. It was reported by **MAYER and EVENARI (1952)** that the activity of 2-pentene-1:4-olid was half that of coumarin. It is conspicuous that this compound (which is only two methyl groups short of the chemical structure of TMB), is considerably less active than coumarin and therefore also TMB. This indicates that the activity of TMB may be manipulated with the addition or removal of chemical substituents.

Although smoke stimulates the germination of a vast array of plant species, the application of smoke-water (SW) at relatively high concentration reduced the germination of certain seeds **(LIGHT *et al.* 2002)**. According to **LIGHT *et al.* (2002)** smoke could play a dual regulatory role during germination, by providing the stimulus to germinate whilst temporarily preventing germination until sufficient water is available. Since SW contains more TMB than KAR_1 **(GHEBREHIWOT *et al.* 2013)** it is possible that the reduced germination associated with the application of higher concentrations of SW is a consequence of the TMB present in the SW. Such a "stop-go" system could potentially have applications in agriculture or horticulture for controlling the timing of germination, particularly since the effect of **2** may be reversed by leaching the treated seeds, whereas the effect of **1** is only reversible by leaching shortly after treatment (within 1-2 h) **(SOÓS *et al.* 2012)**. Furthermore, compounds with opposing actions on germination could be useful for understanding physiological events involved in breaking seed dormancy **(SOÓS *et al.* 2012)**.

Intensive efforts have been made towards elucidation of the mode of action of KAR_1 (**1**) and structurally-related karrikins **(NELSON *et al.* 2009; FLEMATTI *et al.* 2010; NELSON *et al.* 2010; SOÓS *et al.* 2010; NELSON *et al.* 2011; SCAFFIDI *et al.* 2011; SCAFFIDI *et al.* 2012)**, however, relatively little research has been carried out investigating the action of the germination inhibitor **2**, with the exception of one recent study which found that these two compounds do not compete for the same binding site **(SOÓS *et al.* 2012)**. Thus, in order to further study the action of the

naturally occurring inhibitor (**2**), a structure-activity relationship study of synthetic analogues was initiated to determine the influence of substituents with differentiated electronic and steric properties on inhibitory activity. Moreover, substituents were also selected with regard to further functionalization with fluorescent markers. Therefore, several derivatives of **2** were prepared to investigate the effect of related compounds on the germination inhibition of 'Grand Rapids' lettuce seeds.
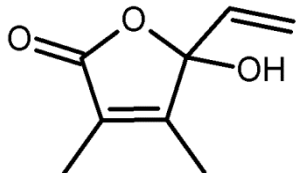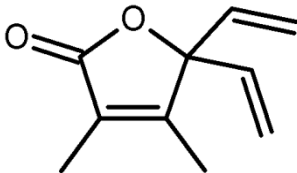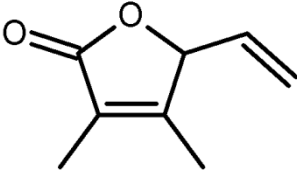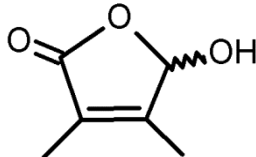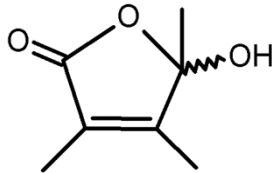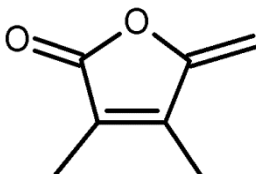
3.2 MATERIALS AND METHODS

3.2.1 Synthesis of analogues of trimethylbutenolide

The analogues used in this study were synthesised by **POŠTA *et al.* (2013)** as listed in Table 3.1. The synthetic analogues were numbered starting with the isolated compounds with **1** = KAR$_1$ and **2** = TMB. Chemical substituents were added to the C-5 position of the furanone ring of **2**. Compound **3**, which has no substituent added to the C-5 position, served as a precursor for the synthesis of the analogues of **2** and was therefore not tested **(POŠTA *et al.* 2013)**. The substituents added consisted of either a butyl group added alone **(5)** or with the addition of a hydroxyl group to produce **4**. A prop-1-ynyl group was also added to C-5 to produce **8** with the addition of either a hydroxyl group (**6**) or another prop-1-ynyl group to produce **7**. The addition of a single vinyl group delivered **11**, which produced **9** with the addition of a hydroxyl group and **10** with the addition of a second vinyl group. Two analogues were produced with the addition of a single hydroxyl group to deliver **12** and with the addition of a methyl group to produce **13**. The last analogue synthesised consisted of a methylene group attached to the C-5 position to produce **14** (Table 3.1).

Table 3. 1 Analogues of trimethylbutenolide with their chemical structures and compound numbers

Substituent added	Chemical name	Compound number	Chemical structure
(Precursor)	2,3-dimethylmaleic anhydride	3	
Butyl	5-Butyl-5-hydroxy-3,4,-dimethyl-furan-2(5*H*)-one	4	
	5-Butyl-3,4,-dimethyl-furan-2(5*H*)-one	5	
Prop-1-ynyl group	5-Hydroxy-3,4-dimethyl-5-(prop-1-ynyl)furan-2(5*H*)-one	6	
	3,4-Dimethyl-5,5-di(prop-1-ynyl)furan-2(5*H*)-one	7	
	3,4-Dimethyl-5-(prop-1-ynyl)furan-2(5*H*)-one	8	

Vinyl group	5-Hydroxy-3,4-dimethyl-5-vinylfuran-2(5H)-one	**9**	
Vinyl group	3,4-Dimethyl-5,5-divinylfuran-2(5H)-one	**10**	
	3,4-Dimethyl-5-vinylfuran-2(5H)-one	**11**	
Hydroxyl group	5-Hydroxy-3,4-dimethylfuran-2(5H)-one	**12**	
Hydroxyl and methyl groups	5-Hydroxy-3,4,5-trimethylfuran-2(5H)-one	**13**	
Methylene group	3,4-Dimethyl-5-methylenefuran-2(5H)-one	**14**	

*The compounds listed in Table 3.1 were synthesised by **POŠTA et al. (2013)** and drawn using ACD/ChemSketch software (Version 12.01).*

3.2.2 'Grand Rapids' lettuce seed germination bioassay

The germination inhibitory activities of the synthesised analogues of **2** were assessed using 'Grand Rapids' *Lactuca sativa* (lettuce) seeds (Stokes, U.S.A., Lot # 211798) in the germination assay described by **LIGHT *et al.* (2010)**. The inhibitory activities of all 11 analogues were tested against one concentration of KAR_1 (10^{-8} M) as this concentration stimulated the germination of lettuce seeds above 95% in the absence of **2** and at low concentrations of **2**. Each synthesised analogue was either applied alone or in combination with 10^{-8} M KAR_1 at 10^{-7} – 10^{-3} M concentrations to four 65-mm Petri dishes each containing 25 seeds. The Petri dishes were lined with two Whatman No. 1 filter paper discs which were moistened with 2.2 mL test solution. Each treatment (consisting of four Petri dishes) was enclosed in an oven roasting bag to prevent cross-contamination of the volatile test compounds between different treatments **(STEWART-JONES and POPPY 2006)** and incubated in light-proof boxes at 25°C for 24 h. Distilled water served as the control while 10^{-8} M KAR_1 was applied alone as the positive control. The experiment was carried out twice (n=8).

3.2.3 Statistical analysis

Germination data were arcsine transformed and analysed using a one-way ANOVA and means separated using Duncan's multiple range test, at a significance level of $P < 0.05$ (Genstat 14th Edition).

3.3 RESULTS

Germination of the water control seeds ranged from 13 – 43% which was consistent with the germination of the control reported by **LIGHT *et al.* (2010)**. The positive control, KAR_1 (10^{-8} M), consistently stimulated lettuce seeds to germinate above 95%. The synthesised TMB molecule (**2**) served as a reference to compare the activity of the analogues and was included in Fig 3.1. This compound produced similar germination inhibitory activity than what was previously reported by **LIGHT *et al.* (2010)**. **2** significantly reduced the germination of lettuce seeds when applied

alone at a rate of 10^{-6} – 10^{-3} M and also significantly reduced the germination promotory activity of **1** when applied simultaneously at the same concentrations, compared to both the water and KAR$_1$ controls (Fig. 3.1). Applying **4** alone or in combination with **1** only produced significantly less germination at 10^{-3} M compared to both controls (Fig. 3.1). **5** significantly reduced lettuce seed germination when applied alone or in combination with **1** at 10^{-4} and 10^{-3} M, compared to the respective controls (Fig. 3.1). **6** exhibited low germination inhibitory activity since it only reduced the germination of lettuce seeds when applied alone at 10^{-4} and 10^{-3} M and when combined with **1** at 10^{-3} M compared to the respective controls (Fig. 3.1).

The prop-1-ynyl substituents of **6** and **7** significantly reduced the germination of lettuce seeds at 10^{-3} M and at 10^{-5} – 10^{-3} M, respectively, when applied in combination with **1** compared to the controls (Fig. 3.2). The seedlings produced by treating lettuce seeds with **6** and **7** stopped elongating after 24 h and were much thicker than the control seedlings and exhibited stunted growth (Fig. 3.3). Treating lettuce seeds with **9** significantly reduced their germination when applied alone at 10^{-3} M and at 10^{-4} and 10^{-3} M when combined with **1**, compared to the respective controls (Fig. 3.2). **10** produced germination inhibitory activity comparable to that of **2** by reducing the germination of lettuce seeds when applied alone at 10^{-6} – 10^{-3} M and when combined with **1** at 10^{-5} – 10^{-3} M, compared to the respective controls (Fig. 3.2).

The inhibitory activity of **11** was most similar to **2** as it produced significantly lower germination when combined with **2** at 10^{-6} – 10^{-3} M, compared to the control. When the substituent consisted of only a hydroxyl group (**12)** lettuce seed germination was only inhibited significantly at 10^{-3} M when applied alone and at 10^{-4} and 10^{-3} M when combined with **1** compared to the respective controls. The addition of a methyl group to the hydroxyl group delivered **13** which significantly reduced germination at 10^{-4} – 10^{-3} M when applied to lettuce seeds alone and at 10^{-5} – 10^{-3} M in combination with **1**, compared to the respective controls. **14** reduced the germination of lettuce seeds at 10^{-4} and 10^{-3} M when applied alone and at 10^{-5} – 10^{-3} M when combined with **1**.
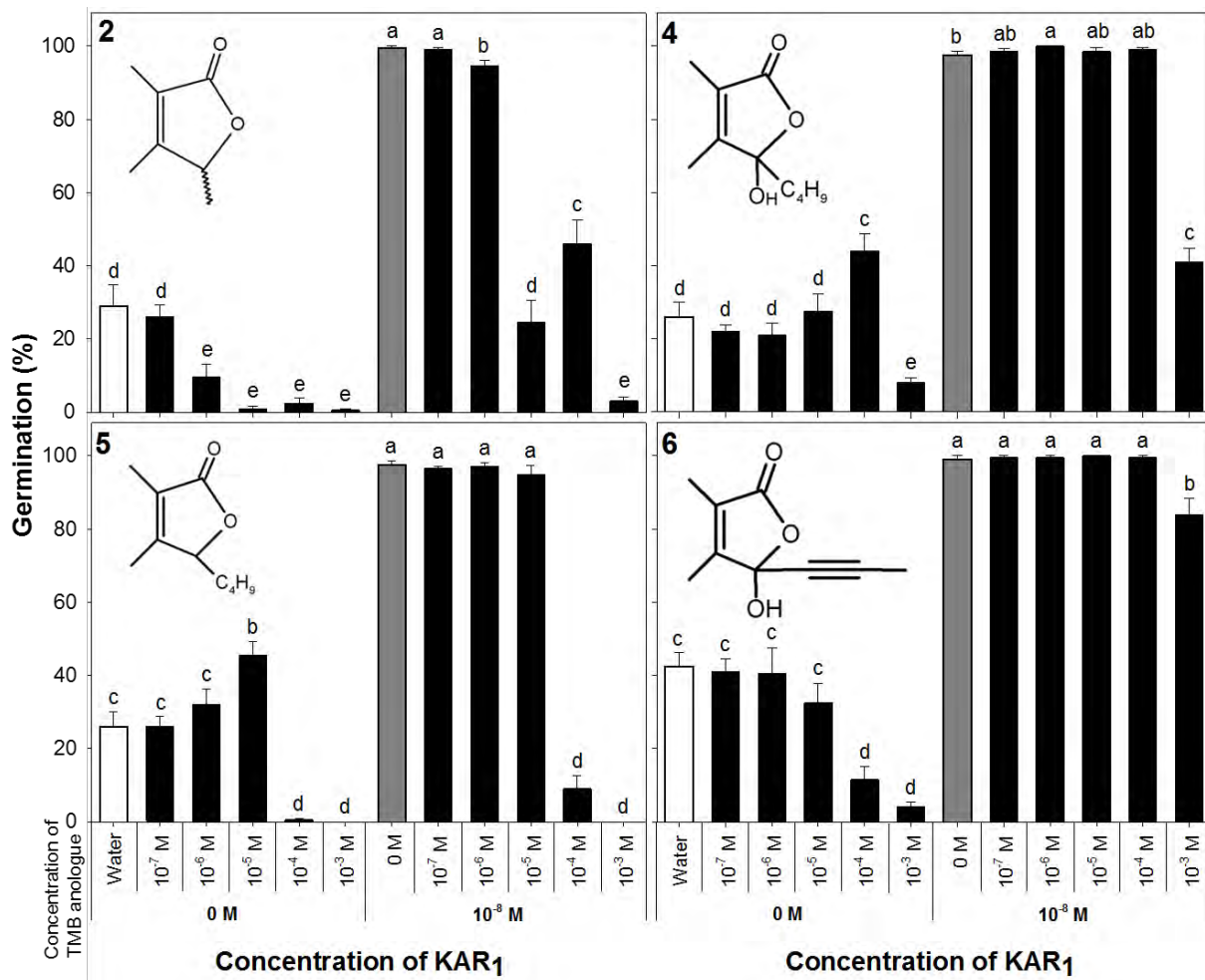
Figure 3. 1 The germination inhibitory activity of TMB (**2**) and the TMB-analogues **4**, **5**, and **6** on 'Grand Rapids' lettuces seeds germinated at 25°C in the dark for 24 h. Solutions contained either the synthesised analogues applied alone, or in combination with **1** (10^{-8} M). Means of germination (bars) ± SE are the average of two separate experiments (n=8). White bars indicate the water controls while grey bars indicate the positive controls (KAR$_1$ 10^{-8} M only). Different letters indicate significant differences between treatments ($P < 0.05$) using Duncan's multiple range test.
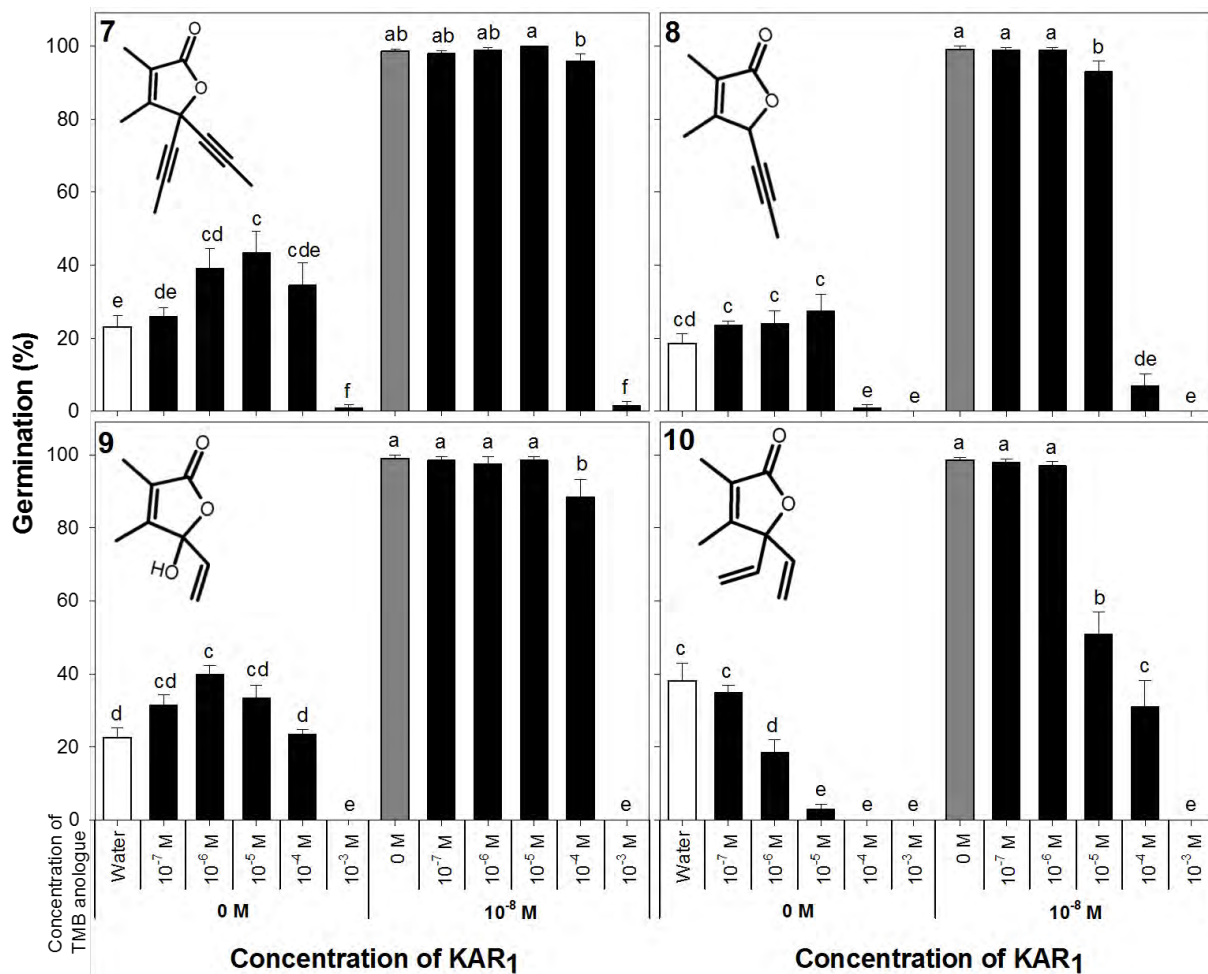
Figure 3. 2 The germination inhibitory activity of the TMB-analogues **7**, **8**, **9** and **10** on 'Grand Rapids' lettuces seeds germinated at 25°C in the dark for 24 h. Solutions contained either the synthesised analogues applied alone, or in combination with **1** (10^{-8} M). Means of germination (bars) ± SE are the average of two separate experiments (n=8). White bars indicate the water controls while grey bars indicate the positive controls (KAR$_1$ 10^{-8} M only). Different letters indicate significant differences between treatments ($P < 0.05$) using Duncan's multiple range test.
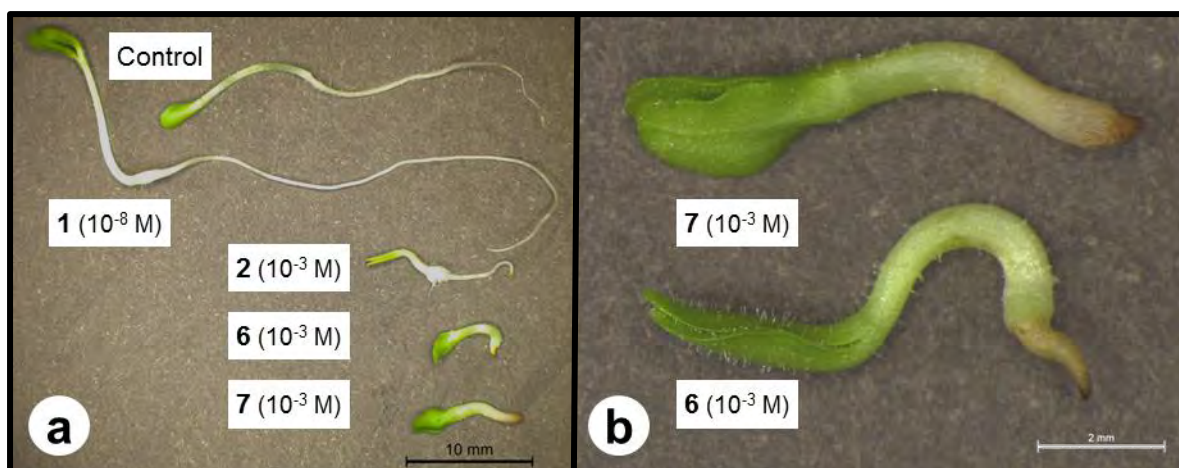
Figure 3. 3 Seedling development of light-sensitive 'Grand Rapids' lettuce seeds germinated with water (control), 10^{-8} M of **1**, or 10^{-3} M of compounds **2**, **6** or **7** after 24 h in the dark and a further 72 h in the light at 25°C. (a) Representative seedlings from each treatment, scale bar = 10 mm. (b) Close-up image of seedlings treated with compounds **6** or **7** showing damage to the radicle, scale bar = 2 mm.
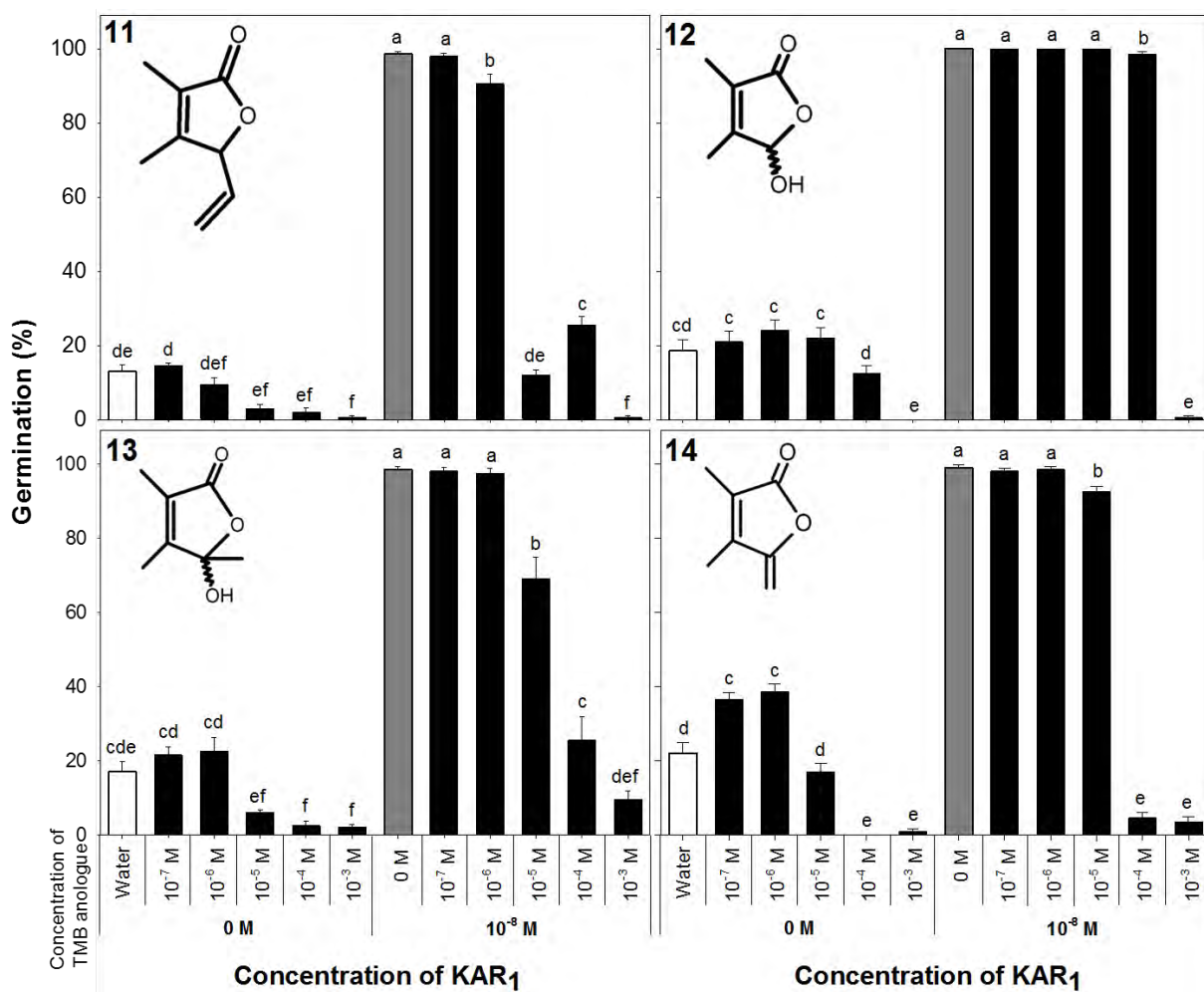
Figure 3. 4 The germination inhibitory activity of the TMB-analogues **11**, **12**, **13** and **14** on 'Grand Rapids' lettuces seeds germinated at 25°C in the dark for 24 h. Solutions contained either the synthesised analogues applied alone, or in combination with **1** (10^{-8} M). Means of germination (bars) ± SE are the average of two separate experiments (n=8). White bars indicate the water controls while grey bars indicate the positive controls (KAR$_1$ 10^{-8} M only). Different letters indicate significant differences between treatments ($P < 0.05$) using Duncan's multiple range test.

3.4 DISCUSSION

The germination inhibitory activities of 11 analogues of **2** were tested using the 'Grand Rapids' lettuce seed germination assay (Figs. 3.1, 3.2 and 3.4) developed by **(DREWES *et al.* 1995)**. This assay was not only fundamental in the isolation of **1** **(FLEMATTI *et al.* 2004; VAN STADEN *et al.* 2004)** and **2 (LIGHT *et al.* 2010)** but has also been used to assess the germination activity of various compounds **(LIGHT 2006)**. The reason why this assay has been used so frequently is because the seeds are light sensitive. The germination of these seeds can therefore be controlled, with only 20-40% of the seeds germinating in the dark when moistened with distilled water. This variety of lettuce seeds germinates rapidly (within 24 h), providing quick results. Furthermore, when these seeds are treated with **1**, at concentrations as low as 10^{-9} M, more than 80% germination is achieved. Since **2** does not compete with the binding site of **1 (SOÓS *et al.* 2012)** but actively reduces the germination promotory activity of **1 (LIGHT *et al.* 2010)**, **1** can be used as a positive control to test the germination inhibitory activities of the synthesised analogues of **2**. Preliminary results indicated that similar results were obtained for the compounds in combination with **1** at 10^{-7}, 10^{-8} and 10^{-9} M. Hence, Figures 3.1, 3.2 and 3.4 show germination activity for the test compounds ranging from $10^{-7} - 10^{-3}$ M, in combination with 10^{-8} M of compound **1**. In all germination tests, 10^{-8} M of **1** alone consistently resulted in germination levels > 95%, and the water control resulted in germination levels ranging from 13% – 43% (Figs. 3.1, 3.2 and 3.4).

Activity for **2** was comparable with the results reported by **LIGHT *et al.* (2010)**, showing significant inhibition at $10^{-6} - 10^{-3}$ M (Fig. 3.1). Although no analogue produced inhibition at concentrations lower than what was reported for **2**, all the analogues significantly reduced germination at 10^{-3} M when combined with 10^{-8} M of compound **1**. Only compounds **4** and **6** did not reduce germination below 20% when applied at 10^{-3} M when combined with **1** (Fig. 3.1). Compound **11** produced the most similar results to **2** by inhibiting germination at $10^{-6} - 10^{-3}$ M when combined with **1** (Fig. 3.4). Compound **10** was also very active inhibiting germination at $10^{-5} - 10^{-3}$ M when combined with **1** (Fig. 3.2). Compounds **5**, **8**, **13** and **14** significantly reduced the germination of lettuce seeds below 40% in the presence of **1** at 10^{-4} and 10^{-3} M (Figs. 3.1, 3.2 and 3.4). Germination was also significantly reduced by **8**, **13** and **14** at 10^{-5} M compared to the positive control when combined with **1** (Figs. 3.2 and 3.4),

but germination was still above 60% and therefore less active than **10**. Compounds **7**, **9** and **12** reduced the germination to less than 5% at 10^{-3} M when applied alone, or in combination with **1** (Figs. 3.2 and 3.4). Compounds **9** and **12**, however, significantly reduced the germination of lettuce seeds when applied at 10^{-4} and 10^{-3} M when combined with **1**. At lower concentrations (10^{-6} and 10^{-5} M), compound **7** showed a slight increase in germination in comparison to the water control, though not to the level as observed with 10^{-8} M of compound **1** (Fig. 3.2). This slight increase in germination, compared to the water control, was also seen with compound **4** (10^{-4} M), compound **5** (10^{-5} M), compound **9** (10^{-6} M) and compound **14** (10^{-7} and 10^{-6} M) (Figs. 3.1, 3.2 and 3.4). Although these slight germination increases were found to be statistically significant (compared to the water control in each case), the germination increase observed was never of the magnitude observed with compound **1** alone (i.e. > 95% germination), and a consistent trend was not observed over the concentration gradient. Overall it can be concluded that these compounds did not act as germination promoters in a similar manner as compound **1**. Compounds **4** and **6** produced the lowest inhibitory activity and only significantly inhibited germination at 10^{-3} M which resulted in about 40% and 80% germination when combined with **1**, respectively (Fig. 3.1).

As mentioned above, **6** and **7** were only effective in reducing germination at 10^{-3} M when combined with **1**. After 24 h the seeds treated with **6** and **7** started germinating. Observations after 72 h revealed that the seedlings that formed after being treated with **6** and **7** were abnormal compared to the control seedlings (Fig. 3.3a). Treating lettuce seeds with 10^{-3} M of either compound **6** or **7,** with or without **1,** produced seedlings with slight radicle tip damage (Fig. 3.3b). These seedlings stopped elongating and never exceeded a length of 10 mm and were noticeably thicker in appearance. It is interesting to note that the addition of a single or double prop-1-ynyl group to the C-5 position of **2** did not only reduce the germination inhibitory activities of these compounds but elicited a morphological change in the germinating seeds/ seedlings.

In terms of the butylene, prop-1-ynyl and vinyl groups that were added to the C-5 position of **2**, it was conspicuous that the addition of a hydroxyl group to any one of the groups disrupted the activity of that specific compound. For instance, the addition of a hydroxyl group to **5** to produce **4**, reduced the activity of **5** considerably

(Fig. 3.1). Similarly, the addition of a hydroxyl group to **8** to produce **6** and to **11** to produce **9**, reduced the inhibitory activities of **8** and **11** considerably (Figs. 3.2 and 3.4). This reduction of inhibitory activity was supported by the activity reported for compound **12** where the substituent consisted of only a hydroxyl group (Fig. 3.4). The activity of **12** was improved by the addition of a methyl group to produce **13** which was clearly effective as a germination inhibitor (Fig. 3.4).

Of all the compounds synthesised the vinyl and divinyl substituents **11** and **10** were the only compounds that retained the germination inhibitory activity that was reported for **2**. Since their germination inhibitory activities were comparable to that of **2**, it makes them ideal candidates for either further functionalization or use in determining the mode of action of this compound. Although the results were only representative of the inhibitory activity of the 11 analogues for a specific period of time (i.e. 24 h), it does give a good indication of the relative activities of the compounds in reducing the germination promotory activity of **1** and also the controls. More experiments need to be conducted to establish the longevity of the effects of these compounds.

The synthesis and biological evaluation of the first series of synthetic derivatives of a germination inhibitor found in plant-derived smoke is reported. Knowledge of the structure-activity relationships of analogues of **2** bearing substituents with differentiated electronic and sterical parameters at C-5 is valuable for the design of similar compounds with improved activity, as well as for synthesis of molecular probes that can be used to elucidate the mode of actions of the smoke-derived compounds.

CHAPTER 4 – USING SMOKE-WATER, KARRIKINOLIDE AND TRIMETHYLBUTENOLIDE TO CONTROL WEED SEED GERMINATION

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4.1 INTRODUCTION

Weeds pose a great problem to farmers worldwide by reducing both the yield and quality of crops **(EHRLICH _et al._ 1993; CARVALHO 2006)**. Controlling weeds demands a high input cost for herbicides and labour **(BROMILOW 2010)**. Due to current environmental regulations, a limited number of herbicides are commercially available (with limited modes of action) to control weeds **(HEAP 2014)**. Furthermore, many weeds have evolved resistance to commercial herbicides **(JASIENIUK _et al._ 1996; BUSI _et al._ 2013)**. New innovative weed management practices are therefore required to control weeds.

After the imbibition of most seeds (seeds with reserves), nutrients need to be released from the reserve storage areas so that germination can take place. To accomplish this, gibberellins mediate the release of hydrolytic enzymes (amylase and protease) which will hydrolyse carbohydrate and protein reserves **(HOPKINS and HÜNER 1995)**. Carbohydrate metabolism (converting starch into reduced sugars by α- and β-amylase) is one of the fundamental processes that occur during germination **(ELLIOTT and LEOPOLD 1953)**. **ELLIOTT and LEOPALD (1953)** showed that an inhibitor of the enzyme amylase was effective in inhibiting the germination of _Avena sativa_ seeds. Alpha-amylase, which is continuously secreted by the embryo, will break down amylose and amylopectin (the constituents of starch), by cleaving the α-(1→4) glucosyl bonds, leaving the disaccharide maltose **(HOPKINS and HÜNER 1995)**. Each maltose molecule will subsequently be converted to two glucose molecules by the enzyme α-glucosidase, which can be used during the germination of seeds **(HOPKINS and HÜNER 1995)**.

Two different weed management strategies are proposed using smoke-water (SW) karrikinolide (KAR$_1$) and trimethylbutenolide (TMB) to manage weed levels. The first

method involves stimulating weed seed germination before sowing crops, followed by physical removal or herbicide treatment of the emerging weeds. Mass germination of the weed seeds in the soil seed bank can be achieved by SW or KAR$_1$ treatment **(KULKARNI *et al.* 2011)**. The second method involves managing the weed seeds in the soil seed bank. Trimethylbutenolide can be applied to the soil after the crop seedlings have emerged. The germination of weed seeds in the soil seed bank can be inhibited with TMB application, creating weed-free periods that would allow sufficient time for crops to establish. Until now, no study has reported on the use of TMB as a method to control weed seeds in the soil seed bank. Before such a mechanism can be utilised, the effect of TMB on weed seed germination needs to be evaluated. The aim of this study was therefore to assess the effects of SW, KAR$_1$ and TMB on five common weed species. A basic understanding on the effects of these compounds on weed seed germination will indicate how these compounds can be utilized to manipulate the germination of weed seeds in the soil seed bank as an organic approach to weed control.

4.2 MATERIALS AND METHODS

4.2.1 Weed seed collection

Five weed species were assessed in this study: *Conyza albida* Willd. ex Sprengel, *Hypochaeris radicata* L., *Solanum mauritianum* Scop., *Spilanthes decumbens* (Sm.) A.H. Moore and *Talinum paniculatum* (Jacq.) Gaertn (Fig. 4.1). Weed seeds were collected from around the outskirts of the University of KwaZulu-Natal Pietermaritzburg campus, South Africa (S29° 37.50', E30° 24.23'). Upon collection, seeds were decontaminated by immersing them in a 0.5% HgCl$_2$ (mercuric chloride) solution for 5 min **(KULKARNI *et al.* 2006b)**. Seeds were then dried and stored at 4°C for 1 year until used.
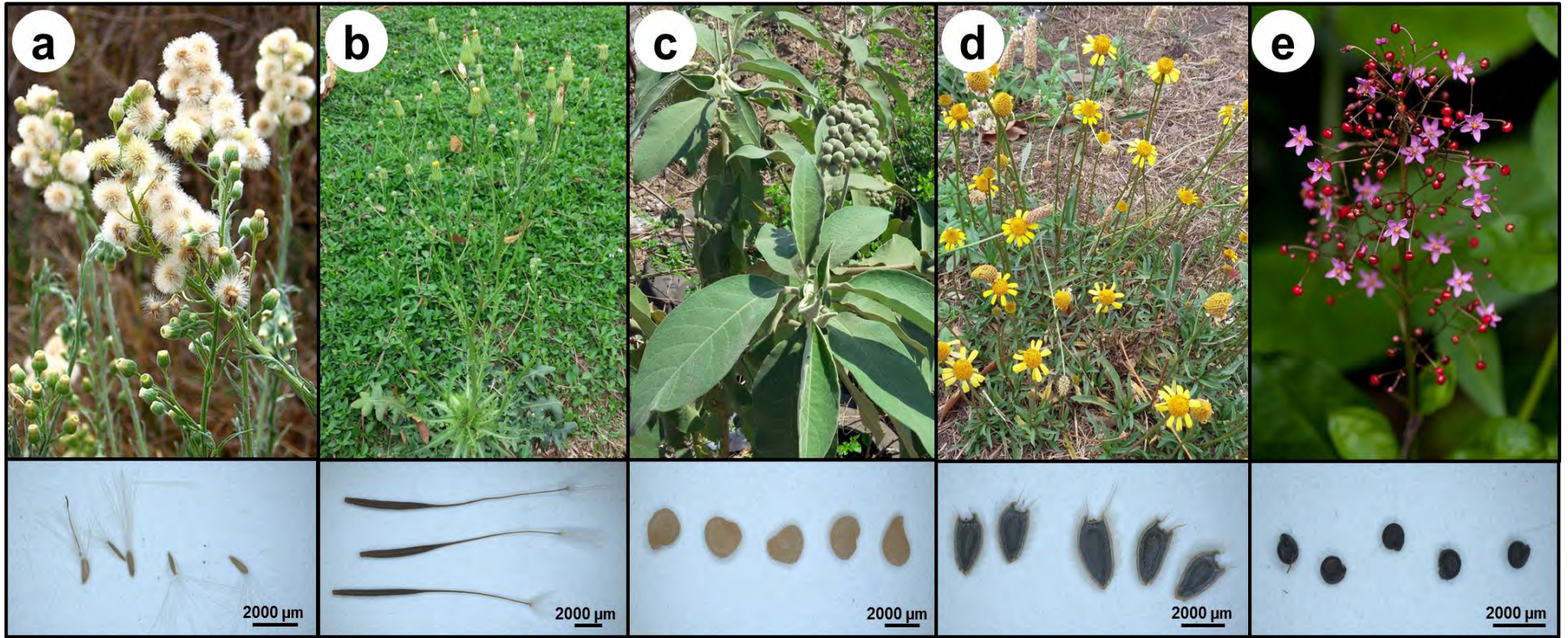
Figure 4. 1 The five weed species assessed in this study with their respective seeds below were a) *Conyza albida*; b) *Hypochaeris radicata*; c) *Solanum mauritianum*; d) *Spilanthes decumbens* and e) *Talinum paniculatum*.

4.2.2 Germination assay

A stock solution of SW was previously prepared by bubbling the smoke from burning plant material (5 kg *Themeda triandra* Forssk. leaf material) through 500 mL distilled water for 45 min as described by **BAXTER *et al.* (1994)**. The SW solution 1:250 (v:v) was prepared by diluting 1 part SW stock solution in 250 parts distilled water. The synthesised KAR_1 (purity > 95%) and TMB (purity > 98%) that were used in this study were prepared according to **FLEMATTI *et al.* (2005)** and **SURMONT *et al.* (2010)**, respectively. In order to investigate the effects of SW, KAR_1 and TMB on the germination of the selected species, 50 seeds per species were placed in each of four 65-mm Petri-dishes lined with two sheets of 70-mm Whatman No. 1 filter paper. For each different experiment, 2.2 mL of the test solutions were added to the Petri dishes. The moisture content in the Petri dishes was maintained by adding 1 mL distilled water every 4 days. In an initial experiment, a range of concentrations of each test solution were applied to the seeds of each weed species to determine the most effective concentrations. The final test solutions used were as follows: KAR_1 10^{-8} M, TMB 10^{-3} M, SW 1:250 (v:v) with distilled water serving as the control. All the experiments were conducted in both complete darkness and a 16 h light/ 8 h dark schedule. These germination trials were also conducted at different temperatures of 20°C, 25°C, 30°C; as well as an experiment where 8 h darkness was held at 20°C and 16 h light at 30°C. The experiment was repeated twice to confirm the results (n=8).

4.2.3 Alpha-amylase activity determination

Alpha-amylase activity was determined in the seeds of all five weed species using the method described by **SADASIVAM and MANICKAM (1996)**. Alpha-amylase activity was determined on the reduction reaction of dinitrosalicylic acid by maltose. To determine the α-amylase activity for each weed species, 200 mg of dry seeds were placed in each of three 65-mm Petri dishes lined with two sheets of 70-mm Whatman No.1 filter paper. The filter paper in each Petri dish was moistened with 2.2 mL test solution. The test solutions consisted of distilled water, SW 1:250 (v:v), KAR_1 (10^{-8} M) and TMB (10^{-3} M). The seeds were incubated at 20-30°C and a 16 h light/ 8 h dark regime. Alpha-amylase activity of *H. radicata* and *S. mauritianum* were

assessed after 10, 15 and 20 days after the addition of the test solutions and after 2, 4 and 6 days for *C. albida*, *S. decumbens* and *T. paniculatum*, which germinated faster than the other two species. After the respective incubation periods, the seeds of each weed species were rinsed with distilled water, blotted dry and weighed. The seeds from each replicate of each treatment were finely ground in 5 mL ice-cold 10 mM calcium chloride using a Janke and Kunkel homogenizer (Ultra-Turrax T25, Germany). The resultant solutions were incubated at 25 ± 1°C for 3 h and then centrifuged at 4°C at 15000 rpm for 20 min using an Avanti J-E centrifuge (Beckman Coulter, USA). The supernatant was used as the enzyme source. From each sample, 1 mL of enzyme was added to test tubes containing 1 mL of a 1% starch solution. The test tubes were then placed in a water bath set at 27°C for 15 min. Thereafter, the reaction was stopped by the addition of 2 mL dinitrosalicylic acid to each test tube which was then placed in boiling water for 5 min. While the tubes were still warm, 1 mL of 40% Rochelle salt solution was added to each test tube and then cooled. The volume of each test tube was made up to 10 mL with distilled water. The absorbance was read at 560 nm using a Cary 50 spectrophotometer (Varian, Germany). The amount of maltose in each sample was calculated on the basis of a standard curve constructed by measuring the absorbance of known quantities of maltose at 560 nm.

4.2.4 Statistical analysis

The germination data were arcsine transformed prior to statistical analysis. For the germination assay and α-amylase activity, significant differences between treatments were determined using one-way ANOVA according to the Duncan's multiple range test ($P < 0.05$) (Genstat 14th Edition).

4.3 RESULTS

4.3.1 Weed seed germination

The effects of KAR_1 and TMB on seed germination were evaluated for five common weed species of crops and gardens. All five weed species required light for optimum germination (Fig. 4.2). Many plant species will only germinate in the presence of light since this relays a message of favourable environmental conditions for germination **(BASKIN and BASKIN 1998)**. If the light requirement of a weed seed can be overcome, it may germinate when conditions are not necessarily optimal for its germination, decreasing its ability to compete with other plants (crops).

The germination of *Conyza albida* seeds was generally low and independent of the light and temperature conditions. Karrikinolide treatment significantly increased the germination of *C. albida* seeds in complete darkness at 30°C (Fig. 4.2a) and in light and dark conditions at 20°C and 25°C compared to the respective controls (Fig. 4.2b and c). Smoke-water also increased the germination of *C. albida* seeds significantly compared to the control at 25°C when exposed to alternating light conditions (Fig. 4.2b). Trimethylbutenolide inhibited the germination of *C. albida* seeds completely when exposed to the alternating temperature treatment (20/30°C) in alternating light conditions (Fig. 4.2d).

Water treated *Hypochaeris radicata* seeds germinated when exposed to alternating light conditions at 20°C and also when exposed to the alternating 20/30°C temperature treatment in alternating light and in complete dark conditions (Fig. 4.2e and h). Smoke-water and KAR_1 treatment significantly increased the germination of *H. radicata* seeds when exposed to alternating light at 20°C and to complete darkness at 20/30°C compared to the respective controls (Fig. 4.2e and h). This result is noteworthy since the germination levels of the SW and KAR_1 treated seeds that were kept in complete dark conditions at 20/30°C was similar to the germination results of the water treated seeds subjected to alternating light at the same temperature treatment (Fig. 4.2h). Smoke-water was the only treatment that stimulated the germination of *H. radicata* seeds at a temperature of 25°C in alternating light conditions compared to the control (Fig. 4.2f). Compared to the respective controls, the germination of *H. radicata* seeds were significantly inhibited

by the TMB treatment at 20°C (alternating light conditions) and at 20/30°C in dark and alternating light conditions (Fig. 4.2e and h).

Water treated *Solanum mauritianum* seeds responded to similar temperature treatments (20°C and 20/30°C) as *H. radicata* (Fig. 4.2i and l). Smoke-water and KAR$_1$ treatment significantly increased the germination of *S. mauritianum* seeds at 20°C, 25°C and at 20/30°C in alternating light compared to the respective water controls (Fig. 4.2i, j and l). The germination of *S. mauritianum* were significantly increased compared to the respective water controls when treated with KAR$_1$ at 20°C and when treated with both SW and KAR$_1$ at 20/30°C in complete darkness (Fig. 4.2i and l). Since SW and KAR$_1$ treatment increased the germination of the dark treated seeds at 20/30°C above the germination level reported for the light treated water control seeds, it seems evident that these treatments overcame the light requirement of *S. mauritianum* seeds (Fig 4.2l). The germination of *S. mauritianum* seeds were significantly reduced (inhibited) at 20°C and at 20/30°C in alternating light and complete dark conditions when compared to the respective water controls (Fig. 4.2i and l).

Water, SW and KAR$_1$ treated *Spilanthes decumbens* seeds produced close to 100% germination at 30°C and at 20/30°C with slightly lower germination at 20°C and at 25°C in alternating light conditions (Fig. 4.2m, n, o and p). Germination of *S. decumbens* seeds were only significantly higher when treated with KAR$_1$ at 20°C compared to the water control in alternating light conditions (Fig. 4.2m). The germination of *S. decumbens* seeds exposed to alternating light conditions were significantly reduced at 20°C, 25°C and 30°C when treated with TMB and in alternating light and complete dark conditions at 20/30°C, compared to the respective water controls (Fig. 4.2m, n, o and p).

Trimethylbutenolide treatment produced significantly lower germination for *Talinum paniculatum* seeds at 30°C (alternating light conditions), and at 25°C and 20/30°C in both alternating light and complete dark conditions when compared to the respective water controls (Fig. 4.2r, s and t). The germination of *T. paniculatum* seeds were only significantly increased by SW treatment when subjected to complete darkness at 25°C and 30°C compared to the respective water controls (Fig. 4.2r and s). Overall the germination of the five weed species with respect to their water controls

germinated optimally when exposed to light condition at 20/30°C. Treating the different weed species with SW and KAR_1 increased the rate of germination while adding TMB decreased the rate of germination compared to the respective water controls (data not shown). When the seeds from the different weeds species were subjected to the 20/30°C temperature treatment, TMB consistently reduced the germination of these seeds below the germination levels of the respective water controls regardless of the light condition (Fig. 4.2d, h, l, p and t).
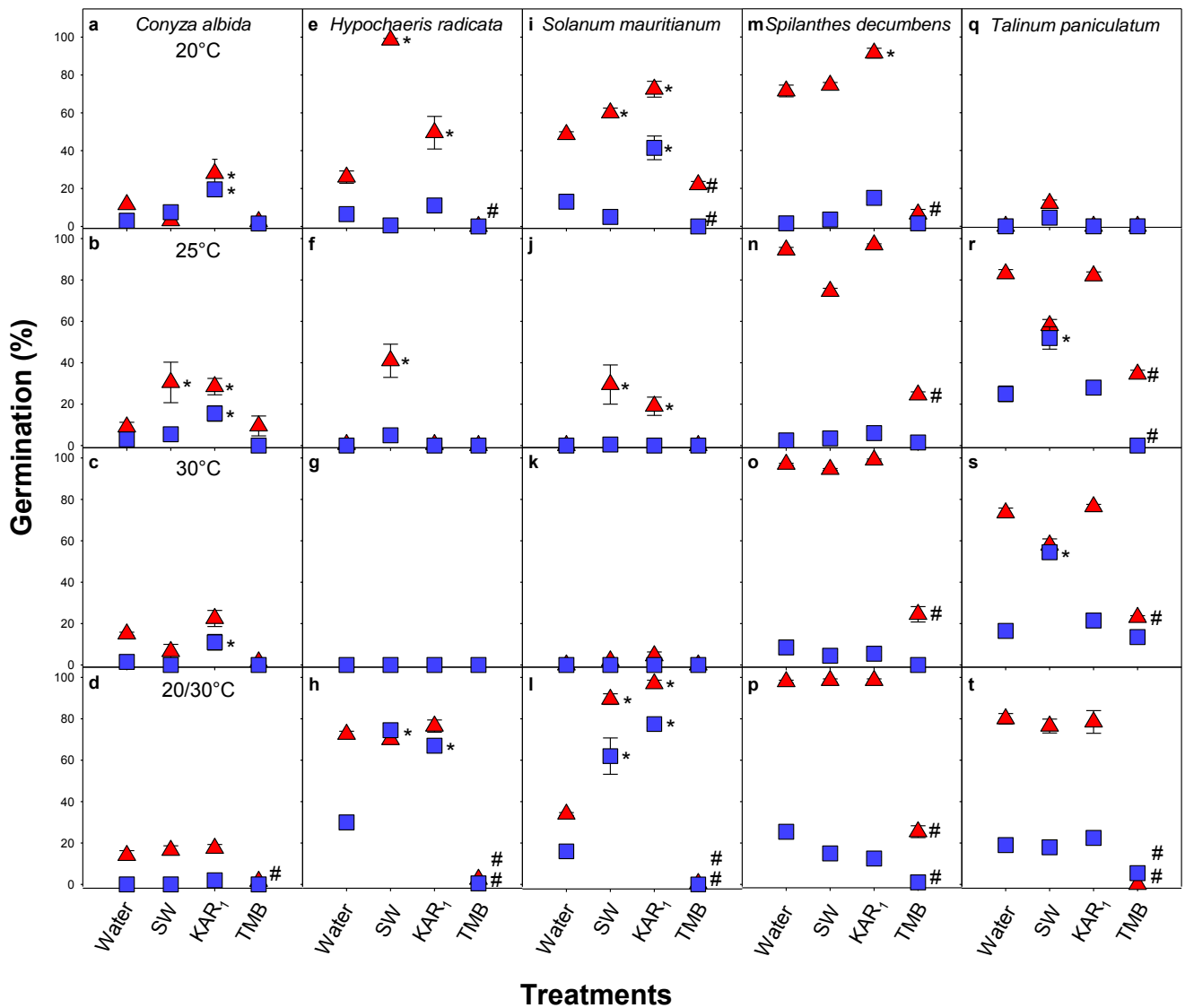
Figure 4. 2 The effect of smoke-water (SW; 1:250 v:v), karrikinolide (KAR$_1$; 10^{-8} M) and trimethylbutenolide (TMB; 10^{-3} M) on the germination of five weed species. Squares (■) ± SE indicate germination of seeds subjected to constant darkness and triangles (▲) ± SE indicate germination of seeds exposed to a 16 h light/ 8 h dark regime. Treatments that produced significantly higher percentage germination compared to the other treatments under each light condition are indicated by an asterisk symbol (*) while the treatments that showed significantly lower percentage germination under each light condition are indicated with a hash symbol (#) according to Duncan's multiple range test ($P < 0.05$) (n=8).

4.3.2. Alpha-amylase activity in germinating weed seeds

Since germination can be controlled by manipulating α-amylase activity **(ELLIOTT and LEOPOLD 1953)**, the effects of SW and the smoke-derived compounds were evaluated in terms of their ability to increase or decrease α-amylase activity. Alpha-amylase activity was assessed in the seeds of all five weed species since it is one of the key metabolic processes during the inception of seed germination and is summarized in Figure 4.3. Karrikinolide treatment significantly increased the α-amylase activity in *C. albida* seeds compared to the water control after 2 and 4 days, while SW treatment only produced a significantly higher α-amylase activity by day 4 (Fig. 4.3a). Treating *C. albida* seeds with TMB significantly reduced α-amylase activity compared to the water treated seeds at day 2, 4 and 6 (Fig. 4.3a). Similarly, TMB treatment also significantly reduced α-amylase activity in *H. radicata* seeds below that of the water control for all the days tested (Fig. 4.3b). At day 15 only KAR_1 produced significantly higher α-amylase activity compared to the water control in *H. radicata* and produced significantly higher α-amylase activities than the water control at day 20 (Fig. 4.3b). Similarly, SW and KAR_1 treatment produced significantly higher α-amylase activities in the seeds of *S. mauritianum* compared to the water control at day 15 while only SW significantly increased α-amylase at day 20 (Fig. 4.3c). Trimethylbutenolide produced significantly lower α-amylase activities at day 15 and 20 in *S. mauritianum* seeds (Fig. 4.3c). No significant differences in α-amylase activity were detected for seeds of *S. decumbens* regardless of the treatment, except for the water control that produced significantly higher α-amylase activity at day 6 (Fig. 4.3d). Trimethylbutenolide treatment, however, significantly reduced α-amylase activity at day 6 when compared to the water controls (Fig. 4.3d). Smoke-water and KAR_1 treatment did not increase α-amylase activity in *T. paniculatum* compared to the water control (Fig. 4.3e). Alpha-amylase was significantly lower than the water control at day 4 and 6 when treated with TMB (Fig. 4.3e).
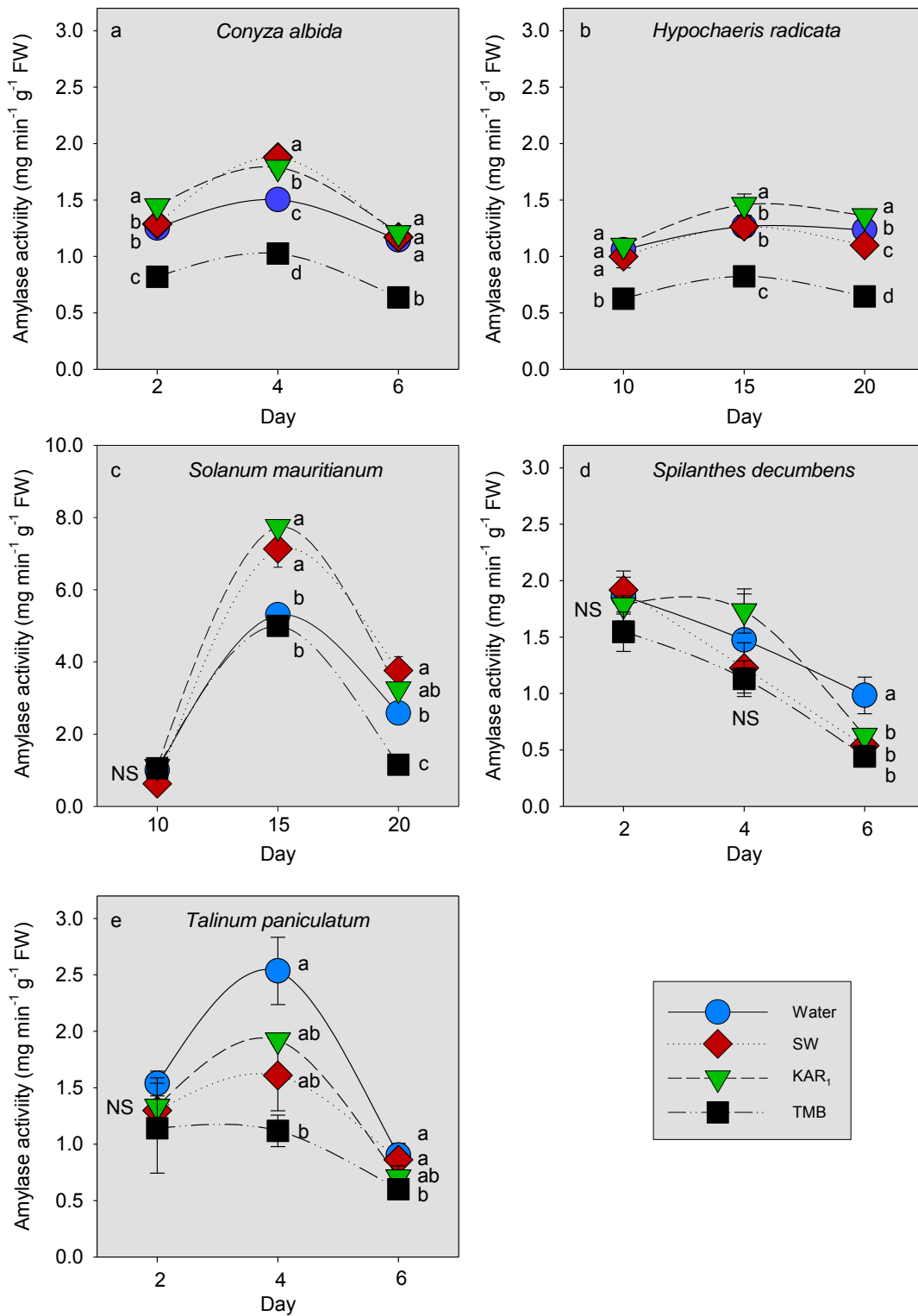
Figure 4. 3 The effect of water (●), smoke-water (1:250, v:v; ♦), karrikinolide (10^{-8} M; ▼) and trimethylbutenolide (10^{-3} M; ■) on the α-amylase activity of five weed species. Significant differences between the treatments at each time period are indicated with different letters according to Duncan's multiple range test ($P < 0.05$). No significant differences are indicated with the letters NS.

4.4 DISCUSSION

Weeds have the potential to reduce crop yields more than any other agricultural pest **(DAYAN *et al.* 2009)**. Not only do herbicides increase production costs **(BROMILOW 2010)** but the number of herbicides that can still effectively manage weeds is diminishing rapidly **(HEAP 2014)**. According to **DAYAN *et al.* (2009)** several natural products have been used to control weeds and these include substances such as acetic acid, corn gluten meal, essential oils and fatty acids. Although the natural products can control weeds to a certain extent, their application is often labour intensive, has low crop selectivity and they need to be applied in large quantities **(DAYAN *et al.* 2009)**. This study describes the use of smoke and the compounds isolated from smoke as natural products for weed control. Although the natural compounds from smoke have low crop selectivity, they can be applied at relatively low amounts which is different to other natural products. The first strategy of weed control proposed in this study makes use of SW and KAR_1 to increase the emergence of weeds so that they can be removed or killed with one treatment. The second strategy makes use of TMB which inhibits seed germination, thereby managing weed seed germination.

Smoke-technology can be utilized as a tool to manage weeds by stimulating their germination with low concentrations of SW and KAR_1 **(KULKARNI *et al.* 2011)**. By stimulating seed germination from the soil seed bank, physical removal and/or herbicide treatments could be more effective in controlling emerging weed seedlings. Several studies have reported that SW and KAR_1 successfully stimulated the germination of weed seeds **(ADKINS and PETERS 2001; BAR-NUN and MAYER 2005; DAWS *et al.* 2007; STEVENS *et al.* 2007)**. In this study, KAR_1 significantly increased germination above the water controls in most of the species tested under alternating light (Fig. 4.2a, b, e, i, j, l and m) and dark conditions (Fig. 4.2a-c, e, h-j, l and m). Smoke-water also significantly increased germination in *C. albida*, *H. radicata*, *S. mauritianum* and *T. paniculatum* under alternating light conditions (Fig. 4.2b, e, f, i, j and l). When subjected to constant darkness, SW treatment produced significantly higher germination in several instances (Fig. 4.2h, l, r and s). The ability of SW and KAR_1 to increase α-amylase activity and stimulate the germination of light sensitive seeds is a very important aspect of this weed management strategy since the weed seeds can be stimulated to germinate when

environmental conditions are not favourable. The subsequent emerging weed seedlings can then be removed before the crops are planted. Any KAR_1 that remains in the soil may also improve the germination of the crop seeds. The concentrations of these compounds in the soil may decrease gradually, since they are polar molecules and could be leached from the soil by rain **(DE LANGE and BOUCHER 1990; BROWN and VAN STADEN 1997; PRESTON and BALDWIN 1999)**. A further advantage of using SW and KAR_1 as tools to stimulate mass seed germination is that the number of weed seeds in the soil seed bank is drastically reduced, thereby reducing subsequent infestation levels.

Smoke-technology can be used to manipulate the weed seeds present in the soil seed bank. The germination inhibitory activity of TMB is illustrated in Figure 4.2. The inhibitory activity of TMB was emphasized by its ability to reduce α-amylase activity (Fig. 4.3) in the germinating seeds of all five weed species tested. The amount of reduced sugars available for successful germination was therefore much lower in the weed seed treated with TMB. According to **SOÓS *et al.* (2012)** TMB blocks fundamental metabolic processes by down-regulating genes associated with metabolism. Trimethylbutenolide also up-regulated abscisic acid and maturation and dormancy related transcripts **(SOÓS *et al.* 2012)**. The low α-amylase activity of seed treated with TMB might therefore be a consequence of its ability to down-regulate the genes associated with the breakdown of storage products. The basis of using TMB as a method of weed eradication rests on the ability of TMB to create weed free periods. It was suggested by **ZIMDAHL (1988)** and **WYSE (1992)** that weed free periods provide crops a window to establish, so that they can compete with the subsequent emerging weeds. Weed seeds in the soil seed bank could be exposed to light, darkness or intermittent light conditions at any time. Although seeds exposed to light would be likely to germinate, TMB could potentially inhibit their germination. Treating soil with TMB after crop establishment may inhibit the germination of weeds, creating weed free periods, which will allow the crops to establish and compete effectively with the weeds if they should emerge thereafter.

Although plant-derived smoke contains TMB and KAR_1, the amounts present are relatively low **(GHEBREHIWOT *et al.* 2013)** and these compounds still need to be synthesised for experimental use. It is therefore much easier to use crude SW as a tool to stimulate seed germination rather than KAR_1. However, the TMB molecule is

small in size (**Chapter 2**, Fig. 2.2), water soluble and can be readily synthesised (**LIGHT *et al.* 2010; POŠTA *et al.* 2013**). If the yield of TMB synthesis can be further increased, it could be used as a tool to inhibit the germination of weed species. Besides reducing the germination of the weed species in this study, the inhibitory activity of TMB has only been tested on lettuce seeds. It is very important to establish the germination inhibitory activity of TMB on a variety of seeds from different genera. Its activity range in terms of inhibiting seed germination will determine if it can be used as a general seed germination inhibitor. The toxicity of TMB also needs to be determined to be able to use it commercially. The interaction of residual KAR_1 and TMB in the soil with crops also needs to be established. If these aspects are met, KAR_1 and TMB might be useful tools in controlling weed emergence. Since this study was conducted under laboratory conditions, field studies are needed to test the new proposed method to manage weed seeds in the soil seed bank.

This is the first report on the potential use of TMB as a tool to minimize weed infestations. Manipulating the germination of weed seeds in the soil could provide crops with ample time to establish and gain a competitive edge against weeds that may be present later. By making use of smoke and the compounds isolated from smoke, the germination of weeds could be controlled, thereby reducing the weed infestation. Since plant-derived smoke (and the compounds that reside in smoke) is easily accessible, it could function as a source for the butenolide compounds. However, the butenolides used in this study (KAR_1 and TMB) were chemically synthesised which is still a costly process. More research is required to make the synthesis of the compounds cheaper and more accessible. If these criteria are met, it may be possible to use SW, KAR_1 and TMB to manipulate weed seed germination.

CHAPTER 5 – INTERACTIONS BETWEEN A PLANT GROWTH-PROMOTING RHIZOBACTERIUM (*BACILLUS LICHENIFORMIS*) AND SMOKE-DERIVED COMPOUNDS AND THEIR EFFECT ON *ABELMOSCHUS ESCULENTUS* (OKRA) GROWTH

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5.1. INTRODUCTION

There is a global trend towards organic farming in order to increase crop productivity to meet the demands of an expanding population while simultaneously improving water use efficiency and mitigating environmental pollution and high energy inputs associated with synthetic fertilizers, pesticides and herbicides **(EHRLICH *et al.* 1993; CARVALHO 2006)**. Conventional farming is also adopting low input, environmentally friendly practices **(GOMIERO *et al.* 2011; DA COSTA *et al.* 2013)**. Areas of research for sustainable and environmentally friendly agricultural practices include the use of plant growth promoting rhizobacteria (PGPR) and the application of natural plant biostimulants.

Soil microbes are essential for maintaining soil nutrient levels that sustain plant growth **(MIRANSARI 2013)**. Plant growth promoting rhizobacteria are a diverse group of rhizosphere colonizing bacteria that enhance plant growth, suppress root diseases and protect plants from environmental stresses such as drought, salinity and heavy metals **(MAYAK *et al.* 2004)**. There are multiple mechanisms by which these PGPR influence plant growth. These include the synthesis of plant growth regulators such as cytokinins, auxins, gibberellins (GAs) and nitric oxide (NO) that promote growth and also stress-related plant growth regulators such as abscisic acid (ABA) and jasmonic acid (JA). It may also include the biosynthesis of antimicrobial compounds and the induction of enhanced plant defence mechanisms, nitrogen fixation, iron sequestration by siderophores, phosphate solubilization and production

of 1-amino-cyclopropane-1-carboxylic acid deaminase (**MAYAK** *et al.* **2004; DA COSTA** *et al.* **2013; BHARTI** *et al.* **2014; CASSÁN** *et al.* **2014)**. The PGPR have been used for many decades to improve crop yields (**MIRANSARI 2013**) and in disease management (**NEGI** *et al.* **2011**). Many PGPR inocula are available commercially where they are applied with either an organic or inorganic carrier to improve the survival of the bacteria and to maintain a threshold PGPR population to ensure positive effects on plants (**BASHAN** *et al.* **2014**). The most common genera include symbiotic *Rhizobium* and the free living *Azospirillum*, *Pseudomonas* and *Bacillus* strains (**BASHAN** *et al.* **2014**).

Smoke derived from the combustion of plant material stimulates germination in a wide range of wild and cultivated species including weeds (**LIGHT** *et al.* **2009**). Apart from stimulating germination, other beneficial effects of smoke treatments include better seedling growth and vigour, increased root growth, improved resistance to salinity, temperature and drought stress, increased flowering and improved crop yield as well as promoting growth in heavy metal contaminated soils (reviewed in **LIGHT** *et al.* **2009; KULKARNI** *et al.* **2011**). Karrikins, the compounds in plant-derived smoke which affect seed germination, are water soluble, thermostable, long-lasting in solution and highly active at very low concentrations (up to 10^{-9} M **LIGHT** *et al.* **2009**) and have no mutagenic and genetic toxic effects (**VERSCHAEVE** *et al.* **2006; TRINH** *et al.* **2010**).

Smoke-water, and the compounds derived from smoke, offers great potential for both conventional and organic agriculture, weed management and land rehabilitation. However, before SW and karrikins can be used on a large-scale, a number of questions need to be addressed including the impact they have on the soil microbial population (**LIGHT** *et al.* **2009**) and in particular, on PGPR. The interaction(s) between a PGPR, namely *Bacillus licheniformis* and SW, KAR_1 and TMB were investigated on the growth of okra (*Abelmoschus esculentus*).

5.2. MATERIALS AND METHODS

5.2.1 Preparation of smoke-water, KAR$_1$ and TMB

Smoke-water was prepared following the method of **BAXTER *et al.* (1994)** by burning 5 kg *Themeda triandra* Forssk. leaf material and bubbling the smoke through 500 mL distilled water for 45 min. The resulting solution was diluted to 1:250 (v:v) to give the stock solution. KAR$_1$ (purity > 95%) and TMB (purity > 98%) were synthesised according to the protocols of **FLEMATTI *et al.* (2005)** and **SURMONT *et al.* (2010)**, respectively.

5.2.2 Bacterial inoculum

Bacillus licheniformis Rt4M10 was isolated from the root surface of *Vitis vinifera* cv. Malbec grown in a commercial vineyard in Mendoza, Argentina. It was characterized biochemically and phylogenetically and identified by 16S rRNA gene sequences **(SALOMON *et al.* 2014)**.

Bacterial inoculum was prepared by growing *B. licheniformis* in 200 mL Luria Broth (LB) media for 2 days at 27°C on an orbital shaker. The optical density (Varian Cary 50 Spectrophotometer) was measured to achieve uniform populations of bacteria of $\approx 10^8$ colony forming units (CFU).mL^{-1}. The inoculum was centrifuged at 7230 rpm for 10 min (Beckman Coulter Avanti J-E Centrifuge) and the pellet rinsed with distilled water to remove traces of LB medium. A bacterial suspension was made using distilled water and adjusted to A$_{660nm}$ − 1.0. This suspension was immediately applied as a soil drench (15 mL) to the pots.

5.2.3 Pot trial

Okra cv. Clemson spineless seeds (*Abelmoschus esculentus*) were purchased from McDonald's Seed Company, Pietermaritzburg, South Africa. New pots (10 cm diameter) were filled with 242 g autoclaved garden soil with one seed sown per pot. Each one of the three replicates (n=3) consisted of five pots giving a total of 15 pots per treatment. The pots were arranged on a metal bench in a greenhouse with a

daily temperature of 22 ± 3°C and midday light intensity of 500-600 $\mu mol.m^{-2}.s^{-1}$. On the day of planting and again 14 days after planting, 15 mL of test solution was added to each pot as a soil drench. The treatments were as follows: (1) control (distilled water); (2) smoke-water [SW (1:500 v:v)]; (3) SW 1:1000 v:v; (4) KAR_1 10^{-7} M; (5) KAR_1 10^{-8} M; (6) TMB 10^{-3} M and (7) TMB 10^{-4} M. Additional treatments consisted of the above listed treatments combined with the bacteria inoculum bringing the total number of treatments to 14. Pots were watered twice a week with tap water to full capacity for the duration of the pot trial.

Plants were harvested five weeks after sowing over a period of three days with five plants pooled as a single replicate. Growth parameters of shoot height, root length, fresh weight of roots and shoots (leaves and stem) and leaf area were recorded. Fresh root and shoot material were taken for the various biochemical analyses and the remaining plant material ground in liquid nitrogen and lyophilized.

5.2.4 Determining chlorophyll content

Chlorophyll (Chl a + b) and carotenoid content in the leaves were determined by extracting 250 mg (FW) leaf material in acetone with a small amount of acid-washed sand. The extract was centrifuged at 3000 rpm for 5 min and the pellet re-extracted. The absorbance of the combined supernatants was measured at 470 nm, 645 nm and 662 nm (Varian Cary 50 Spectrophotometer). The pigment content expressed as $\mu g.g^{-1}$ FW, was estimated using the formulae:

Chl a = $11.23A_{662} - 2.04A_{645}$

Chl b = $20.13A_{645} - 4.19A_{662}$

Chl $a+b$ = $7.05A_{662} + 18.09A_{645}$

Total carotenoids = $(1000A_{470} - 1.90$Chl $a - 63.14$Chl $b)/214$ **(LICHTENTHALER 1987)**.

5.2.5 Determining protein content

Protein content in the leaves was estimated following the method of **LOWRY *et al.* (1951)**. Briefly, 500 mg (FW) leaf material was extracted using 10 mL 20% trichloroacetic acid. The homogenate was centrifuged for 15 min at 600 rpm. The supernatant was discarded and 5 mL 0.1 M NaOH was added to the pellet and centrifuged. The supernatant was separated and made up to 5 mL with 0.1 M NaOH. From this extract, 0.5 mL of the sample was combined with 5 mL alkaline copper solution ($CuSO_4$) and mixed well. This solution was kept for 10 min in the dark, after which 0.5 mL Folin phenol reagent (previously diluted in a 1:1 ratio with distilled water) was added with vigorous mixing and kept in the dark for a further 30 min. The sample was read at 660 nm in the spectrophotometer. A blank was prepared without a protein sample. A standard curve of protein prepared by using Bovine Serum Albumin was used to calculate the protein content ($mg.g^{-1}$ FW).

5.2.6 Determining sugar content

The sugar content was estimated in duplicate using anthrone reagent **(JERMYN 1975)** where 25 mg (DW) shoot material was extracted in 10 mL 80% ethanol for 60 min at 95°C. Following centrifugation at 2000 rpm for 15 min, the supernatant was made up to 10 mL with distilled water. The extract (500 µL) and 3 mL anthrone reagent (freshly made 0.2 g anthrone in 100 mL 95% H_2SO_4) was combined and heated at 100°C for 10 min after which the reaction was stopped by placing the solution on ice. The absorbance was read at 620 nm. A standard curve was prepared with glucose ($0 - 250$ $µg.mL^{-1}$) and used to calculate the sugar content.

5.2.7 Determining α-amylase activity

Alpha-amylase activity was determined in the root and leaf material according to the method described by **SADASIVAM and MANICKAM (1996)**. Alpha-amylase activity was determined on the reduction reaction of dinitrosalicylic acid (DNS) by maltose. Leaf material (1 g FW) was homogenized in a pre-chilled mortar and pestle with 10 mL calcium chloride (10 mM). The resultant solutions were incubated at 25 ± 1°C

for 3 h and then centrifuged at 4°C at 15000 rpm for 20 min using an Avanti J-E centrifuge (Beckman Coulter, USA). The supernatant was used as the enzyme source. From each sample, 1 mL of enzyme was added to test tubes containing 1 mL of a 1% starch solution. The test tubes were then placed in a water bath set at 27°C for 15 min. Thereafter, the reaction was stopped by the addition of 2 mL DNS to each test tube which was then placed in boiling water for 5 min. While the tubes were still warm, 1 mL of 40% Rochelle salt solution was added to each test tube and then cooled. The volume of each test tube was made up to 10 mL with distilled water. The absorbance was read at 560 nm using a Varian Cary 50 spectrophotometer (Germany). The amount of maltose in each sample was calculated on the basis of a standard curve constructed by measuring the absorbance of known quantities of maltose at 560 nm. The activity of α-amylase was expressed as µmoles maltose released mg^{-1} protein min^{-1}.

5.2.8 Estimating soil bacterial abundance

During harvesting, the soil adhering to the roots (rhizosphere soil) was gently shaken into a plastic bag. Bacterial abundance in this rhizosphere sample was estimated using the modified method of **ALAM *et al.* (2013)**. The collected soil (500 mg) was suspended in 50 mL sterile LB medium and shaken on an orbital shaker for 20 min at 27°C. Before sampling, the flasks were gently shaken to suspend the soil and then 1 mL suspension was transferred to a bottle containing 99 mL LB media. This diluted suspension was gently shaken and 1 mL of the homogenous suspension was spread on a Petri dish of LB medium under sterile conditions. The petri dishes were incubated at 27°C in the dark. The bacterial colonies were counted after 24 h using a colony counter (Colony Anderman Counter) and the number of bacterial colony forming units $(CFU).g^{-1}$ soil was calculated.

5.2.9 Statistical analysis

The results were analysed using a one-way ANOVA and the means separated using Duncan's multiple range test at a 5% level of significance ($P < 0.05$). General

ANOVA was also conducted to determine the significant difference of main effects and their interactions ($P < 0.05$; GenStat[®] 14th Edition).

5.3. RESULTS

5.3.1 Effect of bacteria inoculum and/or smoke treatment on okra growth

Application of the bacterium *B. licheniformis* promoted growth of okra with a significant increase in shoot biomass (Fig. 5.1a) as well as an increase in root biomass and leaf area compared to the respective controls (Fig 5.1b and c). Only KAR$_1$ (10^{-7} M) significantly increased all the growth parameters compared to the control when it was applied alone. Although not significantly, SW 1:500 increased all the growth parameters when compared to the control in the absence of the bacteria inoculum. Lower concentrations of KAR$_1$ (10^{-8} M) and SW 1:1000 had no or a slight inhibitory effect on the growth of okra when applied in combination with or without the bacteria inoculum (Fig. 5.1). Application of *B. licheniformis* in combination with KAR$_1$ (10^{-7} M) and SW 1:500 had an antagonistic effect and decreased the growth parameters compared to the control (Fig.5.1). In comparison, the smoke-derived inhibitor TMB had a negative effect on okra growth with the highest TMB concentration (10^{-3} M) significantly decreasing shoot biomass and leaf area and decreasing root biomass compared to the control. Application of *B. licheniformis* inoculum in combination with TMB overcame the inhibitory effects of TMB so that growth was similar to that of the control plants (Fig. 5.1). Similar trends were also measured for the shoot and root length (data not shown).
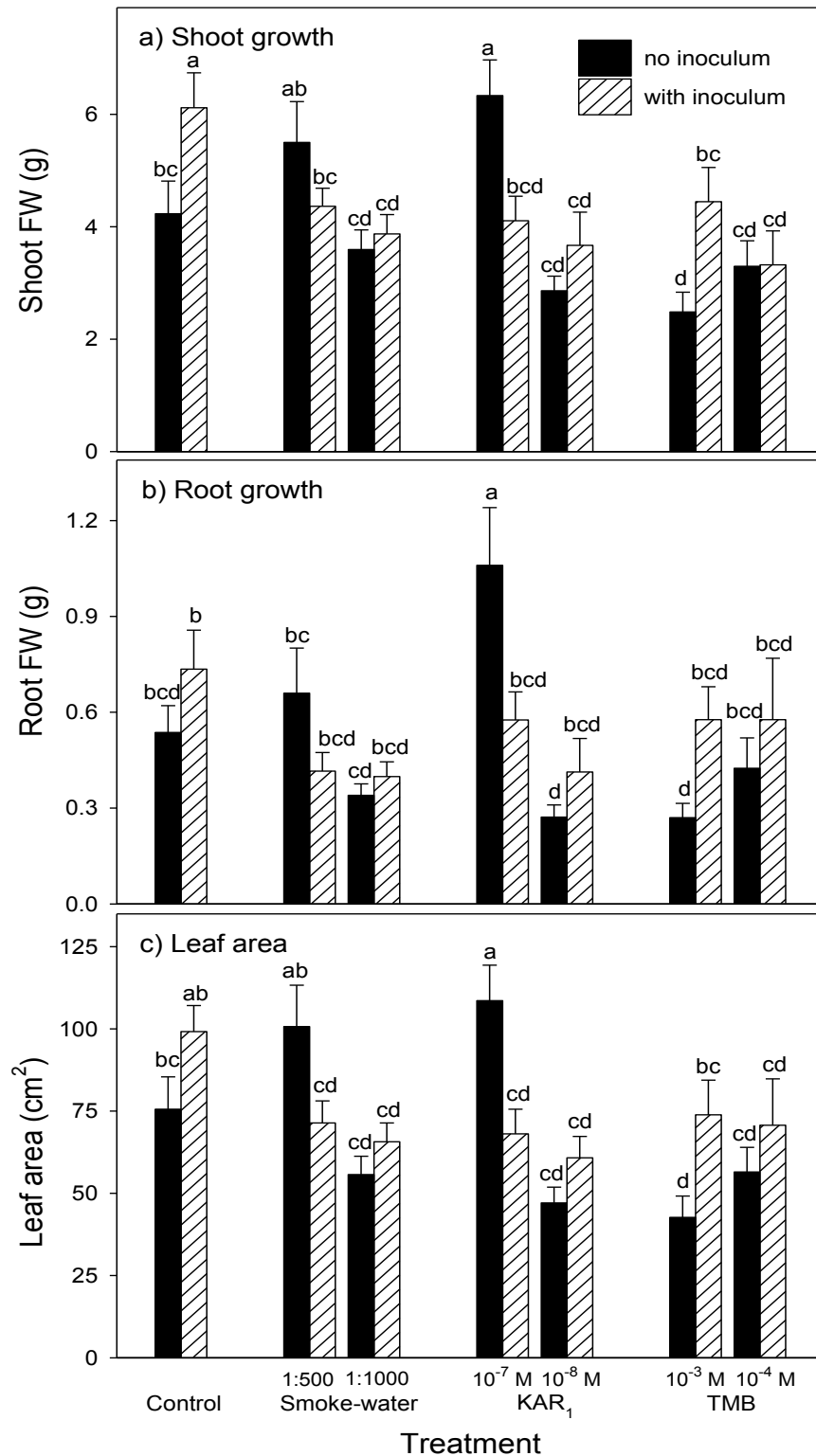
Figure 5. 1 Growth parameters showing a) shoot biomass; b) root biomass and c) leaf area of okra harvested after 5 weeks when treated with smoke-derived compounds and *Bacillus licheniformis* inoculum applied singularly and in combinations. Results are presented as mean ± SE (n=3) with significant differences indicated by different letters ($P < 0.05$). KAR_1 = karrikinolide; TMB = trimethyl-butenolide.

General analyses of variance showed that treatment, concentration and their interaction had a significant effect on okra shoot and root length, shoot fresh weight and plant leaf area. Bacteria alone did not show a significant difference but it should be their interaction with treatment and different concentrations of smoke-derived compounds showed significant differences for all the examined growth parameters (Table 5.1).

Table 5. 1 General analysis of variance of growth parameters of okra plants with main effects and their interactions

Source of variation	Shoot length (mm)		Root length (mm)		Shoot FW (g)		Root FW (g)		Plant leaf area (cm^2)	
	F value	*P* value	*F* value	*P* value	*F* value	*P* value	*F* value	*P* value	*F* value	*P* value
Treatment (T)	14.21	< 0.001	3.02	< 0.031	5.64	< 0.001	2.07	> 0.106	3.91	< 0.010
Concentration (C)	3.97	< 0.001	2.45	< 0.020	3.72	< 0.001	3.43	< 0.002	3.73	< 0.001
Bacteria (B)	0.62	> 0.431	1.14	> 0.287	0.03	> 0.872	0.03	> 0.857	0.00	> 0.981
T X C	7.17	< 0.001	4.42	< 0.002	6.73	< 0.001	6.20	< 0.001	6.74	< 0.001
T X B	4.68	< 0.004	1.21	> 0.309	2.05	> 0.108	2.58	> 0.055	3.20	< 0.025
C X B	2.01	> 0.079	1.93	> 0.092	2.75	< 0.020	2.17	> 0.059	2.92	< 0.015
T X C X B	4.25	< 0.001	2.48	< 0.025	3.44	< 0.003	3.21	< 0.005	4.16	< 0.001

5.3.2 Biochemical quantification

Bacillus licheniformis inoculum and the various smoke-derived compounds applied singularly and in combination, had no significant effect on the total chlorophyll (Fig. 5.2a) and carotenoid (Fig. 5.2b) contents in okra. The one exception was the combined SW 1:1000 and *B. licheniformis* treatment where there was a significant decrease in the chlorophyll content compared to the other treatments (Fig. 5.2a and b).

a) Total chlorophyll

b) Carotenoids
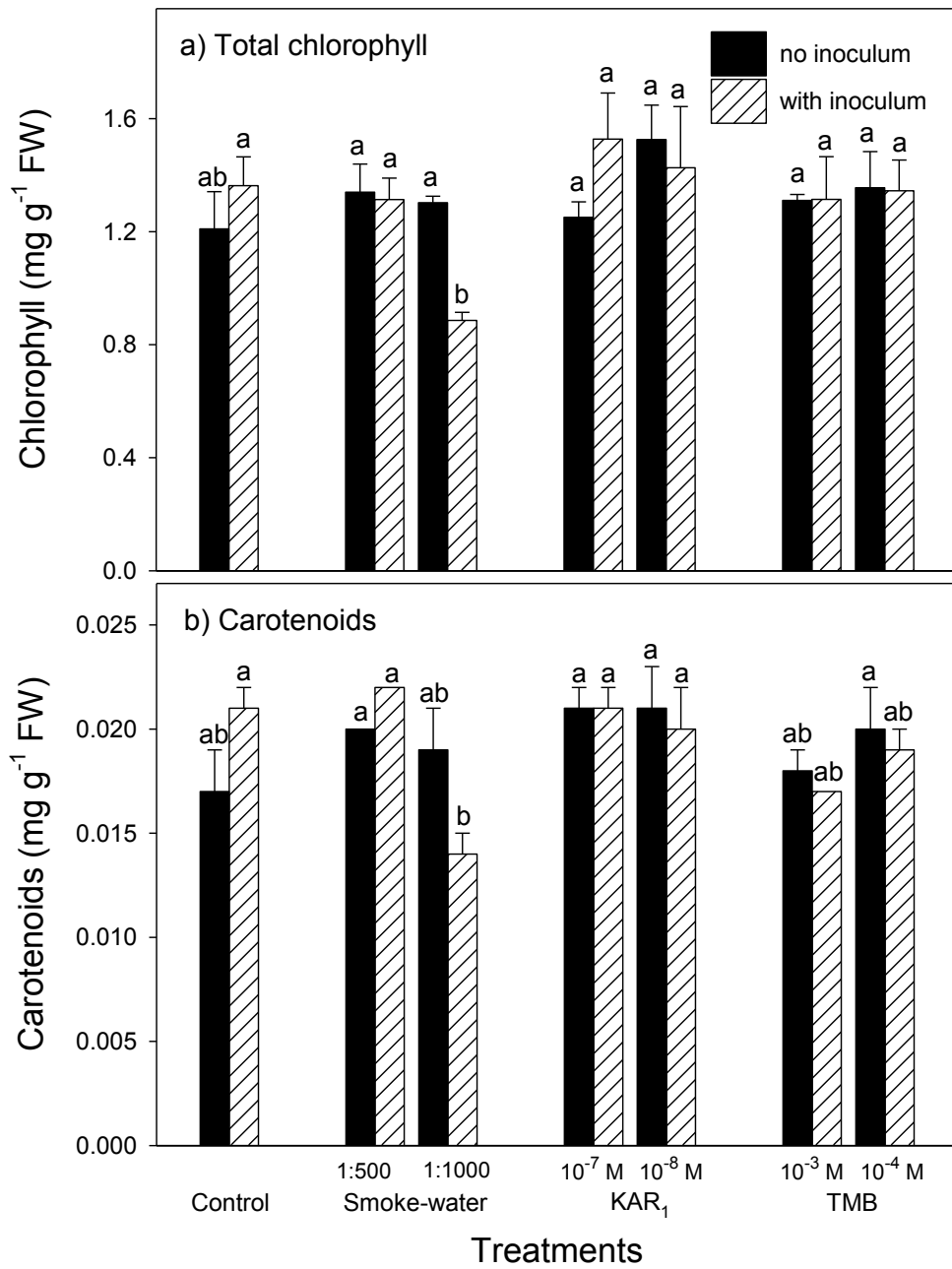
significantly above the control without inoculum (Fig. 5.3b). Lower concentrations of SW (1:1000), KAR_1 (10^{-8} M) and TMB (10^{-3} M and 10^{-4} M) had no measureable effects on the α-amylase activity in the okra roots (Fig. 5.3b). None of the treatments had a significant effect on the protein content in the shoots and roots of okra (Fig. 5.3c and d) and on the sugar content (Fig. 5.3e).
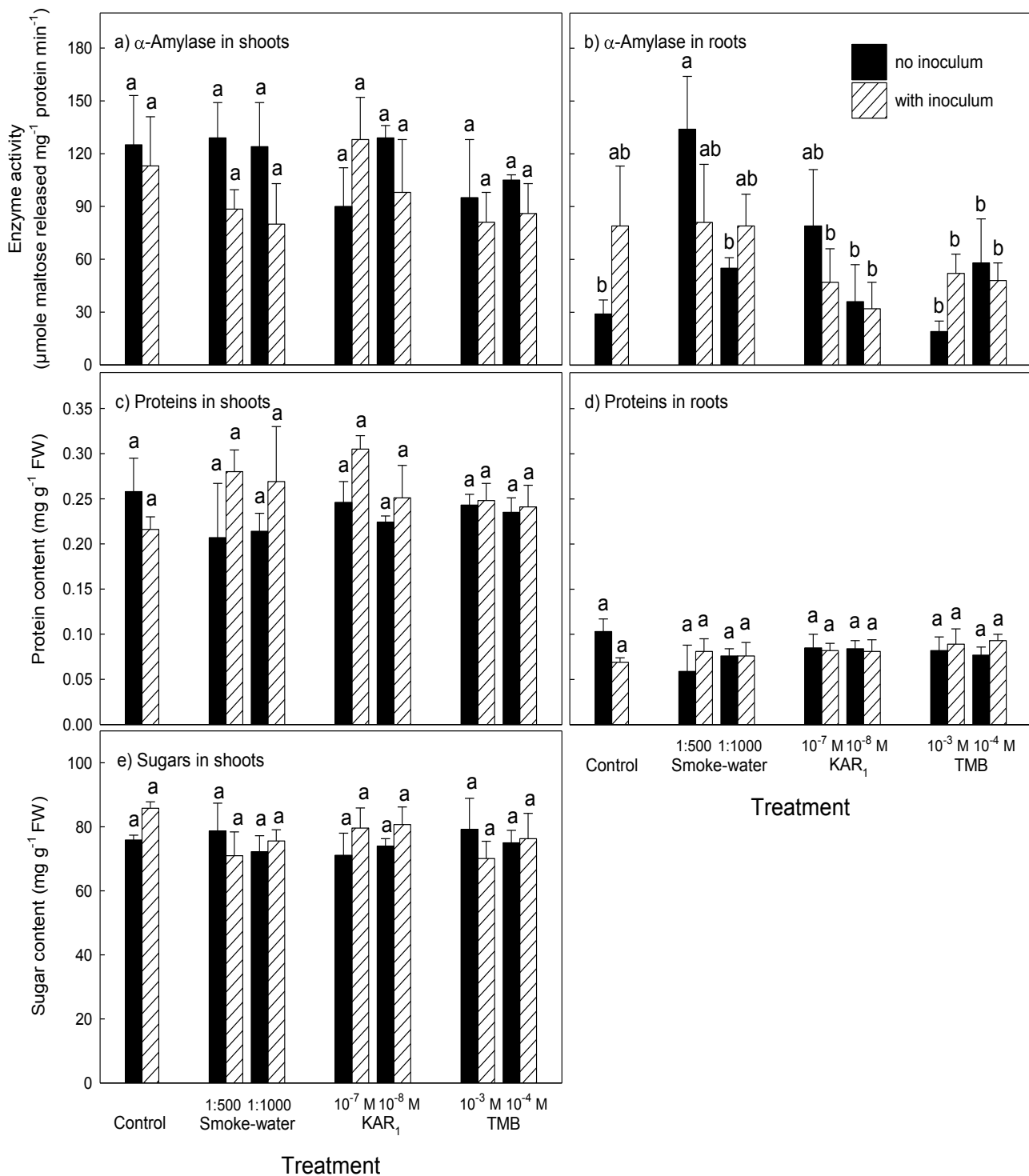
Figure 5. 3 Alpha-amylase activity in a) shoots and b) roots, protein content in c) shoots and d) roots and e) sugar content in shoots of okra harvested after 5 weeks when treated with smoke-derived compounds and *Bacillus licheniformis* inoculum applied singularly and in combinations. Results are presented as mean ± SE (n=3) with significant differences indicated by different letters ($P < 0.05$). KAR_1 = karrikinolide; TMB = trimethylbutenolide.

General analyses of variance of the biochemical parameters did not show significant differences for their main effects and interactions (Data not shown).

5.3.3 Bacterial abundance

Bacterial population abundance in the rhizosphere was quantified at the end of the pot trial. None of the treatments affected bacterial abundance significantly, however, the differences in bacterial abundance among the treatments is mentioned. Generally, treatments where *B. licheniformis* inoculum was added had higher bacterial abundance compared to the corresponding treatments where bacteria were omitted (Fig. 5.4). The exceptions were the TMB treatments where the bacterial populations in the rhizosphere were lower in the pots where *B. licheniformis* was added (Fig. 5.4). Bacterial colonies were also greatly reduced in the SW 1:500 treatment (Fig. 5.4). General analyses of variance of the bacterial abundance did not show significant differences for their main effects and interactions (Data not shown).
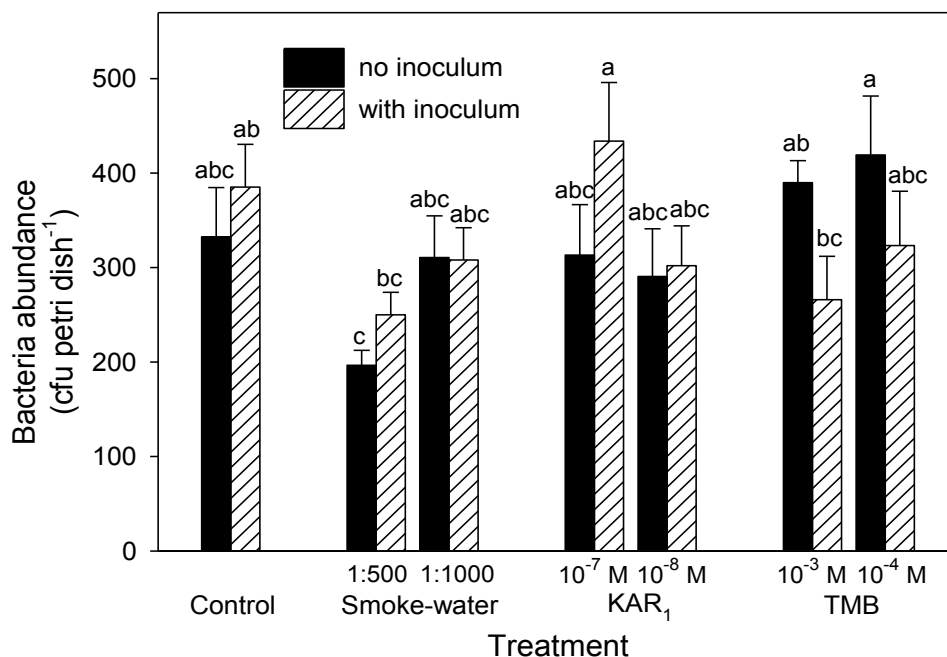
Figure 5. 4 Bacterial population abundance in the rhizosphere of okra when treated with smoke-derived compounds and *Bacillus licheniformis* applied singularly and in combinations. Results are presented as means ± SE (n=3) with significant differences indicated by different letters ($P < 0.05$). KAR_1 = karrikinolide; TMB = trimethylbutenolide.

5.4. DISCUSSION

It is necessary to maintain a threshold number of bacterial cells in the rhizosphere for a positive effect on plant growth to be achieved. A population decline may occur, especially if the inoculum is not applied with a suitable carrier. However, positive results may still be achieved when using PGPR without any formulations in small scale application **(BASHAN *et al.* 2014)** as was done in the present study where the bacterial inoculum was applied twice at a two week interval to the pots. This method of application was successful to ensure a sufficient rhizobacteria population to achieve a positive effect as seen by the improved growth of okra treated with bacterial inoculum (Fig. 5.1).

Plant growth promoting rhizobacteria have been used to improve the growth and yield of a number of crops such as tomato, pepper **(MAYAK *et al.* 2004)**, potato **(GURURANI *et al.* 2013)** and wheat **(KASIM *et al.* 2013)**. These organisms have multiple mechanisms by which they influence plant growth including the ability to synthesise a number of plant hormones with "crosstalk" interactions with the host plant. For example, *Azospirillum* sp. synthesises auxins, GAs, cytokinins, ethylene, ABA, NO and polyamines **(CASSÁN *et al.* 2014)** and *Bacillus pumilus* and *Achromobacter xylosoxidans* synthesise JA, ABA and salicylic acid **(CASTILLO *et al.* 2013)**. The plant hormones ABA, indole-3-acetic acid (IAA) and GAs (GA_1 and GA_3) were previously identified in the *B. licheniformis* strain used in the present study when grown in a chemically-defined medium. It had non-pathogenic characteristics and was able to improve shoot and root length and leaf area in *in vitro Vitis vinifera* cv. Malbec as well as increase the ABA content in the shoots and the IAA content in the roots **(SALOMON *et al.* 2014)**. This strain was also effective in promoting shoot and root growth in the present pot trial with okra (Fig. 5.1).

Smoke-water and KAR_1 improve growth in a wide diversity of plants **(LIGHT *et al.* 2009)** as was the case in the present study with KAR_1 (10^{-7} M) and SW (1:500) which improved both root and shoot biomass and leaf area. The lower concentrations had less effect. Similar to other studies, KAR_1 had a greater effect on the growth of okra than the crude SW extract (Fig. 5.1). The mode(s) of action by which the various smoke-derived compounds influence plant growth have not been fully elucidated although gene expression and protein ubiquitination patterns are

different with SW and KAR_1 treatment **(SOÓS *et al.* 2010; SOÓS *et al.* 2012)**. Physiological studies have shown that there are interactions between the smoke compounds and plant hormones. For example, KAR_1 interact with GAs as well as affecting endogenous GA and ABA contents in various species. In addition, smoke compounds can substitute for strigolactones in stimulating weed germination and can substitute for auxins in somatic embryogenesis **(reviewed in LIGHT *et al.* 2009)**. Karrikinolide stimulated cell division in the soybean callus bioassay which is used to measure cytokinin-like activity. It also stimulated rooting in the mung bean bioassay which is used to measure auxin-like activity. A synergistic effect was observed when KAR_1 was applied in combination with a cytokinin (kinetin) and auxin (indole-3-butyric acid) in the respective bioassays **(JAIN *et al.* 2008)**. Taken together, these studies suggest crosstalk between KAR_1 and plant growth regulators **(reviewed in LIGHT *et al.* 2009)**.

Although the application of *B. licheniformis*, SW (1:500) and KAR_1 (10^{-7} M) enhanced growth in okra when applied singularly, an antagonistic effect was observed when they were applied in combination (Fig. 5.1). A possible explanation for this antagonistic effect may be that *B. licheniformis*, SW and KAR_1 have overlapping modes of action, all being involved in hormone crosstalk with the associated plant with *B. licheniformis* producing hormones **(SALOMON *et al.* 2014)** and KAR_1 having synergistic effects **(JAIN *et al.* 2008)**. Thus, combined applications could potentially disrupt hormone homeostasis in the plant and thus inhibit growth. This interaction between PGPR and SW, KAR_1 and TMB requires further investigation as their interactions for most of the growth parameters were significantly different (Table 5. 1).

As expected, TMB had an inhibitory effect on okra growth (Fig. 5.1). Although not elucidated, the mechanisms of action of TMB are different to those of KAR_1. Trimethylbutenolide reduced the stimulatory effect of KAR_1 in a concentration-dependent manner although they are not competing for the same binding sites **(SOÓS *et al.* 2012)**. Transcriptome analysis showed contrasting expression patterns with KAR_1 suppressing and TMB up-regulating ABA, seed maturation and dormancy-related transcripts **(SOÓS *et al.* 2012)**. According to **SOÓS *et al.* (2012)** the effects of TMB are more easily reversed by washing the seeds while the effects of KAR_1 are more long lasting. In the present study, *B. licheniformis* inoculum was able to

overcome the inhibitory effects of TMB (Fig. 5.1), perhaps by altering the hormone balance although this interaction also requires further investigation.

Plant growth promoting rhizobacteria and smoke treatments can alter the chlorophyll and macromolecule composition in treated plants. For example, mango trees (*Mangifera indica* cv. Ataulfo) inoculated with the ACC-deaminase producing *Burkholderia caribensis* and a hormone producing *Rhizobium* sp. initially had higher nitrogen and carbohydrate content (sucrose, glucose and fructose) in the leaves but this decreased over time as floral development proceeded with more flowers in the inoculated trees **(DE LOS SANTOS-VILLALOBOS *et al.* 2013)**. Similarly, SW and KAR$_1$ improved shoot and root growth in micropropagated "Williams" bananas as well as increasing the photosynthetic pigments (Chl *a*, Chl *b* and carotenoids) and secondary metabolites **(**phenolics, flavonoids and proanthocyanidins **AREMU *et al.* 2012)**. In the present study, neither the bacterial inoculum nor the smoke treatments (SW, KAR$_1$ and TMB) had any effect on the photosynthetic pigments apart from the combined SW (1:1000) and bacteria treatment causing a decrease in the pigment content (Fig. 5.2) and an associated decrease in growth (Fig. 5.1). Similarly, protein and sugar content was not affected by any of the treatments (Fig.5.3 c-e) while α-amylase activity in the roots did increase slightly with *B. licheniformis* inoculation and SW (1:1000) and KAR$_1$ treatments (Fig. 5.3b). Future experiments should perhaps focus on changes in the composition of stress-relieving secondary metabolites to get further insight into the interactions between PGPR and natural smoke-derived biostimulants. There are many reports of PGPR alleviating abiotic stress in plants by altering reactive oxygen species scavenging enzymes and osmolyte content **(**glycine betaine, proline **GURURANI *et al.* 2013; BHARTI *et al.* 2014; SARMA and SAIKIA 2014)**. Smoke treatments also alter the secondary metabolite composition, for example, SW and KAR$_1$ treated *Aloe arborescens* had a significantly higher flavonoid and phenolic content **(KULKARNI *et al.* 2014)** with molecular evidence suggesting that SW can up-regulate the phenylpropanoid pathway and flavonoid related genes **(SOÓS *et al.* 2010)**.

Apart from having a direct influence on the plant, SW and KAR$_1$ also affected the rhizosphere microbial populations where KAR$_1$ (10^{-7} M) promoted bacterial growth while the rhizobacteria population was reduced by the SW (1:500) treatments (Fig. 5.4). Smoke has antimicrobial properties with some traditional agricultural

methods exposing seeds to smoke to reduce microbial contamination during seed storage **(KULKARNI *et al.* 2011)**. There are also examples of smoke compounds applied to crops being able to reduce harmful phytopathogenic bacteria **(**reviewed in **KULKARNI *et al.* 2011)**. These antimicrobial compounds in the crude SW can explain the reduced bacterial abundance in the SW (1:500) treatments in the present pot trail. In contrast, KAR_1 increased the rhizobacteria populations while TMB had no effect on the naturally colonizing rhizobacteria but did appear to inhibit the establishment of the *B. licheniformis* inoculum (Fig 5.4). This provides further indirect evidence supporting the idea of overlapping modes of action of *B. licheniformis* and smoke treatments upsetting the hormone homeostasis. For example, treatments with the highest bacterial populations (i.e. 10^{-7} M KAR_1 + *B. licheniformis* inoculum) had a negative effect on the growth of the okra plants (Fig. 5.1). While TMB partially limited the establishment of *B. licheniformis* populations, these lower rhizobacteria populations were able to overcome the inhibitory effects of TMB on okra growth.

Root exudate consisting of organic compounds such as sugars, polysaccharides, amino acids, peptides, proteins, vitamins and phenolics as well as rhizodeposits (sloughed cells and decaying roots) provide a substrate for the microbial population. The quantity and composition of the exudate is one factor that influences the composition of the rhizobacterial community **(GREGORY 2006; MIRANSARI 2013)**. For example, isoflavanoids and flavonoids activate the *Rhizobium* genes responsible for the nodulation process and vesicular-arbuscular mycorrhiza colonization. Root exudates also contain secondary metabolites and proteins that can act as antimicrobials **(BAIS *et al.* 2004)**. In turn, microbial communities regulate the rate of decomposition of organic matter and thus the availability of plant nutrients **(GREGORY 2006)**. Thus treatments such as SW (1:500) and KAR_1 (10^{-7} M) that significantly alter the root growth (both biomass and root depth) as well as the secondary metabolite content **(KULKARNI *et al.* 2014)** may indirectly affect the microbial population in the rhizosphere by altering the amount and composition of the root exudates. This requires further investigation.

Diverse populations of PGPR provide a better resource for improving plant growth and disease management as each strain has a different mode of action and survival in changing environmental conditions **(NEGI *et al.* 2011)**. Other important factors in selecting PGPR strains are their persistence in the soil and their survival under

different stress conditions **(MIRANSARI 2013)**. Agricultural management practices influence soil microbial diversity and abundance as well as their enzyme activities **(SHEN *et al.* 2010)**. Thus before natural biostimulants such as smoke-derived compounds can be used in agriculture, their effect on microbial diversity and enzyme activity needs to be elucidated to ensure healthy soil. The present study is the first study to show that interactions between a hormone-producing PGPR and various smoke-derived compounds do occur. There were negative effects on plant growth when *B. licheniformis* was applied in combination with SW or KAR_1 while the bacterial inoculum alleviated the inhibitory effects of TMB on okra growth. A possible explanation for this antagonistic effect may be overlapping modes of action of *B. licheniformis* and SW or KAR_1 which disrupts hormone homeostasis. In addition, the different treatments also affected the rhizosphere microbial populations with crude SW displaying mild antimicrobial activity and KAR_1 increasing the rhizosphere microbial populations. While this study showed that interactions do occur between PGPR and natural smoke-derived biostimulants, more in-depth experiments are now required to fully elucidate this relationship.

CHAPTER 6 – THE EFFECTS OF SMOKE-WATER, KAR$_1$ AND TMB ON *IN VITRO ANSELLIA AFRICANA* ORCHID SEED GERMINATION

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6.1 INTRODUCTION

The germination of an orchid seed in a natural system requires not only favourable environmental conditions but also a symbiotic fungus **(ARDITTI 1979; CHANG 2006)**. The symbiotic fungus supplies nutrients and water to the germinating seed since the seed has virtually no resources of its own **(ARDITTI 1979; BASKIN and BASKIN 1998; CHANG 2006)**. Of the millions of dust like seeds that are released from an orchid seed pod, only a couple of seeds will germinate and grow into adult plants **(KNUDSON 1922)**. For commercial growers and hybridizers of orchids, this process is far too lengthy and unproductive. The ability to germinate and grow orchid seeds using tissue culture techniques made it possible to create large numbers of orchids from a single orchid pod **(KNUDSON 1922; CHUGH *et al.* 2009)** and thereby revolutionized the orchid industry.

Ansellia africana Lindl., leopard orchid, is an African orchid and the only species in the genus *Ansellia* (Fig. 6.1). This orchid is becoming endangered because of overharvesting by traditional healers **(POOLEY 1998; GOLDING and BANDEIRA 2002)**. Some studies reported on cultivating this species using tissue culture techniques in order to produce large numbers of plants that can be introduced back into the wild **(BYTEBIER *et al.* 1996; ZOBOLO 2010)**. In an attempt to increase the numbers of plants produced using tissue culture techniques, **VASUDEVAN and VAN STADEN (2010; 2011)** reported that plantlet generation from *A. africana* seeds can be enhanced by treating (sterilizing) the seeds with a bleach solution and by introducing cytokinins into the growing media.
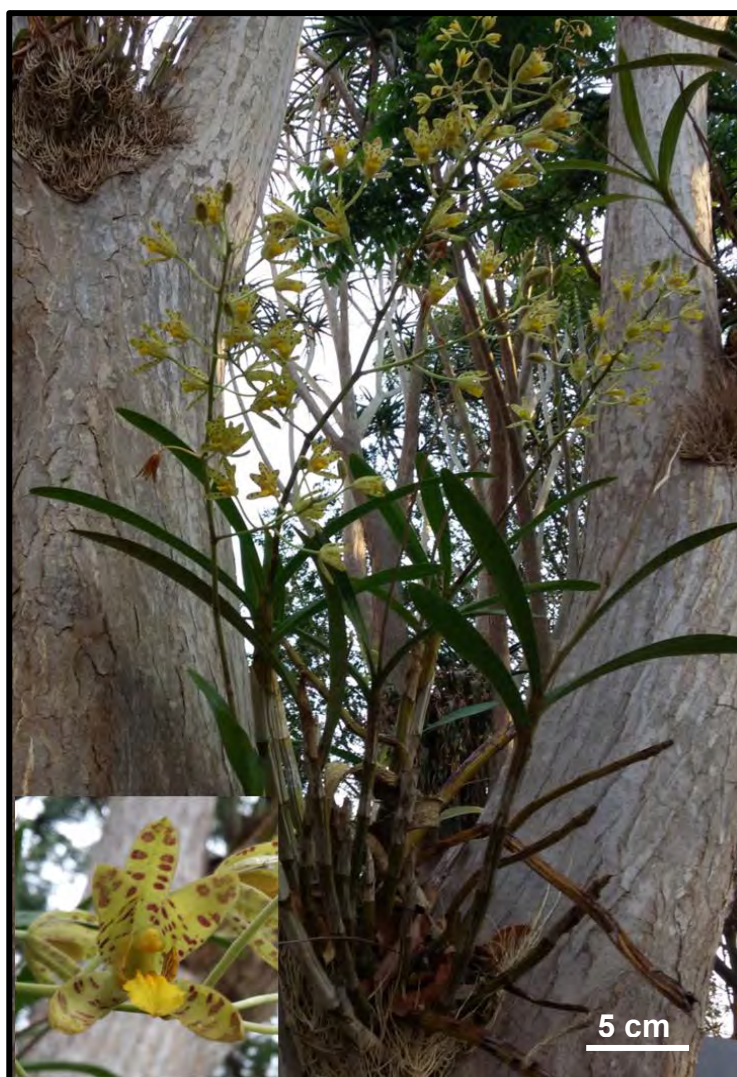
Figure 6. 1 Flowering *Ansellia africana* orchid. Picture taken at the University of KwaZulu-Natal Botanical Gardens, Pietermaritzburg. Scale bar = 5 cm.

Smoke and the karrikins isolated from smoke have significant effects on the germination and growth of plants. Several studies have reported that SW and KAR_1 can be used to stimulate the germination of seeds from different plant families and also increase the growth of plants **(reviewed in LIGHT *et al.* 2009; KULKARNI *et al.* 2011)**. Trimethylbutenolide was shown to inhibit the germination of lettuce seeds **(LIGHT *et al.* 2010)** and also the seeds of five weed species assessed in **Chapter 4**. A molecular study by **SOÓS *et al.* (2012)** indicated that KAR_1 down-regulates and TMB up-regulates abscisic acid (ABA), seed maturation and dormancy-related transcripts. It was reported in **Chapter 4**, that TMB has an inhibitory effect on α-amylase activity of the seeds of five weed species which has a direct bearing on the mobilization of seed reserves.

The orchid *A. africana* grows epiphytically on trees in the tropical areas of Africa and could be exposed to smoke which is common during the winter months. The effect of smoke and the smoke-isolated compounds on orchid seed germination has never been documented. Furthermore, it will be interesting from a physiological perspective to determine how the smoke treatments affect orchid seed germination and growth since these seeds have limited to no reserves. Therefore the effects of SW, KAR_1 and TMB on orchid seed germination and development were investigated.

6.2 MATERIALS AND METHODS

6.2.1 Collection of *Ansellia africana* pods

Mature seed pods of *Ansellia africana* Lindl., were harvested from plants grown in the Botanical Garden of the University of KwaZulu-Natal (S 29° 37.50', E 30° 24.23'), Pietermaritzburg, South Africa during May 2014, 9 months after flowering had occurred. The pods were surface sterilized by immersing the pods in full strength commercial bleach (NaOCl) for 20 min. The pods were then blotted dry and stored at 4°C in sealed plastic containers with desiccant until used.

6.2.2 Preparation of the growth media

Before removing the seeds, one *A. africana* seed pod was placed in a desiccator containing silica gel until the pod changed from a green colour to a light yellow colour (before dehiscence). The intact pod was surface sterilized by immersing it in 100% NaOCl for 20 min. The pod was then immersed in 100% EtOH and set alight. After the fire was quenched, the pod was again immersed in 100% EtOH, set alight a second time and placed on a sterile laminar flow bench. The pod was cut open with a sterile scalpel and the seeds removed.

A stock solution of smoke-water (SW) was prepared as described by **BAXTER *et al.* (1994)**. The synthesised KAR_1 (purity > 95%) and TMB (purity > 98%) that were used in this study were prepared according to the protocols of **FLEMATTI *et al.* (2005)** and **SURMONT *et al.* (2010)**, respectively.

Half-strength **MURASHIGE and SKOOG (1962)** medium (MS) containing 3% sucrose was used as the growing medium. In order to test the effects of the various treatments, appropriate amounts of concentrated SW, KAR_1 and TMB solutions were added to individual conical flasks which were subsequently made up to a volume of 100 mL with ½ strength MS medium. The treatments were as follows: (1) control; (2) SW 1:250 (v:v); (3) SW 1:500 (v:v); (4) SW 1:750 (v:v); (5) SW 1:1000 (v:v); (6) KAR_1 (10^{-7} M); (7) KAR_1 (10^{-8} M); (8) KAR_1 (10^{-9} M); (9) TMB (10^{-3} M); (10) TMB (10^{-4} M); (11) TMB (10^{-5} M); (12) KAR_1 (10^{-7} M) + TMB (10^{-3} M); (13) KAR_1 (10^{-7} M) + TMB (10^{-4} M); (14) KAR_1 (10^{-7} M) + TMB (10^{-5} M); (15) KAR_1 (10^{-8} M) + TMB (10^{-3} M); (16) KAR_1 (10^{-8} M) + TMB (10^{-4} M); (17) KAR_1 (10^{-8} M) + TMB (10^{-5} M); (18) KAR_1 (10^{-9} M) + TMB (10^{-3} M); (19) KAR_1 (10^{-9} M) + TMB (10^{-4} M) and (20) KAR_1 (10^{-9} M) + TMB (10^{-5} M). The pH of the media in the individual conical flasks was adjusted to 5.8. The medium was solidified using 0.6% (w/v) agar (Bacteriological, Agar No. 1, Oxoid Ltd, Bastingstoke, England) and sterilized by autoclaving at 121°C for 20 min. The medium of each conical flask was dispersed in 5 Petri dishes (90 mm). After the medium cooled and solidified, 200 to 400 seeds were sown per Petri dish, and then sealed with parafilm. The Petri dishes were incubated at 25°C and were subjected to a 16 h light/ 8 h dark regime with a photosynthetic photon flux (area) density of 30 $\mu mol.m^{-2}.s^{-1}$. Germination rate index (GRI) and developmental rate index (DRI) were calculated on the data of two separate experiments (n=10).

6.2.3 Determining germination rate and protocorm development rate

After six weeks the seeds started germinating and five different growth stages were recorded using a dissecting microscope (Kyowa Optical, Japan). Seeds were considered germinated and were differentiated from the ungerminated seeds (Fig. 6.2a) with the formation of rhizoids (Stage 1; Fig. 6.2b). The next stage of development was depicted by seeds which broke free from their testae completely (Stage 2; Fig. 6.2c). A seed that was enlarged and started turning green was considered a protocorm body (Stage 3; Fig.6.2d). Enlarged protocorms were characterized by an increase in size and a cone-shape, a pronounced green colour and numerous rhizoids (Stage 4; Fig. 6.2e). The last developmental stage considered for this study was characterized by the formation of large protocorms that

could be discerned without the use of a dissecting microscope (Stage 5; Fig. 6.2f). The germination of *A. africana* seeds were recorded for an 8 week period (13 weeks after sowing of seeds).

The germination rate index (GRI) was determined as described by **BOUTON *et al.* (1976)** and modified by **FOWLER (1991)**:

$$\mathrm{GRI} = \frac{G1}{1} + \frac{G2}{2} + \cdots + \frac{G8}{8}$$

Where G1 is equal to the germination percentage x 100 at the first count after sowing; G2 is equal to the germination percentage x 100 at the second count after sowing etc.

The GRI reflects the percentage of germination at each count throughout the germination period (week 6 – 13). This calculation was also adopted to indicate the developmental rate index (expressed as DRI) of each stage of development of the seeds (later protocorms) and was calculated in the same manner as described above:

$$\mathrm{DRI} = \frac{D1}{1} + \frac{D2}{2} + \cdots + \frac{D8}{8}$$

Where D1 is equal to the percentage of developed seeds/protocorms of a particular developmental stage (Stage 2 – 5) x 100 at the first count after sowing; D2 is equal to the percentage of developed seeds/protocorms of a particular developmental stage (Stage 2 – 5) x 100 at the second count after sowing etc.

6.2.4 Statistical analysis

Values that were significantly different to the respective controls were determined according to a two-sample T-test ($P < 0.05$) (GenStat[®], Edition 14).
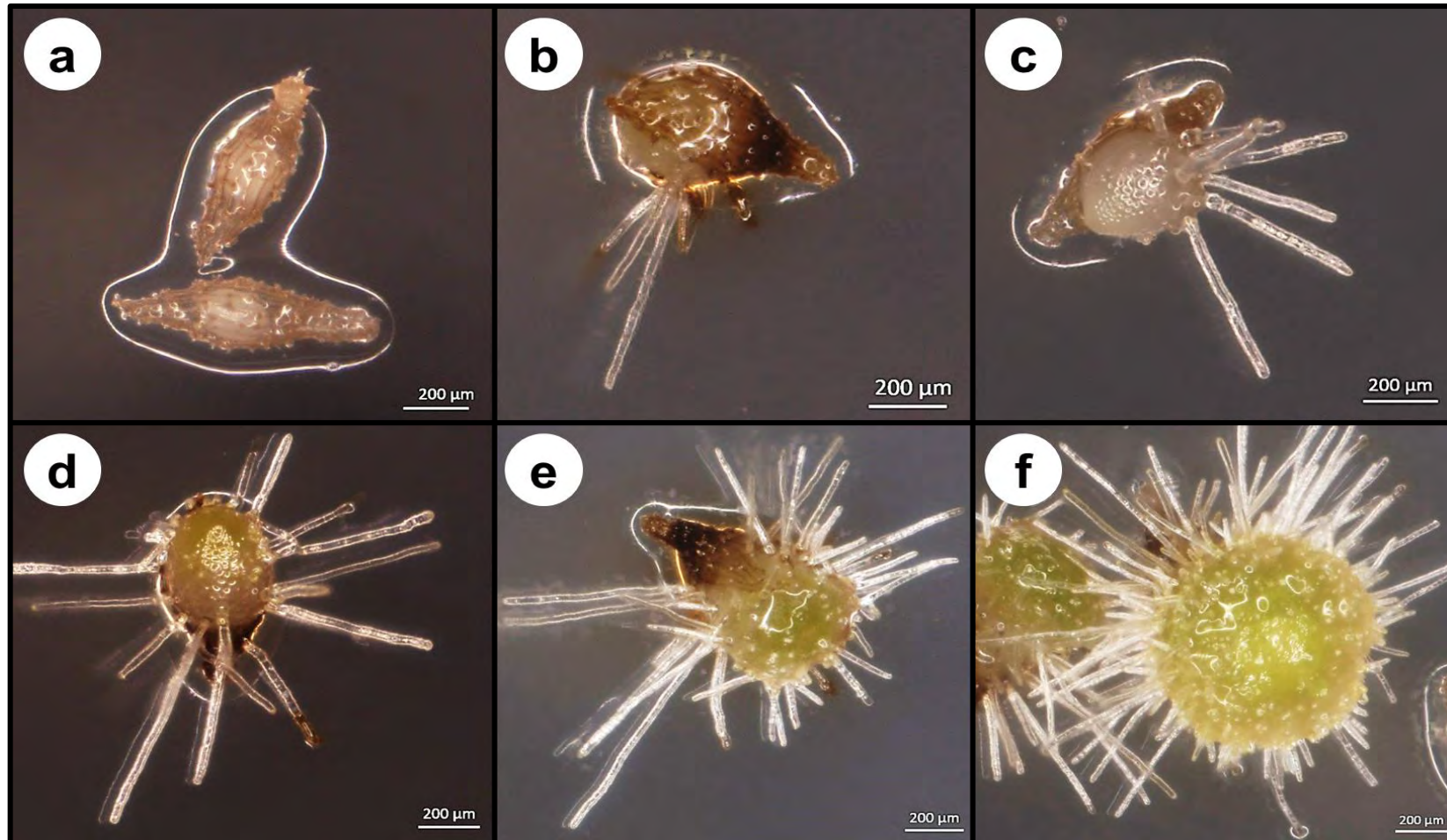
Figure 6. 2 Developmental stages of germinating *Ansellia africana* orchid seeds. The different stages were defined as a) ungerminated seeds; b) Stage 1 – germinated seed with rhizoids emerging; c) Stage 2 – seed without testa; d) Stage 3 – protocorm body with a green colour; e) Stage 4 – enlarging protocorm with many rhizoids (cone-shaped); f) Stage 5 – large protocorm with many rhizoids (visible with naked eye). Scale bars = 200 µm.

6.3 RESULTS

In order to assess the average percentage of germination and development together with the time of germination and time to reach a specific developmental stage, the GRI and DRI was calculated according to the formula described by **BOUTON *et al.* (1976)** and modified by **FOWLER (1991)**. The GRI and DRI are summarized in Table 6.1. The GRI of *A. africana* seeds sown on MS medium was only significantly higher when the medium was supplemented with SW 1:250 compared to the control. Supplementing the media with TMB (10^{-5} M) significantly reduced the GRI compared to the control. The GRI was also significantly reduced with KAR_1 10^{-7} M + TMB (10^{-3} and 10^{-4} M), KAR_1 10^{-8} M + TMB (10^{-3} – 10^{-5} M) and KAR_1 10^{-9} M + TMB 10^{-4} M compared to the control. Smoke-water 1:250 significantly increased the number of seeds that broke free of their testae and also significantly increased the numbers of protocorms at developmental stages 3, 4 and 5 compared to their respective controls. Compared to the control, supplementing the media with SW 1:1000 significantly increased the DRI of protocorms that started turning green (Stage 3). The DRI of large protocorms (Stage 5) was significantly increased by all the SW treatments compared to the control. Supplementing the media with TMB (10^{-3} and 10^{-5} M), KAR_1 10^{-7} M + TMB (10^{-3} and 10^{-4} M), KAR_1 10^{-8} M + TMB (10^{-4} and 10^{-5} M) and KAR_1 10^{-9} M + TMB (10^{-4} M) significantly reduced the DRI of seeds at stage 2 and protocorms at stages 3 and 4 compared to the controls. The same set of treatments with the addition of TMB 10^{-4} M, KAR_1 10^{-8} M + TMB 10^{-3} M and KAR_1 10^{-9} M + TMB 10^{-5} M significantly reduced the DRI of large protocorms (Stage 5) compared to the control. Supplementing the medium with KAR_1 had no significant effect on the GRI or the DRI at any developmental stage. The promotory effects of SW 1:250 and the inhibitory effects of TMB 10^{-3} M on the development of *A. africana* seeds 10 weeks after being sown can clearly be seen in Figure 6.3.

The average number of seeds that developed into large protocorms is summarized in Figure 6.4. Smoke-water 1:250 produced on average significantly more large protocorms compared to the control. Trimethylbutenolide 10^{-3} – 10^{-5} M significantly reduced the average number of stage 5 protocorms compared to the control. The combined treatments KAR_1 10^{-7} + TMB (10^{-3} – 10^{-5} M), KAR_1 10^{-8} M + TMB (10^{-4} and 10^{-5} M) and KAR_1 10^{-9} M + TMB 10^{-4} M significantly reduced the average number of stage 5 protocorms produced compared to the control.

Table 6. 1 Germination rate index (GRI) and developmental rate index (DRI) of *Ansellia africana* seeds sown on half-strength MS medium supplemented with either smoke-water, karrikinolide or trimethylbutenolide over a 13 week period

Developmental phases: Treatments	Stage 1 GRI (% per week)	Stage 2 DRI (% per week)	Stage 3 DRI (% per week)	Stage 4 DRI (% per week)	Stage 5 DRI (% per week)
Control	70.8 ± 6.5	41.7 ± 4.0	20.2 ± 2.2	7.8 ± 1.6	7.1 ± 1.0
SW 1:250	$\mathbf{91.6 \pm 3.2^{*}}$	$\mathbf{56.9 \pm 3.7^{*}}$	$\mathbf{37.9 \pm 2.3^{*}}$	$\mathbf{18.7 \pm 1.1^{*}}$	$\mathbf{31.4 \pm 2.1^{*}}$
SW 1:500	59.0 ± 8.4	34.1 ± 4.8	22.8 ± 3.1	9.1 ± 1.2	$\mathbf{12.4 \pm 1.8^{*}}$
SW 1:750	55.4 ± 6.1	33.1 ± 2.6	21.9 ± 1.3	8.0 ± 1.0	$\mathbf{12.2 \pm 1.2^{*}}$
SW1:1000	74.7 ± 4.6	43.9 ± 2.0	$\mathbf{26.6 \pm 0.6^{*}}$	9.2 ± 0.6	$\mathbf{11.8 \pm 0.9^{*}}$
KAR 10^{-7} M	59.0 ± 3.4	31.3 ± 1.9	18.2 ± 1.2	5.3 ± 0.4	6.4 ± 0.6
KAR 10^{-8} M	62.0 ± 2.5	34.1 ± 1.2	18.0 ± 1.1	4.8 ± 0.4	6.2 ± 0.6
KAR 10^{-9} M	55.3 ± 2.3	30.2 ± 4.8	18.4 ± 2.3	5.3 ± 1.1	4.9 ± 1.2
TMB 10^{-3} M	57.8 ± 7.6	$\mathbf{30.6 \pm 1.8^{\#}}$	$\mathbf{12.7 \pm 0.9^{\#}}$	$\mathbf{3.1 \pm 0.3^{\#}}$	$\mathbf{2.4 \pm 0.3^{\#}}$
TMB 10^{-4} M	69.5 ± 5.3	39.9 ± 3.4	16.6 ± 1.7	3.9 ± 0.6	$\mathbf{3.5 \pm 0.6^{\#}}$
TMB 10^{-5} M	$\mathbf{32.3 \pm 4.8^{\#}}$	$\mathbf{16.0 \pm 2.5^{\#}}$	$\mathbf{9.4 \pm 1.6^{\#}}$	$\mathbf{2.9 \pm 0.7^{\#}}$	$\mathbf{2.5 \pm 0.8^{\#}}$
KAR$_1$ 10^{-7} + TMB 10^{-3} M	$\mathbf{24.0 \pm 1.6^{\#}}$	$\mathbf{13.0 \pm 1.4^{\#}}$	$\mathbf{7.6 \pm 0.9^{\#}}$	$\mathbf{2.5 \pm 0.3^{\#}}$	$\mathbf{2.5 \pm 0.4^{\#}}$
KAR$_1$ 10^{-7} + TMB 10^{-4} M	$\mathbf{39.7 \pm 5.7^{\#}}$	$\mathbf{19.6 \pm 2.2^{\#}}$	$\mathbf{11.8 \pm 1.6^{\#}}$	$\mathbf{3.4 \pm 0.6^{\#}}$	4.7 ± 1.1
KAR$_1$ 10^{-7} + TMB 10^{-5} M	79.1 ± 10.7	40.8 ± 4.1	22.3 ± 1.9	6.7 ± 0.9	7.9 ± 1.2
KAR$_1$ 10^{-8} + TMB 10^{-3} M	$\mathbf{33.7 \pm 3.8^{\#}}$	27.3 ± 4.7	14.8 ± 1.2	4.6 ± 0.5	$\mathbf{4.1 \pm 0.6^{\#}}$
KAR$_1$ 10^{-8} + TMB 10^{-4} M	$\mathbf{35.7 \pm 6.2^{\#}}$	$\mathbf{20.1 \pm 3.2^{\#}}$	$\mathbf{13.5 \pm 0.7^{\#}}$	$\mathbf{3.2 \pm 0.4^{\#}}$	$\mathbf{3.2 \pm 0.8^{\#}}$
KAR$_1$ 10^{-8} + TMB 10^{-5} M	$\mathbf{40.4 \pm 1.7^{\#}}$	$\mathbf{21.1 \pm 0.5^{\#}}$	$\mathbf{13.7 \pm 0.8^{\#}}$	$\mathbf{3.6 \pm 0.3^{\#}}$	$\mathbf{2.5 \pm 0.4^{\#}}$
KAR$_1$ 10^{-9} + TMB 10^{-3} M	80.7 ± 6.9	41.7 ± 4.9	27.4 ± 4.1	9.1 ± 1.5	4.9 ± 1.1
KAR$_1$ 10^{-9} + TMB 10^{-4} M	$\mathbf{38.2 \pm 5.2^{\#}}$	$\mathbf{20.0 \pm 3.5^{\#}}$	$\mathbf{11.2 \pm 2.1^{\#}}$	$\mathbf{2.3 \pm 0.4^{\#}}$	$\mathbf{1.1 \pm 0.3^{\#}}$
KAR$_1$ 10^{-9} + TMB 10^{-5} M	66.4 ± 10.8	36.9 ± 7.2	23.2 ± 4.5	4.8 ± 0.8	$\mathbf{3.3 \pm 1.1^{\#}}$

Values that were significantly different to the control treatment were indicated in bold. Values that were significantly higher than the control treatments are indicated with an asterisk symbol (*) while values that were significantly lower than the control treatments were indicated with a hash symbol (\#) according to a two-sample T-test (P < 0.05).
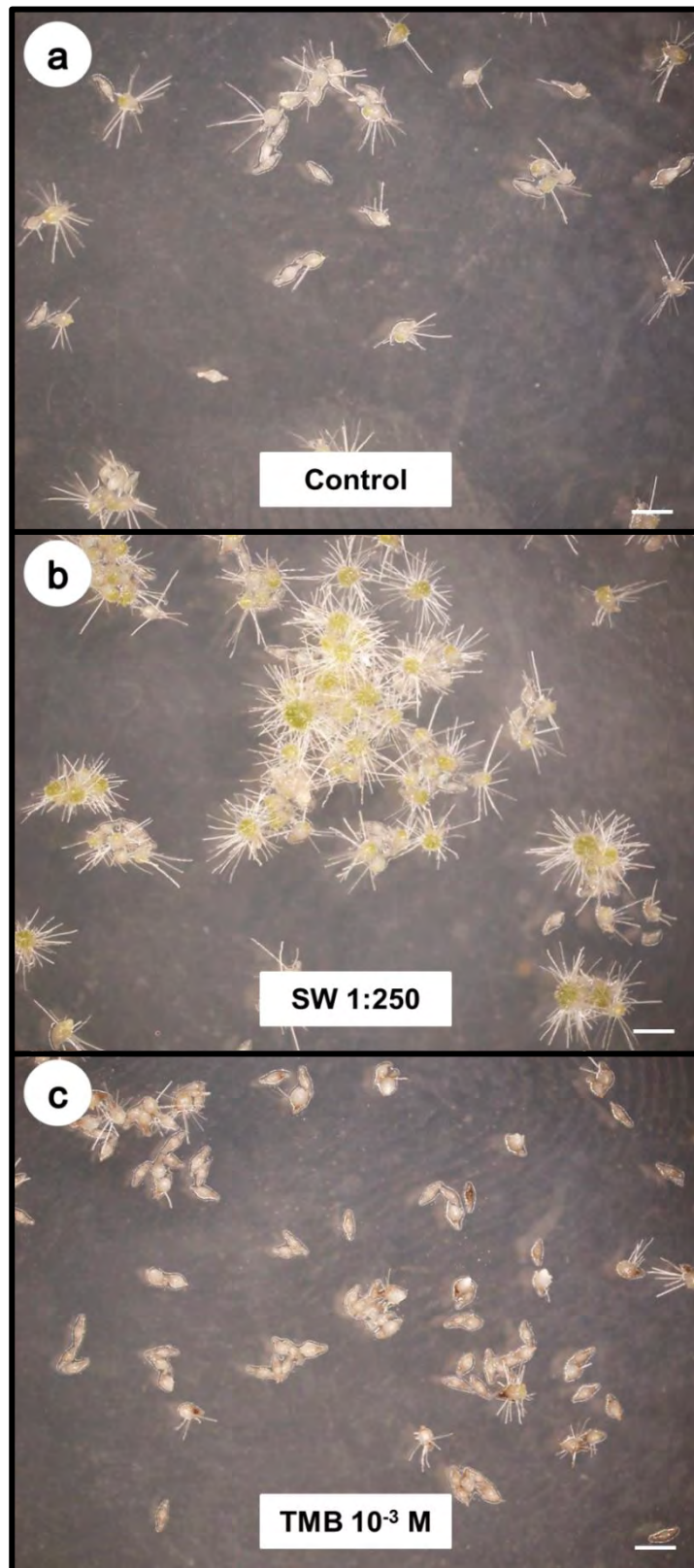
Figure 6. 3 The development of *Ansellia africana* orchid seeds 10 weeks after being sown on a) half-strength MS media, which was also supplemented with b) SW 1:250 and c) TMB 10^{-3} M. Scale bars = 1000 µm.
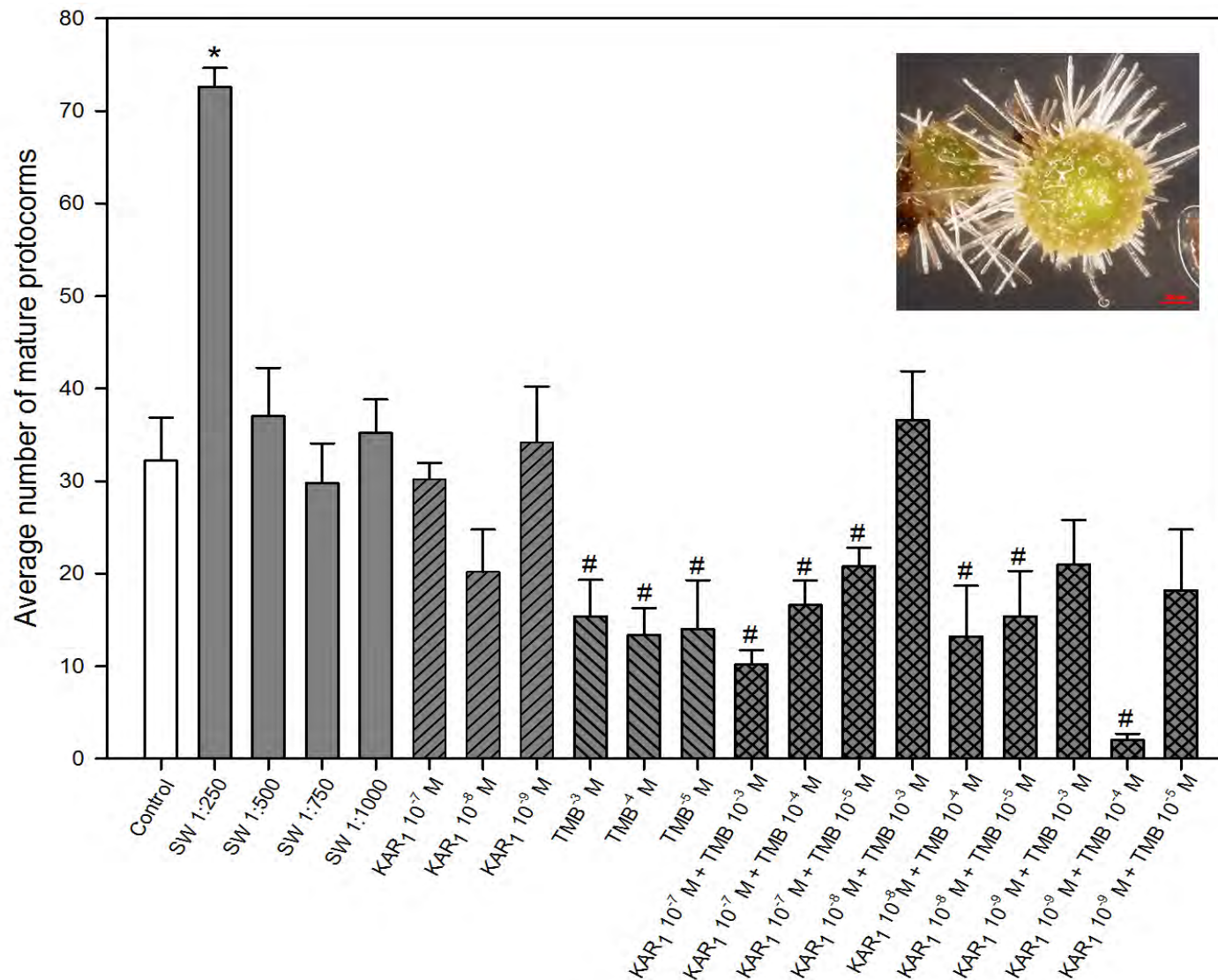
Figure 6. 4 Average number of large *Ansellia africana* protocorms (Stage 5) formed after 13 weeks of culture. Values that are significantly higher than the control are indicated by asterisk symbols (*) while values that are significantly lower than the control are indicated by hash symbols ($^\#$) according to the two-sample T-test ($P < 0.05$).

6.4 DISCUSSION

It is common practice to germinate orchid seeds on a growth medium like MS medium that was used in this study **(CHUGH *et al.* 2009)**. Using tissue culture techniques many plantlets can be generated from the seed of one seed pod. Culturing orchids from seed is, however, very time consuming, with most species taking between 5 to 7 years to reach maturity and produce their first flower(s). Shortening this process at any point, from germination to growth, could be very beneficial for orchid growers and breeders.

From a visual inspection it was evident that the seeds that were sown on media containing SW 1:250 developed much faster (Fig. 6.3) than that of the control treatment. The SW 1:250 treatment did not only significantly increase the average number of large protocorms compared to the control treatment (Fig. 6.4) but also increased the GRI and DRI of every stage of development (Table 6.1). All the SW treatments significantly increased the DRI of large protocorms (Stage 5, Table 6.1). These results indicate that smoke has a stimulatory role on the growth and development of *A. africana* seeds. It is therefore possible that the smoke from fires may stimulate *A. africana* seed development in natural systems. More importantly, smoke could increase the rate of development of orchid seeds grown *in vitro*. More orchid species will need to be evaluated to determine if SW can be incorporated into the growth media of orchid seeds.

Karrikinolide have been used to stimulate germination in seeds from many plant species. It was reported by **DOWNES *et al.* (2010)** that the seeds of some plant species may be stimulated to germinate by SW and not by KAR_1 or by KAR_1 and not by SW. In this instance SW stimulated the germination and growth of *A. africana* seeds while KAR_1 did not have any effect. This might be due to the fact that orchid seeds do not contain any nutrient reserves that can be mobilized since the activity of KAR_1 is associated with up-regulating the activity of hydrolytic enzymes [**(SINGH *et al.* 2014)** also see **Chapter 4**]. This result correlates well to a study conducted by **PAPENFUS *et al.* (2014)** who showed that SW stimulated pollen growth in three Amaryllidaceae species and that the effect of KAR_1 on pollen growth was not as consistent as the SW treatment.

It is possible that the other karrikins or combinations of karrikins present in plant-derived smoke **(FLEMATTI *et al.* 2007)** may be responsible for the stimulatory effect of the SW 1:250 treatment. It was, however, reported by **FLEMATTI *et al.* (2007)** that KAR_1 is the most active and most abundant karrikin in plant-derived smoke. It is therefore possible that other compounds may be present in plant-derived smoke with biological activity and that these compounds were responsible for the increased germination and development in pollen grains **(PAPENFUS *et al.* 2014)** and *A. africana* seed germination and development. It may therefore be worthwhile to develop a bioassay using pollen or orchid seeds to identify other compounds with biological activity in plant-derived smoke.

Similarly to what was recorded for seeds that contain nutrient reserves, TMB reduced the rate of development of *A. africana* orchid seeds (Table 6.1). Although KAR_1 did not have any notable effect on the germination or growth of *A. africana* seeds, it was very interesting to note that TMB reduced the GRI and DRI of the seeds (Table 6.1 and Fig. 6.3). **SOÓS *et al.* (2012)** reported that KAR_1 and TMB do not compete for the same binding site when applied to 'Grand Rapids' lettuce seeds. It is therefore possible that TMB can reduce the growth of *A. africana* seeds while KAR_1 has no effect. The same study by **SOÓS *et al.* (2012)** showed that TMB up-regulated dormancy-related transcripts when applied to 'Grand Rapids' lettuce seeds. The effect of TMB on *A. africana* seed germination and growth could be due to the fact that TMB up-regulates dormancy-related transcripts. More in depth studies are required to elucidate the role of TMB in orchid seed germination studies.

CHAPTER 7 – THE EFFECTS OF SMOKE-WATER, KAR$_1$ AND TMB ON POLLEN GERMINATION AND POLLEN TUBE ELONGATION

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7.1. INTRODUCTION

The ability to germinate and grow pollen grains in an *in vitro* system made it possible to determine the specific growth requirements of pollen grains and how pollen germination and growth are affected by atmospheric chemicals (air pollutants) **(COX 1984; KAPPLER and KRISTEN 1987; WOLTERS and MARTENS 1987; ABBOTT *et al.* 1991; MUNZUROĞLU and GÜR 2000; TUNA *et al.* 2002; GÜR and TOPDEMIR 2005)** and abiotic factors **(DHINGRA and VARGHESE 1985; WANG *et al.* 2006; ACAR and KAKANI 2010)**. The ability to germinate and grow pollen grains *in vitro* was largely made possible by **BREWBAKER and KWACK (1963)** who reported that pollen grains require calcium (Ca) and boric acid in addition to sucrose to germinate and sustain growth in an *in vitro* environment. After this phenomenon was reported numerous pollen growth studies were conducted, regularly reporting on the specific amounts of sucrose, Ca and boric acid required by pollen of particular plant species **(KHATUN and FLOWERS 1995; SATO *et al.* 1998; BOLAT and PIRLAK 1999; FRANKLIN-TONG 1999; TUINSTRA and WEDEL 2000; WANG *et al.* 2004; KUMARI *et al.* 2009; LYRA *et al.* 2011; ABDELGADIR *et al.* 2012)**. Several studies also reported on the use of plant growth regulators (PGR) to enhance pollen growth **(SMITH 1942; BAMZAI and RANDHAWA 1967; HEWITT *et al.* 1985; VOYIATZIS and PARASKEVOPOULOU-PAROUSSI 2000; SINGH *et al.* 2002)**. It is therefore not only possible to grow pollen grain *in vitro*, but also increase their growth by supplementing the solutions with PGRs. It is therefore possible to determine how pollen grains may react to chemical compounds by dissolving them into the basal sucrose medium.

Smoke is regarded as an environmental pollutant and is a common factor studied in fire ecology **(BAXTER and VAN STADEN 1994)**. Plant-derived smoke does,

however, have several beneficial effects on seed germination and overall plant growth **(BROWN and VAN STADEN 1997; KULKARNI *et al.* 2011)**. Smoke [(applied mainly as smoke-water (SW)] has been used extensively to stimulate the germination of seeds in a wide variety of plant species **(LIGHT *et al.* 2002; BROWN and BOTHA 2004; LIGHT *et al.* 2009)**. The active principles in smoke have been reported to exhibit both cytokinin-like and auxin-like activities and are now regarded as a new group of naturally occurring PGRs **(CHIWOCHA *et al.* 2009)**. This is supported by the study conducted by **JAIN *et al.* (2008)** who reported that karrikinolide (KAR_1) increases the rate of cell division of soybean callus. According to **JAIN *et al.* (2008)** this could explain the accelerated radicle emergence in germinating seeds treated with KAR_1.

Since large amounts of smoke are generated and released into the air during wildfires, it is possible that smoke from these fires may affect the reproductive success of plants at the pollen level. In this study, the effect of SW and two smoke-derived compounds were tested on pollen germination and pollen tube growth of 10 plant species from 8 different families.

7.2 MATERIALS AND METHODS

7.2.1 Flower and pollen collection

A pilot study was conducted using three Amaryllidaceae species namely *Clivia gardenii* Hook., *Cyrtanthus mackenii* Hook.f. subsp. *mackenii* and *Scadoxus multiflorus* (Martyn) Raf. subsp*. multiflorus*. After the initial study, seven more species form different plant families were also evaluated. The species were *Aechmea kertesziae* Reitz (Bromeliaceae), *Aloe maculata* All. (Asphodelaceae), *Kniphofia uvaria* (L.) Oken (Xanthorrhoeaceae), *Lachenalia aloides* (L.f.) Engl. var. *aloides* (Hyacinthaceae), *Nematanthus crassifolius* (Schott) Wiehler (Gesneriaceae), *Paeonia lactiflora* Pall. (Paeoniaceae) and *Tulbaghia simmleri* P. Beauv. (Alliaceae). All the flowers were collected from the Botanical Garden of the University of KwaZulu-Natal (S 29° 37.50', E 30° 24.23'), Pietermaritzburg, South Africa between 07:00 and 09:00 in the morning. Inflorescences were placed in conical flasks filled

with tap water and kept at room temperature (24 $\pm$ 2ºC). Fresh pollen grains were collected from each inflorescence after anther dehiscence had occurred.

7.2.2 Assessing pollen viability using three fluorescent staining techniques

Three pollen staining methods were used to differentiate between viable and dead pollen. For all three staining methods, a small quantity of pollen grains were transferred to a drop of dye, mixed thoroughly to a homogenous pollen suspension using a pin and covered with a cover slip. Pollen grains were observed with an Olympus AX70 fluorescence microscope (Camera Nikon DS-Ri1, Japan). The number of viable pollen grains was recorded in each field of view out of the total number of pollen grains. Pollen grains were observed in four different fields of view (replicated four times). The dyes used were: (1) Aqueous 2,3,5-triphenyl tetrazolium chloride (TTC, Merck) [pollen grains that turned red under fluorescence were considered viable **(HAUSER and MORRISON 1964; STANLEY and LINSKENS 1974; KHATUN and FLOWERS 1995; ABDELGADIR *et al.* 2012)**]. (2) Aniline blue-lactophenol (ANB, Merck) staining solution consisting of 5 mL phenol, 20 mL lactic acid, 40 mL glycerol, and 20 mL distilled water **(KEARNS and INOUYE 1993)** [pollen grains were considered viable if they fluoresced blue **(KEARNS and INOUYE 1993; KHATUN and FLOWERS 1995; WANG *et al.* 2004)**]. (3) Fluorescein diacetate [FDA (Sigma-Aldrich)] dissolved in acetone (2 mg.mL^{-1}) and used in combination with 10^{-6} M sucrose solution [pollen grains that fluoresced brightly were taken as viable **(HESLOP-HARRISON and HESLOP-HARRISON 1970; SHIVANNA and HESLOP-HARRISON 1981; HESLOP-HARRISON *et al.* 1984; JAIN and SHIVANNA 1988; KEARNS and INOUYE 1993; KHATUN and FLOWERS 1995; WANG *et al.* 2004)**]. The microscope slides for all three staining methods were kept in humidity chambers (> 90% RH) and placed in the dark for 1 h at 25°C.

7.2.3 Preparation of the different test solutions

All test solutions were evaluated for activity individually and in combination with either Brewbaker and Kwack's (BWK) medium or sucrose and boric acid (SB) medium. The BWK medium was prepared by making a 10% sucrose solution to

which 100 mg.L^{-1} boric acid, 300 mg.L^{-1} calcium nitrate, 100 mg.L^{-1} potassium nitrate and 200 mg.L^{-1} magnesium sulphate were added **(BREWBAKER and KWACK 1963; SHIVANNA and RANGASWAMY 1992)**. The SB medium consisted of a 10% sucrose solution with 100 mg.L^{-1} boric acid **(LINSKENS 1967; SHIVANNA and RANGASWAMY 1992; KUMARI et al. 2009)**.

The specific concentrations of the test solutions used were determined in a preliminary study. Smoke-water (SW) was prepared according to the methods described by **BAXTER et al. (1994)** by burning 5 kg dry *Themeda triandra* (Poaceae) leaf material in a 20 L metal drum and bubbling the smoke through 500 mL distilled water for 45 min. The SW solutions, 1:1000 (v:v) and 1:2000 (v:v), were prepared by diluting 1 part SW in 1000 and 2000 parts distilled water. The synthetic karrikinolide (KAR$_1$) and trimethylbutenolide (TMB) used in this experiment were synthesised according to the methods described by **FLEMATTI et al. (2005)** and **SURMONT et al. (2010)**, respectively. The different liquid media used were as follow: (1) distilled water; (2) BWK; (3) SB; (4) SW (1:1000); (5) SW (1:2000); (6) BWK + SW (1:1000); (7) BWK + SW (1:2000); (8) SB + SW (1:1000); (9) SB + SW (1:2000); (10) KAR$_1$ (10^{-6} M); (11) KAR$_1$ (10^{-7} M); (12) BWK + KAR$_1$ (10^{-6} M); (13) SB + KAR$_1$ (10^{-6} M); (14) TMB (10^{-3} M); (15) BWK + TMB (10^{-3} M) and (16) SB + TMB (10^{-3} M). Since TMB (10^{-3} M) gave the best results, no other concentrations were therefore included. Similarly, only KAR$_1$ (10^{-6} M) was used in the pilot study with the three Amaryllidaceae species. All chemicals that are not otherwise specified were obtained from Sigma-Aldrich.

7.2.4 Assessing *in vitro* pollen germination and pollen tube elongation

Six hanging drop slides were prepared for each test solution (replicated four times). A thin film of petroleum jelly was applied to the rim of the cavity of each cavity slide to prevent evaporation of the test solutions. A consistent amount of pollen grains were transferred from the anthers to each hanging drop slide and mixed into a homogenous pollen suspension using a pin. Preparation of the slides was scheduled in a manner that allowed for the images to be captured exactly 1 h following incubation at 25°C. Images were taken using a compound microscope (Olympus AX70; Camera Nikon DS-Ri1, Japan). Pollen grains were considered germinated

when the pollen tubes were half the length of the pollen grains. The percentage of germinated pollen grains was calculated by counting the number of germinated pollen grains out of the total number of pollen grains in each of two fields of views of 6 slides. Mean pollen tube lengths were determined by measuring 20 pollen tubes in each of six slides using the on-board NIS elements BR4.00.016 software.

7.2.5 Statistical analysis

Before statistical analysis was conducted the germination and viability data (percentages) were arcsine transformed. All the data was analysed using a one-way analysis of variance (ANOVA). Significant differences between treatments were determined using Duncan's multiple range test ($P < 0.05$) (GenStat$^{®}$, Edition 14).

7.3 RESULTS

7.3.1 Pollen viability

The three staining methods, TTC, ANB and FDA successfully stained and indicated viable pollen grains. No significant differences were found between the three staining methods used (Table 7.1).

7.3.2 Effects of SW, KAR$_1$ and TMB on pollen germination and pollen tube elongation of three Amaryllidaceae species

In vitro pollen germination was initiated within the first 30 min with BWK and SB media. In the absence of the media (BWK medium and SB medium), low concentrations of SW (1:1000 and 1:2000) significantly increased pollen germination and also pollen tube length in all three Amaryllidaceae species tested (Fig. 7.1a, d and g). The significant stimulatory effect of SW (1:1000) in the absence of media can be seen in Fig 7.2. In the presence of both media, low concentrations of SW (1:1000 and 1:2000) significantly increased germination in *C. gardenii* and *S. multiflorus* (Fig. 7.1b, c, h and i) and produced significantly longer pollen tubes in all three Amaryllidaceae species when combined with BWK medium (Fig. 7.1b, e and h) and

SB medium (Fig. 7.1c, f and i) compared to their respective controls as can be seen in Figure 7.3.

Karrikinolide (10^{-6} M) showed significantly greater pollen germination in all three species compared to the controls in the absence of media (Fig. 7.1a, d and g) and produced significantly higher germination in *C. gardenii* when incubated with both media (Fig. 7.1b and c). In the absence of media, KAR_1 (10^{-6} M) significantly increased *C. gardenii* pollen tube lengths compared to the other treatments (Fig. 7.1a) and produced significantly longer pollen tubes in *S. multiflorus* in the presence of both media (Fig. 7.1h and i).

Trimethylbutenolide showed significantly higher pollen germination percentages for *S. multiflorus* and *C. gardenii* and produced significantly longer pollen tubes in *S. multiflorus* compared to their respective controls in the absence of media (Fig. 7.1a and g). Trimethylbutenolide produced significantly longer pollen tubes in *C. mackenii* and *S. multiflorus* when combined with both media (Fig. 7.1e, f, h and i). Combining TMB with BWK and SB medium produced significantly lower pollen germination in *C. mackenii* and also in *S. multiflorus* when combined with BWK medium compared to the respective controls (Fig. 7.1e, f and h). Trimethylbutenolide significantly reduced the pollen tube lengths of *C. gardenii* when combined with BWK medium compared to the control (Fig. 7.1b).

Table 7. 1 Pollen viability test of various plant species determined by three fluorescent staining methods (*n*=16)

Plant family	Plant species	Stained pollen grains (%)			Probability level (*P* = 0.05)
		ANB	FDA	TTC	
Indigenous					
	Clivia gardenii	92.2 ± 1.7	94.2 ± 0.5	92.3 ±0.7	NS
Amaryllidaceae	*Cyrtanthus mackenii*	85.7 ± 2.7	90.1 ± 1.3	76.9 ± 5.8	NS
	Scadoxus multiflorus	91.0 ± 1.0	75 ±12.0	79.1 ±1.0	NS
Asphodelaceae	*Aloe maculata*	92.8 ± 0.3	93.0 ± 0.3	94.0 ± 0.2	NS
Xanthorrhoeaceae	*Kniphofia uvaria*	81.1 ± 0.9	83.2 ± 0.6	82.7 ± 0.7	NS
Hyacinthaceae	*Lachenalia aloides*	944 ± 0.7	85.8 ± 6.2	91.9 ± 0.8	NS
Alliaceae	*Tulbaghia simmleri*	95.7 ± 0.4	90.1 ± 0.3	90.7 ± 0.7	NS
Exotic					
Bromeliaceae	*Aechmea kertesziae*	95.7 ± 3.3	93.8 ± 0.3	93.3 ± 0.5	NS
Gesneriaceae	*Nematanthus crassifolius*	86.3 ± 0.1	88.3 ± 0.6	90.7 ± 0.9	NS
Paeoniaceae	*Paeonia lactiflora*	91.0 ± 0.3	89.6 ± 0.2	91.3 ± 0.3	NS

Note: ANB = Aniline blue; FDA = Fluorescein diacetate; TTC = 2,3,5-Triphenyl tetrazolium chloride; NS = Not significantly different at 5% level of significance according to Duncan's multiple range test.
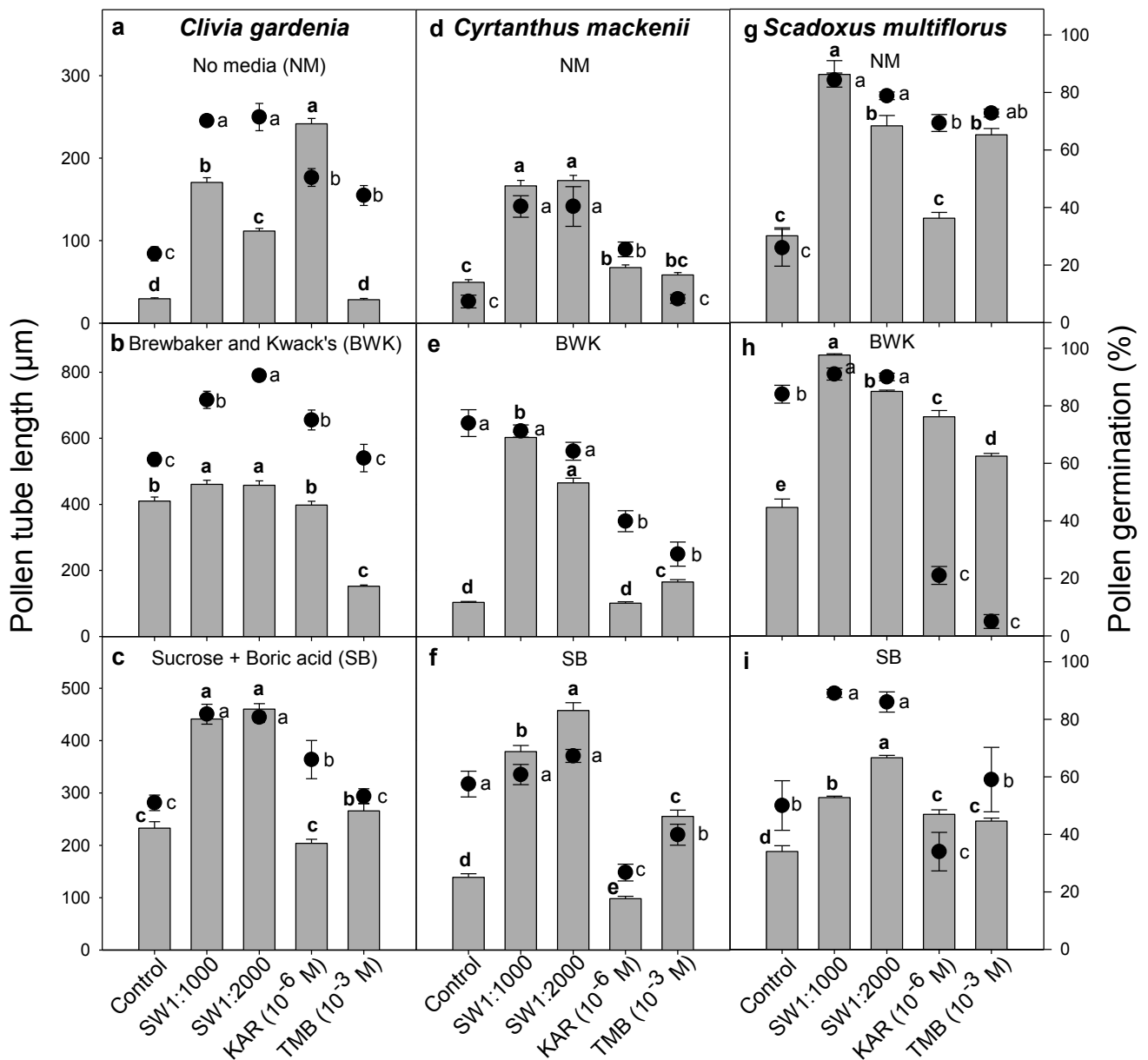
Figure 7. 1 a-i The effect of different concentrations of smoke-water (SW), karrikinolide (KAR$_1$) and trimethylbutenolide (TMB) on pollen germination (dots) and pollen tube length (bars) of three Amaryllidaceae species. Bars (± SE) with different letters (bold face, on top or slightly left of bars) and dots (± SE) with different letters (to the rights of dots), for each species and medium, are significantly different according to Duncan's multiple range test [(P < 0.05) n=24].
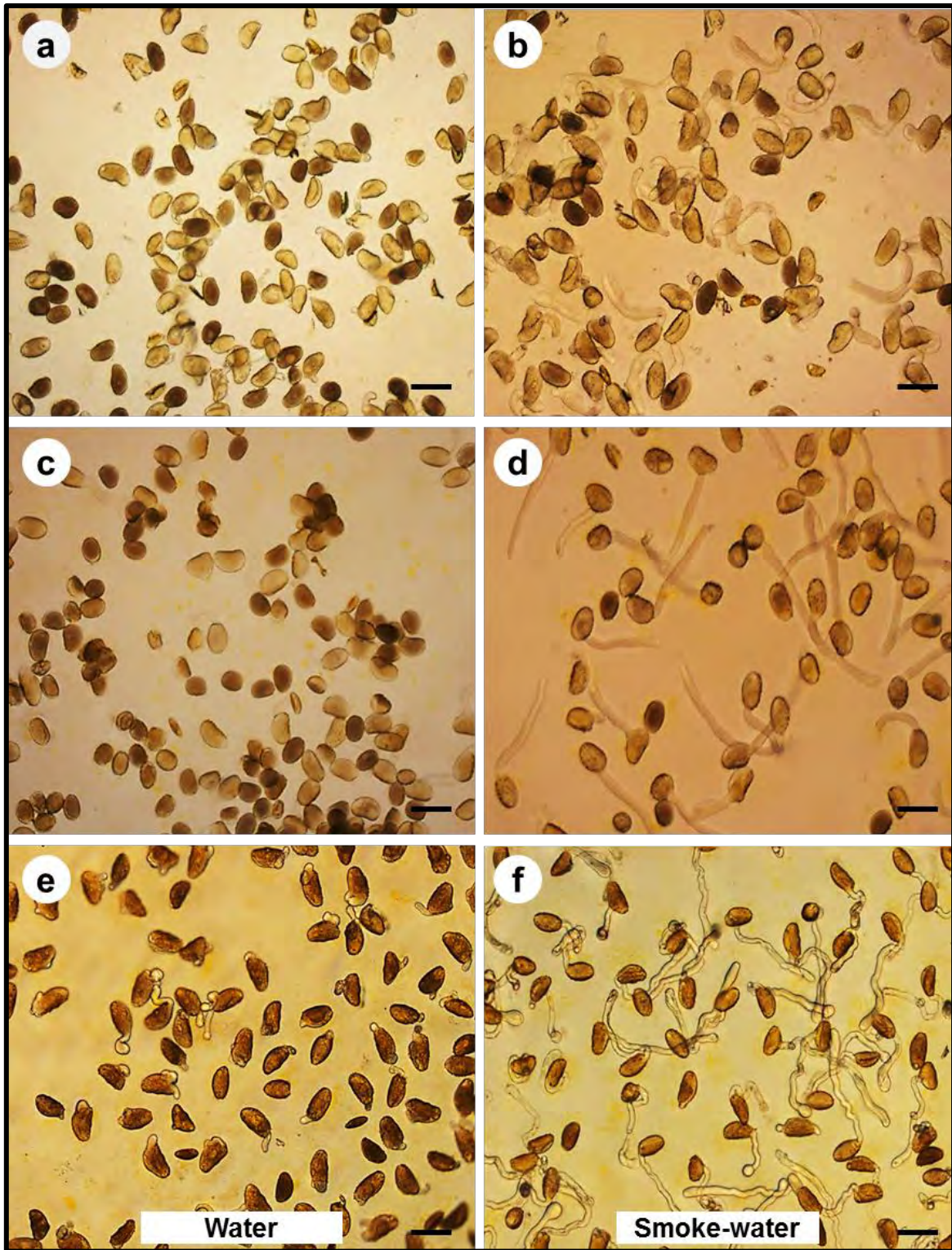
Figure 7. 2 The effect of water (control) and smoke-water (1: 1000) on *in vitro* pollen tube growth of *Clivia gardenii* (a, b), *Cyrtanthus mackenii* (c, d) and *Scadoxus multiflorus* (e, f) in the absence of media. Scale bar = 100 µm.
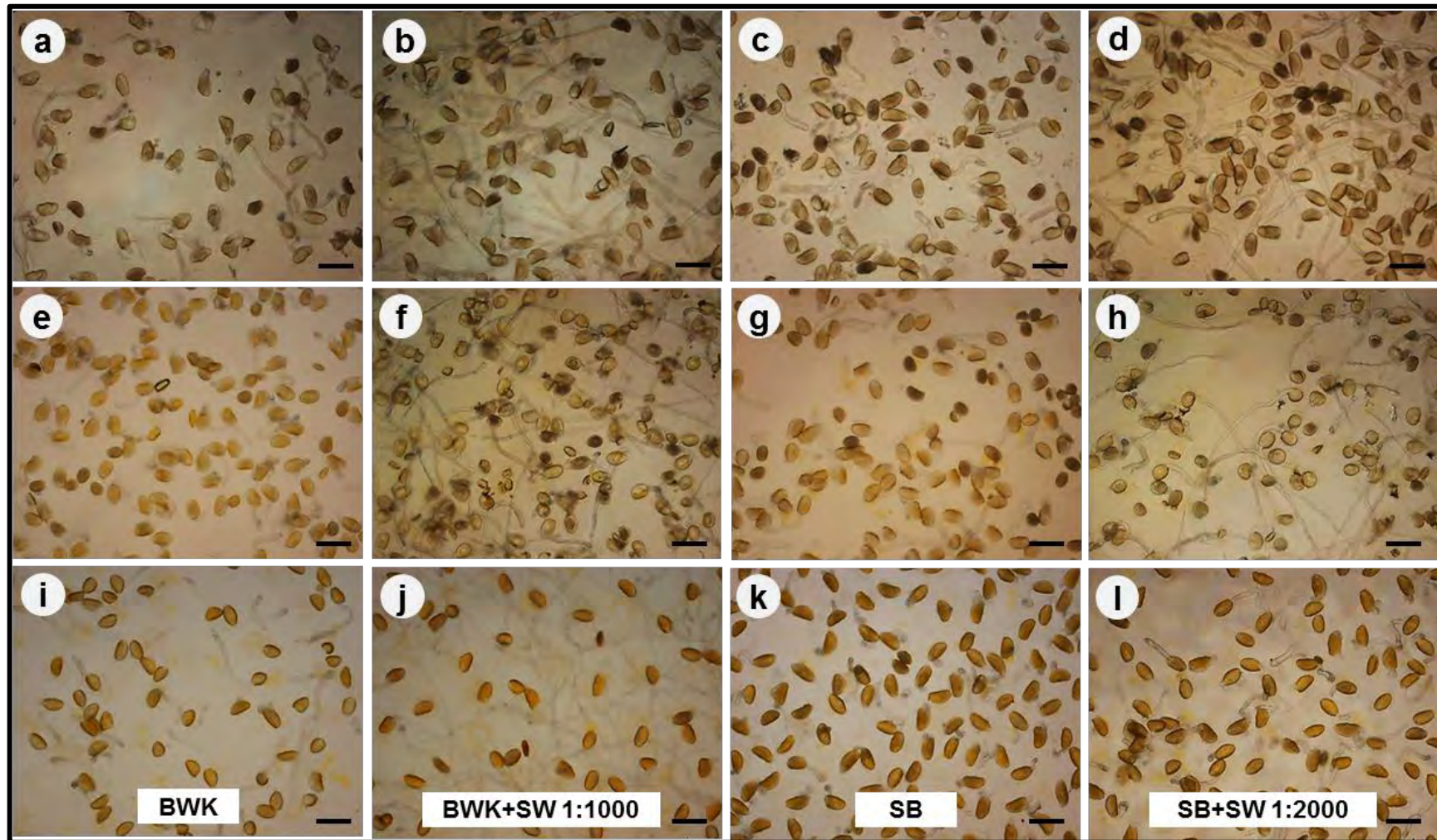
Figure 7. 3 The effects of Brewbaker and Kwack's (BWK) and sucrose and boric acid (SB) media with and without smoke-water (SW) on *in vitro* pollen tube growth of *Clivia gardenia* (a-d), *Cyrtanthus mackenii* (e-h) and *Scadoxus multiflorus* (i-l). Scale bar = 100 μm.

7.3.3 Effects of SW, KAR$_1$ and TMB on pollen germination and pollen tube growth of plant species from different families

The effects of SW, KAR$_1$ and TMB on pollen growth were investigated for various plant species indigenous and exotic to South Africa. In the absence of media, pollen germination of *Aloe maculata* was significantly higher than the control when combined with the KAR$_1$ (10^{-6} and 10^{-7} M) treatments (Fig. 7.4a). Smoke-water (1:1000 and 1:2000) and KAR$_1$ (10^{-6} M) significantly increased pollen germination when combined with the SB medium when compared to the control (Fig. 7.4c), while all the treatments that were combined with the BWK medium produced significantly higher germination compared to the control (Fig. 7.4b). Pollen tube elongation of *A. maculata* was significantly greater than the respective controls when treated with SW, KAR$_1$ and TMB in the presence or absence of the media (Fig. 7.4a - c). The stimulatory effect of KAR$_1$ on pollen tube elongation of *A. maculata* was remarkable and can be observed in Figure 7.5. In the absence of the sucrose media, all the treatments produced significantly higher pollen germination and longer pollen tubes in *Kniphofia uvaria* compared to the respective controls (Fig. 7.4d). Only the SW 1:1000 treatment increased pollen germination in the presence of the SB medium in *K. uvaria* (Fig. 7.4f). All the treatments did, however, produce significantly longer pollen tubes in the same species (Fig. 7.4d - f). Pollen grains of *Lachenalia aloides* showed significantly higher germination compared to the controls when treated with KAR$_1$ (10^{-6} M, without media) and SW 1:1000 + SB medium (Fig. 7.4g and i). Most of the treatments produced longer pollen tubes than the controls in *L. aloides* (Fig. 7.4g - i). Karrikinolide (10^{-7} M without medium) and SW 1:2000 + SB produced higher germination percentages and increased the lengths of pollen tubes of *Tulbaghia simmleri* significantly (Fig. 7.4j and l).

The plant species exotic to South Africa were also responsive to the various smoke treatments. In comparison to the control, KAR$_1$ (10^{-6} M and 10^{-7} M) significantly increased pollen germination, while the same treatments together with TMB produced significantly longer pollen tubes in *Aechmea kertesziae* in the absence of media (Fig. 7.6a). In the presence of media only the KAR$_1$ (10^{-6} M) + BWK medium produced significantly longer pollen tubes in *A. kertesziae* compared to the control (Fig. 7.6b). All the treatments produced longer pollen tubes in *Nematanthus crassifolius* compared to the control in the absence of media (Fig. 7.6d). Karrikinolide

(10^{-6} M) treatment gave significantly higher germination compared to the control and produced the longest pollen tubes in *N. crassifolius* (Fig. 7.6d). In the presence of media, most of the treatments produced significantly higher germination and longer pollen tubes compared to the respective controls (Fig. 7.6e and f). *Paeonia lactiflora* pollen incubated without media showed significantly higher germination when treated with all the tested concentrations of SW and smoke derivatives when compared to the controls (Fig. 7.6g). The various treatments also produced significantly longer pollen tubes in combination with or without BWK medium, compared to the respective controls (Fig. 7.6g and h).
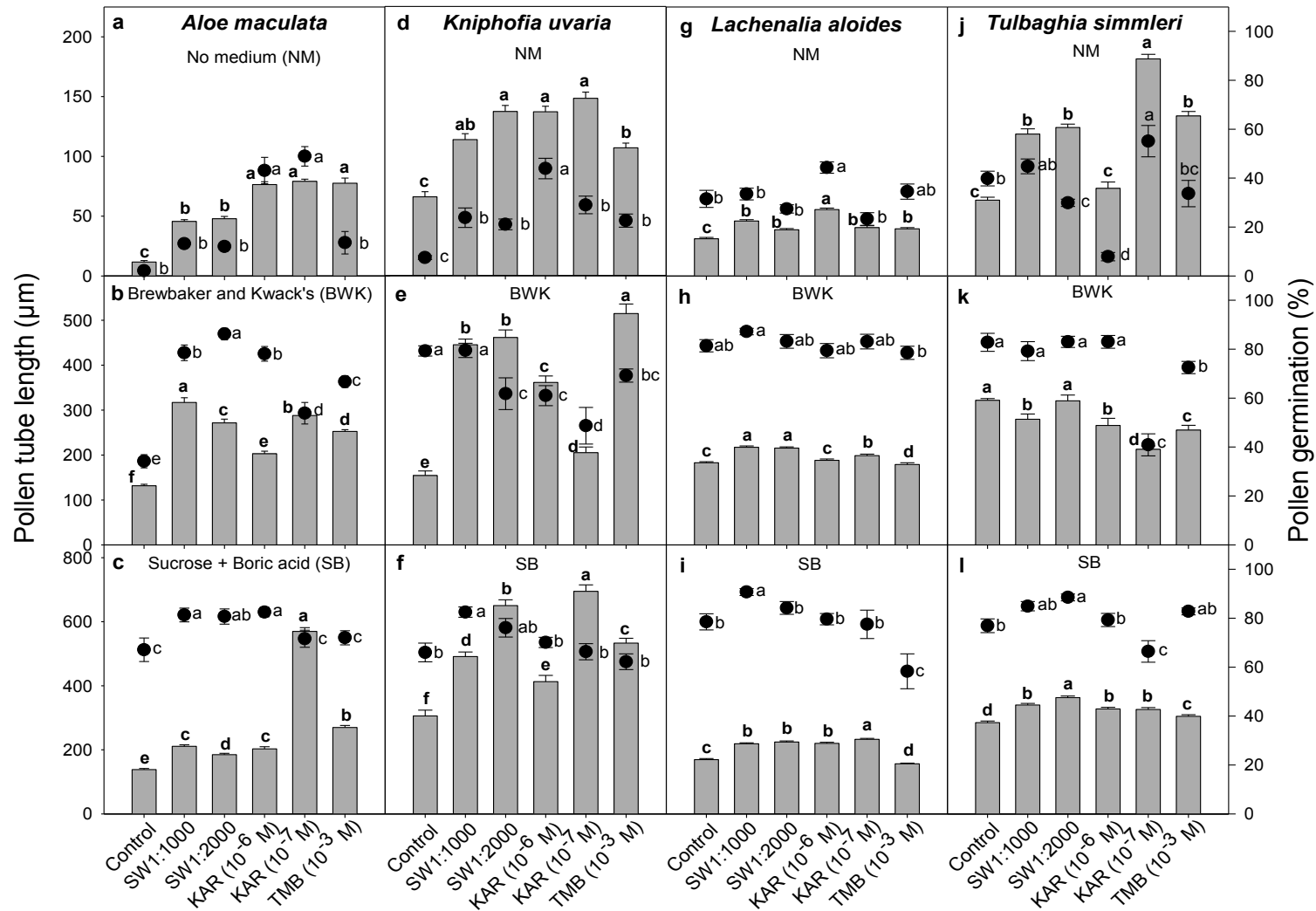
Figure 7. 4 a-l. The effects of different concentrations of smoke-water (SW), karrikinolide (KAR_1) and trimethylbutenolide (TMB) on pollen germination (dots) and pollen tube elongation (bars) of various plant species. Bars (± SE) with different letters (bold face, on top or slightly left of bars) and dots (± SE) with different letters (to the rights of dots), for each species and medium, are significantly different according to Duncan's multiple range test [($P < 0.05$) n=24].
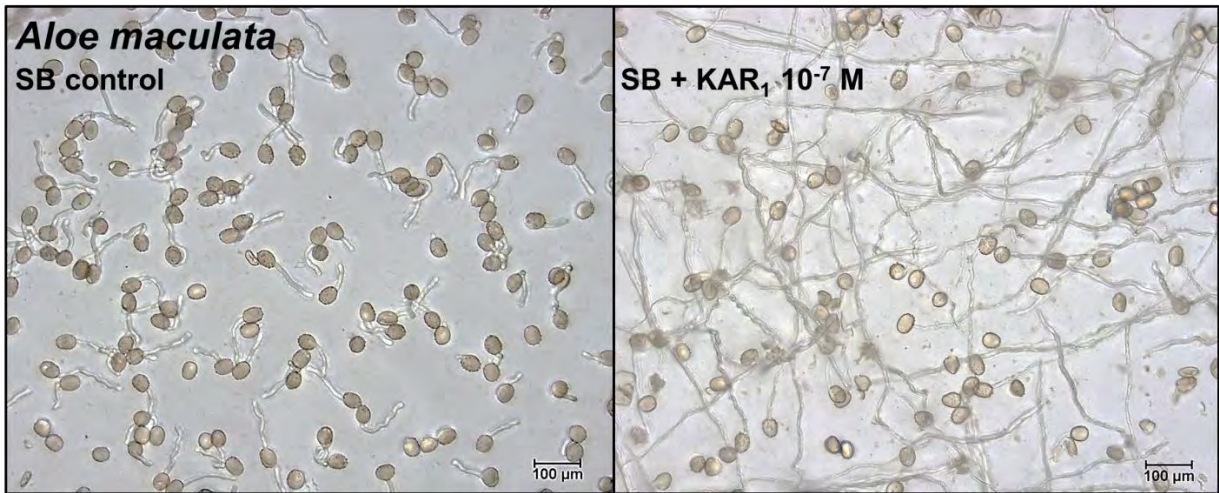
Figure 7. 5 Illustration of the stimulatory effect of KAR$_1$ on the growth of pollen tubes from *Aloe maculata*. The scale bars indicated in the bottom right corner of the pictures are equal to 100 µM.
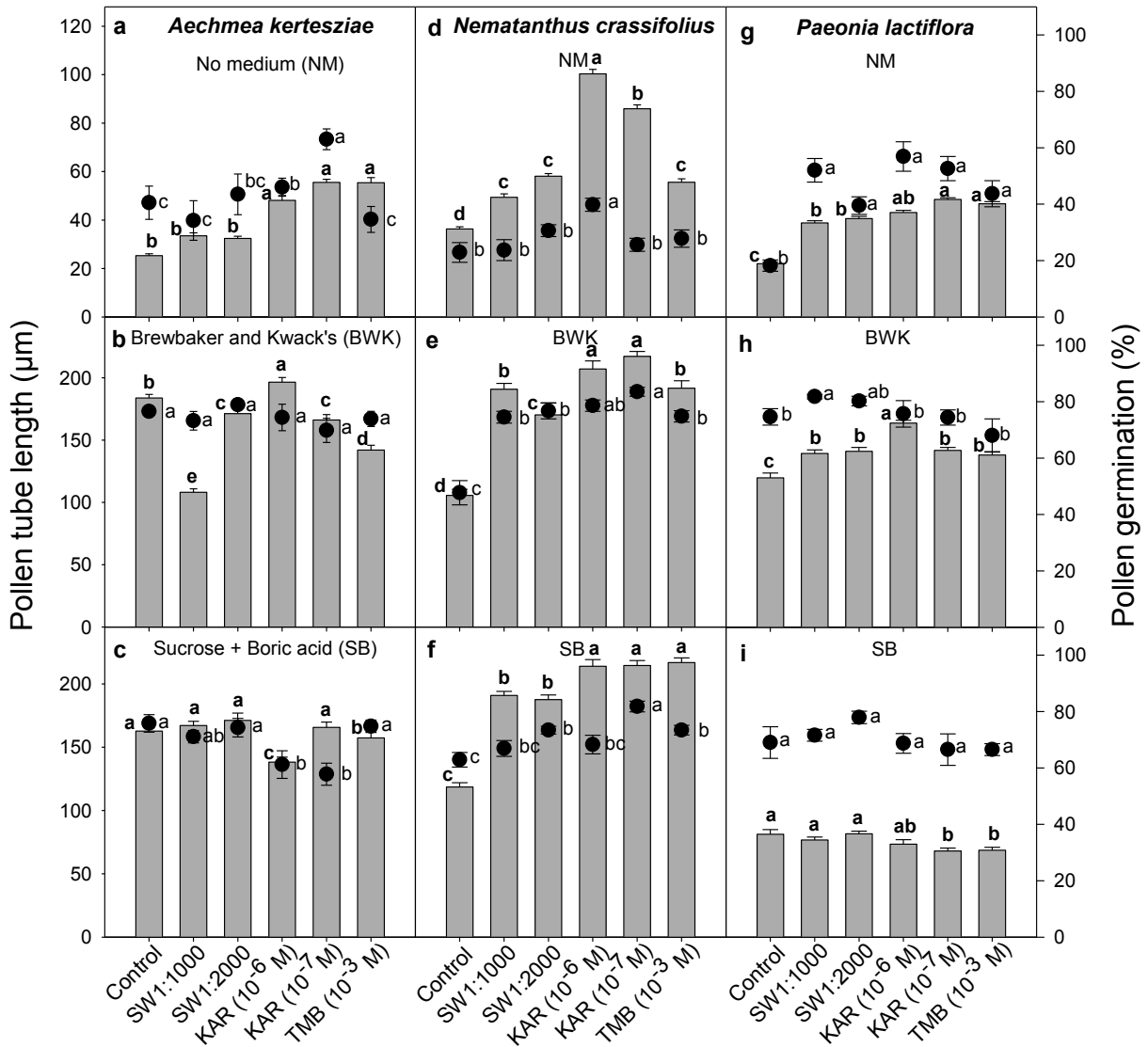
karrikinolide (KAR_1) and trimethylbutenolide (TMB) on pollen germination (dots) and pollen tube elongation (bars) of various plant species. Bars (± SE) with different letters (bold face, on top or slightly left of bars) and dots (± SE) with different letters (to the rights of dots), for each species and medium, are significantly different according to Duncan's multiple range test [($P < 0.05$) n=24].

7.4. DISCUSSION

The effects of environmental and chemical factors on *in vitro* pollen germination and pollen tube elongation have been investigated for many years. These factors include temperature **(ACAR and KAKANI 2010)**, UV-B radiation **(WANG *et al.* 2006)**, PGRs **(DHINGRA and VARGHESE 1985)**, polyamines **(SORKHEH *et al.* 2011)**, cadmium and copper **(SABRINE *et al.* 2010)**. Due to increases in pollution levels, the number of studies concerned with the effects of air pollutants on pollen growth of various plant species have become of paramount importance. A review by **WOLTERS AND MARTENS (1987)** reported that the stimulation and inhibition of pollen grains were dependant on the concentration of the chemical pollutants. These studies revealed that chemicals released into the atmosphere have the ability to effect pollen growth. One of the constituents of atmospheric pollution which has been neglected up until recently is plant-derived smoke.

Plant-derived smoke is one of the major ecological entities that revitalizes vegetation of many ecosystems. This is the first report on the use of SW and smoke-derived compounds to promote pollen germination and pollen tube growth. In the absence of sucrose containing media, low concentrations of SW (1:1000 and 1:2000) significantly increased pollen germination and pollen tube elongation in all three Amaryllidaceae species (Fig. 7.1a, d and g) and also in six of the 7 plant species reported in Figures 7.4 and 7.5 with *A. kerteszie* being the only exception. This is an important result since these solutions contained no added sucrose, calcium (Ca) or boron which are known prerequisites for successful *in vitro* pollen germination and pollen tube elongation **(BREWBAKER and KWACK 1963)**. The role of Ca in the germination and subsequent growth of pollen grains is to establish polarity in the pollen grain **(STEER and STEER 1989; MILLER *et al.* 1992)**. According to **STEER and STEER (1989)** Ca is taken in and regulated at the pollen tube tip where most of the Ca channels are located and often functions as a chemotropic agent. At the tip of the pollen tube, various components are continually incorporated into the elongating cell wall and plasma membrane **(STEER and STEER 1989)**. Many pollen grains of all the species tested ruptured after being placed in pure water for a short while. The pollen grains of the smoke-sensitive species treated with only the SW solutions germinated and produced growing pollen tubes (an illustration of this effect can be observed of the three Amaryllidaceae species tested; Fig. 7.2). This indicates that

SW has the ability to stimulate pollen germination and pollen tube growth, while maintaining pollen tube wall integrity in the absence of added Ca and boric acid. A similar result was obtained when growing *A. maculata* pollen grains in SB medium. All the compounds significantly increased pollen tube elongation and was especially evident with the KAR$_1$ (10^{-7} M) which significantly increased the elongation of *A. maculata* pollen tubes (Fig. 7.4c).

Maximal pollen germination and pollen tube lengths were found when the pollen grains of the three Amaryllidaceae species were treated with low concentrations of SW in the presence of BWK medium (Fig. 7.1b, e and h). Although the significant effect was first observed in the three Amaryllidaceae as part of a pilot study, similar results were also recorded in the indigenous *A. maculata*, *K. uvaria*, *L. aloides* and *T. simmleri* (Fig. 7.4b, e, h and k) and also in the exotic species *N. crassifolius and P. lactiflora* (Fig. 7.6e and h). Although the BWK medium supplied the necessary sucrose, Ca and boric acid, SW (1:1000 and 1:2000) still resulted in higher pollen germination percentages and longer pollen tubes in all nine of the smoke-sensitive species listed above compared to the respective controls. This was supported by the SB + SW (1:2000) results (Fig. 7.1c, f and i), which showed that pollen tube lengths were doubled compared to the respective SB controls in all three Amaryllidaceae species. Considering that the SB medium contained no added Ca, these results indicate that SW has the ability to overcome the Ca requirement for germination and tube growth of *in vitro* grown pollen. No cell division occurs during pollen tube elongation, SW could function in stimulating the mobilization of the sucrose rich reserves in the pollen grains. It is possible that compounds present in SW mobilize Ca present inside the pollen grains. This phenomenon needs to be further investigated, as any predictions of what happens inside the smoke-stimulated pollen grains are speculative at this point.

The seed germination stimulating effect documented for KAR$_1$ **(CHIWOCHA *et al.* 2009; LIGHT *et al.* 2009)** and the inhibitory effects of TMB **(LIGHT *et al.* 2010)** were not analogous to the effects found when these compounds were applied to *in vitro* grown pollen. Compared with a promotory effect of KAR$_1$ on seed germination, this compound reduced pollen germination for *C. mackenii* and *S. multiflorus* (Fig. 7.1e and f, h and i) and only stimulated germination in *C. gardenii* (Fig. 7.1b and c). Similarly, TMB reduced pollen germination in *C. mackenii* and *S. multiflorus*

(Fig. 7.1e, f and h) compared to the respective controls but had no effect on *C. gardenii* pollen (Fig. 7.1b and c). However, in the absence of media KAR_1 and TMB produced significantly higher pollen germination in two of the Amaryllidaceae species (Fig. 7.1a and g). The seed germination inhibitory effect of TMB was not evident in the germination of pollen grains in some of the species evaluated in this study.

In terms of their ability to stimulate the elongation of pollen tubes, KAR_1 and TMB produced significantly longer pollen tubes in many of the plant species examined. In particular, the species *A. maculata*, *K. uvaria* (Fig. 7.4a - f) and *N. crassifolius* (Fig. 7.6d - f) showed a remarkable improvement in pollen tube growth indicating positive responses to the smoke-derivatives. Although not as consistent as with the SW treatments, KAR_1 and TMB do stimulate pollen tube elongation and may play a role in stimulating the growth of pollen tubes in other plant species. Since the effects of SW treatment were more consistent than that of KAR_1 and TMB treatment, it is possible that either (a) other related karrikins are involved in stimulating the pollen grains; (b) that the various karrikins in smoke function synergistically to produce the stimulatory effect or (c) an entirely different group of compounds are responsible for the stimulatory effects observed with SW treatment. This is another avenue of research that must still be followed. It might also be helpful to test the various karrikins or isolated fractions of smoke with an *in vitro* pollen germination assay with pollen grains of one of the Amaryllidaceae species as reported in this study.

It is also important to note that the various treatments used also stimulated significant growth differences in the three plant species *A. kertesziae, N. crassifolius* and *P. lactiflora* that originate from outside South Africa. The phenomenon of smoke-stimulated pollen growth is therefore not restricted to plant species from South Africa but may be applicable to plants from different areas of the world, similar to the phenomenon of smoke-stimulated seed germination.

Flowers produced by the Amaryllidaceae family have great potential for hybridization and commercialization **(NIEDERWIESER *et al.* 2002)**. According to the same author the main constraints of hybridizing *Cyrtanthus* spp. are irregular flowering and difficulty in manipulating flowering. Since low concentrations of SW (1:1000 and 1:2000) consistently showed significantly higher pollen germination percentages and

longer pollen tubes in this study, such treatments have potential to increase the reproductive success of plant species from the Amaryllidaceae family and also in other plant families. The findings of this study are crucial for investigating post-fire flowering of smoke-responsive and non-responsive plant species. The present study indicated that SW, KAR_1 and TMB could be used as potent substitutes for PGRs in pollen germination and pollen tube elongation. Smoke-water, KAR_1 and TMB stimulated a response in the pollen grains of all the plant species to various degrees. Although KAR_1 and TMB did not produce a consistent response among the plant species tested, each species did respond to at least one of the treatments. This might reflect on the evolutionary modification of each species to smoke as an environmental cue.

Pollen performance is mainly measured in its ability to germinate and form a stable growing pollen tube, which contributes to the success of fertilization and subsequent seed-set in flowering plants **(MONDAL and GHANTA 2012)**. Although this is a basic concept, the inability of pollen grains to germinate and grow has devastating effects on seed production and could potentially decrease the production of seed crops. It is therefore of utmost importance to distinguish between factors that could potentially benefit pollen growth and those which could be detrimental. It may be possible to use SW, KAR_1 and TMB to enhance the pollen-pistil interaction and subsequent seed set of flowers of crops (especially seed crops like maize and wheat) and horticultural important plant species. The phenomenon of smoke-stimulated pollen germination may be useful to develop integrated procedures to overcome pre- and post-fertilization barriers.

CHAPTER 8 – GENERAL CONCLUSIONS

The roles of two of the active principles in smoke, namely, karrikinolide (KAR_1) and trimethylbutenolide (TMB), have been modelled around seed dormancy and germination. Today we know that smoke and the compounds isolated from smoke do not only have an effect on germination but that they also have an effect on the growth of plants. Several questions in terms of smoke and the smoke-isolated compounds remain unanswered. Does smoke affect biological processes other than seed germination and plant growth? Can smoke and the compounds isolated from smoke be used in agriculture? If smoke and the smoke-derived compounds are applied to soil in agricultural practices, how do these chemicals affect the rhizosphere microbial organisms? To answer these questions, this study was conducted to get a better understanding on the use of smoke, KAR_1 and TMB in agricultural and horticultural practices.

The structural similarity between TMB and the germination inhibitors that were previously isolated from plant material (mainly seeds) brings about a very interesting phenomenon in terms of the evolution of chemical dormancy in seeds. The characteristic formyl (CHO) chemical group, which is regarded as the group responsible for the germination inhibitory activity of the germination inhibitors, also makes up part of the TMB molecule. It is possible that the germination inhibitors that are found in some seeds have evolved, with TMB acting as the blueprint. If these isolated inhibitors evolved from TMB, it is possible that other plant-related material would also be affected by TMB in some way or another. An example of this is the significant effects of smoke-water (SW), KAR_1 and TMB on pollen growth. There might therefore be other similar plant-related entities that may also be affected by smoke and the compounds isolated from smoke. As more and more studies are conducted on smoke and the smoke-isolated compounds, it is becoming increasingly clear that smoke plays an integral part in the processes that occur in natural environments.

Whether the karrikins (which include KAR_1-KAR_6 and also TMB) were originally derived from smoke or from another means, the roles these compounds play as plant growth regulators (PGRs) are evident and supported by the findings produced from this study. Since KAR_1 has a stimulatory effect on plant growth, **CHIWOCHA et al. (2009)** suggested that karrikins function as PGRs. Considering the germination data collected on TMB and also on some of the synthesised analogues of TMB when combined with KAR_1, a concentration dependent trend can be distinguished. In several instances TMB and some of its analogues have higher activity at 10^{-5} M than at 10^{-4} M when combined with KAR_1. This trend suggests that TMB can also be considered a PGR.

The isolated inhibitors do not only exist as furanone rings such as TMB and 2-pentene-1:4-olid but contain other chemical substituents, which affect their germination inhibitory activities. In an attempt to increase the activity of TMB, several analogues of TMB were synthesised. The aim of this was to determine the structure activity relationship when different substituents were attached to TMB and also to identify compounds with increased inhibitory activity. Although no compounds were found with increased inhibitory activity, two analogues of TMB did retain inhibitory activity comparable to the activity of TMB. These analogues of TMB could be used as building blocks in creating compounds with increased activity. Another possibility is to attach a fluorescent probe to the molecule. This fluorescent-TMB molecule will indicate the fate of TMB in the seed, which may provide some information on the mode of action of this compound.

The application of SW, KAR_1 and TMB in previous studies was largely aimed at increasing the germination of crops, weeds and ecologically important plant species in rehabilitating mine lands. The results from this study have in a sense extended the applicability of SW, KAR_1 and TMB in agricultural and horticultural practices. The results indicated that SW and KAR_1 can be used to increase the germination of weed species so that the germinating weeds can be removed in one treatment. It was also shown that TMB significantly inhibited the germination of the five weed species tested. Trimethylbutenolide also reduced the α-amylase activity in the seeds which indicated that TMB act on at least one class of hydrolytic enzymes. The ability to manipulate the germination of weed seeds in the soil seed bank may be important for future weed control practices. Investigating the interactions of smoke and the

compounds isolated from smoke with the bacteria present in the soil is also very important. It is clear from the results that the interaction between the smoke solution and bacteria are intricate and that this topic must be further investigated. It was, however, interesting to note that the plant growth promoting bacterium could overcome some of the inhibitory effects of TMB on okra seed germination and growth. This indicates that the stable TMB molecule may be metabolised in the soil by bacteria and that it will not remain in the soil indefinitely. It was reported by **GHEBREHIWOT et al. (2013)** that the soil from an area of land that was not burnt for over 60 years contained trace amounts of the karrikin molecules. The microbes present in the soil may therefore remain unaffected by the smoke-derived compounds at the concentrations that they are present at in the soil. It is possible that the compounds may be produced by another source such as bacteria or fungi, however, this still needs to be investigated. It is therefore necessary to investigate the interactions of various different micro-organisms (bacteria and fungi) in the soil with smoke and karrikins.

The importance of smoke and the smoke-derived compounds in horticultural practices was also emphasized by the results produced from this study. It is clear that smoke and the smoke-derived compounds have an effect on the germination and growth of angiosperm pollen. Pollen grain germination and subsequent elongation of pollen tubes were greatly enhanced by the SW treatment and also by KAR_1 and TMB. In some instances pollen germination and pollen tube growth could be sustained in the absence of boric acid, calcium and sucrose. This result emphasizes the role of karrikins as PGRs. The implication of this phenomenon is vast. The application of smoke and the plant-derived compounds may improve the pollen-pistil interaction. This may then result in increased success of ovule fertilization which has implication on the yield of seed crops. It may also be incorporated as a breeding tool. If pollen grains can be stimulated to germinate and grow, incompatibility barriers may be overcome. New varieties of flowers and crops could therefore be produced which could be economically very beneficial.

Applying SW in the growth medium of orchid seeds has a significant effect on the growth and development of *Ansellia africana* orchid seeds. Smoke-water increased the rate of germination and development and ultimately produced significantly more large protocorms compared to the control. The ability of SW to decrease the time of

development of orchid seeds is very important since orchids take several years to develop into flowering plants from seeds. If any part of the development of the orchid from a germinating seed to the mature flowering plant can be reduced, it will be economically very advantageous. It is therefore important to determine if a similar response can be achieved in other orchid species with the SW treatment. It is noteworthy that TMB significantly reduced the rate of development of the orchid seeds compared to the control, especially since KAR_1 did not have any significant effect on the orchid seeds. This could be that the binding site of KAR_1 is not present. It may be possible that TMB reduces the germination of the *A. africana* orchid seeds until the time that the symbiotic fungus provides it with the nutrients it needs to germinate. This, however, is speculative and needs to be investigated further.

It is interesting that SW produced higher germination and more growth when applied to the orchid seeds and pollen grains compared to the other compounds tested. Smoke contains thousands of chemical compounds **(MAGA 1988)**. It may be possible that the increased germination and growth associated with the SW treatment may be due to other related karrikins or an entirely different compound with biological activity. To investigate this, it will be necessary to develop a bioassay using pollen grains so that different fractions of smoke can be efficiently assessed for biological activity.

The role of smoke in a natural environment seems to be more complex than initially imagined. Not only does smoke affect seed germination but also overall plant growth, pollen growth and bacteria population numbers. It is possible that smoke may affect other physiological processes in plants and also other biological processes. The agricultural and horticultural benefits of smoke are becoming more and more apparent. Food security is one of the biggest concerns for an ever growing human population. To insure sufficient food supply in the future, crop yield must be increased and growing crops protected from diseases and pests such as weeds. Implementing smoke and the compounds derived from smoke in agricultural practices will aid in overcoming these obstacles.

REFERENCES

Abbott JD, Bruton BD and Patterson CL. 1991. Fungicidal inhibition of pollen germination and germ-tube elongation in muskmelon. *Hortscience 26*, 529-530.

Abdelgadir HA, Johnson SD and Van Staden J. 2012. Pollen viability, pollen germination and pollen tube growth in the biofuel seed crop *Jatropha curcas* (Euphorbiaceae). *South African Journal of Botany 79*, 132-139.

Abeles FB and Lonski J. 1969. Stimulation of lettuce seed germination by ethylene. *Plant Physiology 44*, 277-280.

Acar I and Kakani VG. 2010. The effects of temperature on *in vitro* pollen germination and pollen tube growth of *Pistacia* spp. *Scientia Horticulturae 125*, 569-572.

Adkins SW and Peters NCB. 2001. Smoke derived from burnt vegetation stimulates germination of arable weeds. *Seed Science Research 11*, 213-222.

Akkerman AM and Veldstra H. 1947. The chemical nature of Köckemann's blastocholine from *Lycopersicon esculentum* Mill. *Recueil des Travaux Chimiques des Pays-Bas 66*, 411-412.

Alam MZ, Braun G, Norrie J and Hodges DM. 2013. Effect of *Ascophyllum* extract application on plant growth, fruit yield and soil microbial communities of strawberry. *Canadian Journal of Plant Science 93*, 23-36.

Arditti J. 1967. Factors affecting the germination of orchid seeds. *The Botanical Review 33*, 1-97.

Arditti J. 1979. Aspects of the Physiology of Orchids. In: Advances in Botanical Research. Woolhouse HW (ed) *Academic Press, London* pp. 421-655.

Arditti J, Ernst R, Yam TW and Glabe C. 1990. The contribution of orchid mycorrhizal fungi to seed germination: a speculative review. *Lindleyana 5*, 249-255.

Aremu AO, Bairu MW, Finnie JF and Van Staden J. 2012. Stimulatory role of smoke–water and karrikinolide on the photosynthetic pigment and phenolic contents of micropropagated 'Williams' bananas. *Plant Growth Regulation 67*, 271-279.

Ashton FM and Crafts AS. 1981. Mode of Action of Herbicides. *John Wiley & Sons, New York* p. 525.

Bais HP, Park S-W, Weir TL, Callaway RM and Vivanco JM. 2004. How plants communicate using the underground information superhighway. *Trends in Plant Science 9*, 26-32.

Baldwin IT, Staszak-Kozinski L and Davidson R. 1994. Up in smoke. I. Smoke-derived germination cues for post-fire annual, *Nicotiana attenuata* Torr. ex. Watson. *Journal of Chemical Ecology 20*, 2345-2371.

Bamzai RD and Randhawa GS. 1967. Effects of certain growth substances and boric acid on germination, Tube growth and storage of grape pollen (*Vitis* ssp.). *Vitis 6*, 269-277.

Bar-Nun N and Mayer AM. 2005. Smoke chemicals and coumarin promote the germination of the parasitic weed *Orobanche aegyptiaca*. *Israel Journal of Plant Sciences 53*, 97-101.

Bashan Y, De-Bashan LE, Prabhu SR and Hernandez J-P. 2014. Advances in plant growth-promoting bacterial inoculant technology: formulations and practical perspectives (1998–2013). *Plant and Soil 378*, 1-33.

Baskin CC and Baskin JM. 1998. Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination. *Academic Press, San Diego* p. 666.

Baxter BJM, Granger JE and Van Staden J. 1995. Plant-derived smoke and seed germination: is all smoke good smoke? That is the burning question. *South African Journal of Botany 61*, 275-277.

Baxter BJM and Van Staden J. 1994. Plant-derived smoke: an effective seed pre-treatment. *Plant Growth Regulation 14*, 279-282.

Baxter BJM, Van Staden J, Granger JE and Brown NAC. 1994. Plant-derived smoke and smoke extracts stimulate seed germination of the fire-climax grass *Themeda triandra. Environmental and Experimental Botany 34*, 217-223.

Berrie AMM, Hendrie MR, Parker W and Knights BA. 1967. Induction of light sensitive dormancy in seed of *Lactuca sativa* L. (lettuce) by patulin. *Plant Physiology 42*, 889-890.

Bewley JD and Black M. 1982. Physiology and biochemistry of seeds in relation to germination: Viability, dormancy and environmental control. *Springer-Verlag*, *Berlin* p. 375.

Bewley JD and Black M. 1994. Seeds: physiology of development and germination. *Plenum Press*, *New York/ London* p. 446.

Bharti N, Barnawal D, Awasthi A, Yadav A and Kalra A. 2014. Plant growth promoting rhizobacteria alleviate salinity induced negative effects on growth, oil content and physiological status in *Mentha arvensis*. *Acta Physiologiae Plantarum 36*, 45-60.

Bolat I and Pirlak L. 1999. An investigation on pollen viability, germination and tube growth in some stone fruits. *Turkish Journal of Agriculture and Forestry 23*, 383-388.

Bouton JH, Dudeck AE and Smith RL. 1976. Germination in freshly harvested seed of centipedegrass. *Agronomy Journal 68*, 991-992.

Bradow JM and Connick WJ. 1988. Seed-germination inhibition by volatile alcohols and other compounds associated with *Amaranthus palmeri* residues. *Journal of Chemical Ecology 14*, 1633-1648.

Brewbaker JL and Kwack BH. 1963. The essential role of calcium ion in pollen germination and pollen tube growth. *American Journal of Botany 50*, 859-865.

Bromilow C. 2010. Problem plants and alien weeds of South Africa. *Briza*, *Pretoria* p. 424.

Brown NAC. 1993a. Promotion of germination of fynbos seeds by plant-derived smoke. *New Phytologist 123*, 575-583.

Brown NAC. 1993b. Seed germination in the fynbos fire ephemeral, *Syncarpha vestita* (L) B-Nord is promoted by smoke, aqueous extracts of smoke and charred wood derived from burning the ericoid-leaved shrub, *Passerina vulgaris* Thoday. *International Journal of Wildland Fire 3*, 203-206.

Brown NAC and Botha PA. 2004. Smoke seed germination studies and a guide to seed propagation of plants from the major families of the Cape Floristic Region, South Africa. *South African Journal of Botany 70*, 559-581.

Brown NAC, Jamieson H and Botha PA. 1994. Stimulation of seed germination in South African species of Restionaceae by plant-derived smoke. *Plant Growth Regulation 15*, 93-100.

Brown NAC and Van Staden J. 1997. Smoke as a germination cue: a review. *Plant Growth Regulation 22*, 115-124.

Brown NAC, Van Staden J, Daws MI and Johnson T. 2003. Patterns in the seed germination response to smoke in plants from the Cape Floristic Region, South Africa. *South African Journal of Botany 69*, 514-525.

Busi R, Vila-Aiub MM, Beckie HJ, Gaines TA, Goggin DE, Kaundun SS, Lacoste M, Neve P, Nissen SJ, Norsworthy JK, Renton M, Shaner DL, Tranel PJ, Wright T, Yu Q and Powles SB. 2013. Herbicide-resistant weeds: from research and knowledge to future needs. *Evolutionary Applications 6*, 1218-1221.

Bytebier B, Simiyu SW and Pearce TR. 1996. Conservation and *in vitro* propagation of rare Kenyan Orchidaceae, a common goal for conservation and horticulture. In: The Biodiversity of African Plants. Van Der Maesen LJG, Van Der Burgt XM and Van Medenbach De Rooy JM (eds). *Springer, Wageningen* pp. 310-312.

Carvalho FP. 2006. Agriculture, pesticides, food security and food safety. *Environmental Science & Policy 9*, 685-692.

Cassán F, Vanderleyden J and Spaepen S. 2014. Physiological and agronomical aspects of phytohormone production by model plant-growth-promoting rhizobacteria (PGPR) belonging to the genus *Azospirillum*. *Journal of Plant Growth Regulation 33*, 440-459.

Castillo P, Escalante M, Gallardo M, Alemano S and Abdala G. 2013. Effects of bacterial single inoculation and co-inoculation on growth and phytohormone production of sunflower seedlings under water stress. *Acta Physiologiae Plantarum 35*, 2299-2309.

Chang DCN. 2006. Research and application of orchid mycorrhiza in Taiwan. XXVII International Horticultural Congress International Symposium on Ornamentals, 299-306.

Chiwocha SDS, Dixon KW, Flematti GR, Ghisalberti EL, Merritt DJ, Nelson DC, Riseborough J-AM, Smith SM and Stevens JC. 2009. Karrikins: A new family of plant growth regulators in smoke. *Plant Science 177*, 252-256.

Chugh S, Guha S and Rao IU. 2009. Micropropagation of orchids: a review on the potential of different explants. *Scientia Horticulturae 122*, 507-520.

Chumpookam J, Lin H-L and Shiesh C-C. 2012. Effect of smoke-water on seed germination and seedling growth of papaya (*Carica papaya* cv. Tainung No. 2). *Hortscience 47*, 741-744.

Commander LE, Merritt DJ, Rokich DP, Flematti GR and Dixon KW. 2008. Seed germination of *Solanum* spp. (Solanaceae) for use in rehabilitation and commercial industries. *Australian Journal of Botany 56*, 333-341.

Cox RM. 1984. Sensitivity of forest plant reproduction to long range transported air pollutants: *in vitro* and *in vivo* sensitivity of *Oenothera parviflora* L. pollen to simulated acid rain. *New Phytologist 97*, 63-70.

Da Costa PB, Beneduzi A, De Souza R, Schoenfeld R, Vargas LK and Passaglia LM. 2013. The effects of different fertilization conditions on bacterial plant growth promoting traits: guidelines for directed bacterial prospection and testing. *Plant and Soil 368*, 267-280.

Daws MI, Davies J, Pritchard HW, Brown NAC and Van Staden J. 2007. Butenolide from plant-derived smoke enhances germination and seedling growth of arable weed species. *Plant Growth Regulation 51*, 73-82.

Dayan FE, Cantrell CL and Duke SO. 2009. Natural products in crop protection. *Bioorganic & Medicinal Chemistry 17*, 4022-4034.

De Lange JH and Boucher C. 1990. Autecological studies on *Audouinia capitata* (Bruniaceae). I. Plant-derived smoke as a seed germination cue. *South African Journal of Botany 56*, 700-703.

De los Santos-Villalobos S, De Folter S, Délano-Frier JP, Gómez-Lim MA, Guzmán-Ortiz DA and Peña-Cabriales JJ. 2013. Growth promotion and flowering induction in mango (*Mangifera indica* L. cv "Ataulfo") trees by *Burkholderia* and *Rhizobium* inoculation: morphometric, biochemical, and molecular events. *Journal of Plant Growth Regulation 32*, 615-627.

DeBano LF and Conrad CE. 1978. The effect of fire on nutrients in a chaparral ecosystem. *Ecology* 489-497.

Dhingra HR and Varghese TM. 1985. Effect of growth regulators on the *in vitro* germination and tube growth of maize (*Zea mays* L.) pollen from plants raised under sodium chloride salinity. *New Phytologist 100*, 563-569.

Dixon KW, Merritt D, Flematti G and Ghisalberti EL. 2009. Karrikinolide – a phytoreactive compound derived from smoke with applications in horticulture, ecological restoration and agriculture. *Acta Horticulturae 813*, 155-170.

Dixon KW and Roche S. 1995. The role of combustion products (smoke) in stimulating *ex situ* and *in situ* germination of western Australian plants. Combined Proceedings-International Plant Propagators Society, 53-56.

Dixon KW, Roche S and Pate JS. 1995. The promotive effect of smoke derived from burnt native vegetation on seed germination of Western Australian plants. *Oecologia 101*, 185-192.

Doherty LC and Cohn MA. 2000. Seed dormancy in red rice (*Oryza sativa*). XI. Commercial liquid smoke elicits germination. *Seed Science Research 10*, 415-421.

Downes KS, Lamont BB, Light ME and Van Staden J. 2010. The fire ephemeral *Tersonia cyathiflora* (Gyrostemonaceae) germinates in response to smoke but not the butenolide 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one. *Annals of Botany 106*, 381-384.

Drewes FE, Smith MT and Van Staden J. 1995. The effect of a plant-derived smoke extract on the germination of light-sensitive lettuce seed. *Plant Growth Regulation 16*, 205-209.

Dyer WE. 1995. Exploiting weed seed dormancy and germination requirements through agronomic practices. *Weed Science 43*, 498-503.

Egley GH and Dale JE. 1970. Ethylene, 2-chloroethylphosphonic acid, and witchweed germination. *Weed Science 18*, 586-589.

Ehrlich PR, Ehrlich AH and Daily GC. 1993. Food security, population and environment. *Population and Development Review 19*, 1-32.

Elliott BB and Leopold AC. 1953. An inhibitor of germination and of amylase activity in oat seeds. *Physiologia Plantarum 6*, 65-77.

Evenari M. 1949. Germination inhibitors. *The Botanical Review 15*, 153-194.

Flematti GR, Ghisalberti EL, Dixon KW and Trengove RD. 2005. Synthesis of the seed germination stimulant 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one. *Tetrahedron Letters 46*, 5719-5721.

Flematti GR, Ghisalberti EL, Dixon KW and Trengrove RD. 2004. A compound from smoke that promotes seed germination. *Science 305*, 977.

Flematti GR, Goddard-Borger ED, Merritt DJ, Ghisalberti EL, Dixon KW and Trengove RD. 2007. Preparation of 2*H*-furo[2,3-*c*]pyran-2-one derivatives and evaluation of their germination-promoting activity. *Journal of Agricultural and Food Chemistry 55*, 2189-2194.

Flematti GR, Merritt DJ, Piggott MJ, Trengove RD, Smith SM, Dixon KW and Ghisalberti EL. 2011. Burning vegetation produces cyanohydrins that liberate cyanide and stimulate seed germination. *Nature Communications 2*, 360.

Flematti GR, Scaffidi A, Goddard-Borger ED, Heath CH, Nelson DC, Commander LE, Stick RV, Dixon KW, Smith SM and Ghisalberti EL. 2010. Structure − activity relationship of karrikin germination stimulants. *Journal of Agricultural and Food Chemistry 58*, 8612-8617.

Fletcher F. 1910. Effect of previous heating of the soil on the growth of plants and the germination of seeds. *Cairo Scientific Journal 4*, 81-86.

Fowler JL. 1991. Interaction of salinity and temperature on the germination of crambe. *Agronomy Journal 83*, 169-172.

Franklin-Tong VE. 1999. Signaling and the modulation of pollen tube growth. *The Plant Cell 11*, 727-738.

Friedman J, Rushkin E and Waller GR. 1982. Highly potent germination inhibitors in aqueous eluate of fruits of bishop's weed (*Ammi majus* L.) and avoidance of autoinhibition. *Journal of Chemical Ecology 8*, 55-65.

Friedman J and Waller GR. 1983. Seeds as allelopathic agents. *Journal of Chemical Ecology 9*, 1107-1117.

Gallandt ER. 2006. How can we target the weed seedbank? *Weed Science 54*, 588-596.

Gatford KT, Eastwood RF and Halloran GM. 2002. Germination inhibitors in bracts surrounding the grain of *Triticum tauschii*. *Functional Plant Biology 29*, 881-890.

Ghebrehiwot HM, Kulkarni MG, Kirkman KP and Van Staden J. 2008. Smoke-water and a smoke-isolated butenolide improve germination and seedling vigour of *Eragrostis tef* (Zucc.) Trotter under high temperature and low osmotic potential. *Journal of Agronomy and Crop Science 194*, 270-277.

Ghebrehiwot HM, Kulkarni MG, Szalai G, Soós V, Balázs E and Van Staden J. 2013. Karrikinolide residues in grassland soils following fire: Implications on germination activity. *South African Journal of Botany 88*, 419-424.

Gill AM and Groves RH. 1981. Fire regimes in heathlands and their plant-ecological effects. In: Heathlands and related shrublands: analytical studies (Ecosystems of the world). Specht RL (ed) *Elsevier, Amsterdam* pp. 61-84.

Golding JS and Bandeira SO. 2002. Southern African plant red data lists. Southern African botancial diversity network report. *SABONET, Pretoria* p. 226.

Gomiero T, Pimentel D and Paoletti MG. 2011. Environmental impact of different agricultural management practices: conventional vs. organic agriculture. *Critical Reviews in Plant Sciences 30*, 95-124.

Gregory PJ. 2006. Roots, rhizosphere and soil: the route to a better understanding of soil science? *European Journal of Soil Science 57*, 2-12.

Gür N and Topdemir A. 2005. Effects of heavy metals (Cd, Cu, Pb, Hg) on pollen germination and tube growth of quince (*Cydonia oblonga* M.) and plum (*Prunus domestica* L.). *Fresenius Environmental Bulletin 14*, 36-39.

Gururani MA, Upadhyaya CP, Baskar V, Venkatesh J, Nookaraju A and Park SW. 2013. Plant growth-promoting rhizobacteria enhance abiotic stress tolerance in *Solanum tuberosum* through inducing changes in the expression of ROS-scavenging enzymes and improved photosynthetic performance. *Journal of Plant Growth Regulation 32*, 245-258.

Hauser EJP and Morrison JH. 1964. Cytochemical reduction of nitroblue-tetrazolium as an index of pollen viability. *American Journal of Botany 51*, 748-753.

Heap I. Accessed on: 24 January 2014. The International Survey of Herbicide Resistant Weeds. Accessed at http://www.weedscience.org/summary/home.aspx.

Helga W. 2009. The World of Organic Agriculture 2009: Summary. In: The World of Organic Agriculture. Statistics and Emerging Trends 2009. Helga W and Kilcher L (eds). *FIBL-IFOAM, Geneva* pp. 19-24.

Hendershot WF, Hesseltine CW, Pridham TG, Benedict RG and Jackson RW. 1962. Ramulosin: Inhibitory effect against plant seeds and various fungi. *Archives of Biochemistry and Biophysics 96*, 166-170.

Heslop-Harrison J and Heslop-Harrison Y. 1970. Evaluation of pollen viability by enzymatically induced fluorescence; intracellular hydrolysis of fluorescein diacetate. *Biotechnic & Histochemistry 45*, 115-120.

Heslop-Harrison J, Heslop-Harrison Y and Shivanna KR. 1984. The evaluation of pollen quality, and a further appraisal of the fluorochromatic (FCR) test procedure. *Theoretical and Applied Genetics 67*, 367-375.

Hewitt FR, Hough T, O'Neill P, Sasse JM, Williams EG and Rowan KS. 1985. Effect of brassinolide and other growth regulators on the germination and growth of pollen tubes of *Prunus avium* using a multiple hanging-drop assay. *Functional Plant Biology 12*, 201-211.

Hodge JE. 1953. Dehydrated foods, chemistry of browning reactions in model systems. *Journal of Agricultural and Food Chemistry 1*, 928-943.

Hopkins WG and Hüner NPA. 1995. Introduction to plant physiology. *Wiley New York* p. 560.

Jäger AK, Light ME and Van Staden J. 1996. Effects of source of plant material and temperature on the production of smoke extracts that promote germination of light-sensitive lettuce seeds. *Environmental and Experimental Botany 36*, 421-429.

Jain A and Shivanna KR. 1988. Storage of pollen grains in organic solvents: Effects of organic solvents on leaching of phospholipids and its relation to pollen viability. *Annals of Botany 61*, 325-330.

Jain N, Stirk WA and Van Staden J. 2008. Cytokinin-and auxin-like activity of a butenolide isolated from plant-derived smoke. *South African Journal of Botany 74*, 327-331.

Jain N and Van Staden J. 2007. The potential of the smoke-derived compound 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one as a priming agent for tomato seeds. *Seed Science Research 17*, 175-181.

Janeš D and Kreft S. 2008. Salicylaldehyde is a characteristic aroma component of buckwheat groats. *Food Chemistry 109*, 293-298.

Jasieniuk M, Brûlé-Babel AL and Morrison IN. 1996. The evolution and genetics of herbicide resistance in weeds. *Weed Science 44*, 176-193.

Jefferson L, Pennacchio M and Havens-Young K. 2014. Ecology of Plant-Derived Smoke: Its Use in Seed Germination. *Oxford University Press, New York* p. 316.

Jeffery DJ, Holmes PM and Rebelo AG. 1988. Effects of dry heat on seed germination in selected indigenous and alien legume species in South Africa. *South African Journal of Botany 54*, 28-34.

Jermyn MA. 1975. Increasing the sensitivity of the anthrone method for carbohydrate. *Analytical Biochemistry 68*, 332-335.

Kappler R and Kristen U. 1987. Photometric quantification of *in vitro* pollen tube growth: A new method suited to determine the cytotoxicity of various environmental substances. *Environmental and Experimental Botany 27*, 305-309.

Kasim WA, Osman ME, Omar MN, El-Daim IAA, Bejai S and Meijer J. 2013. Control of drought stress in wheat using plant-growth-promoting bacteria. *Journal of Plant Growth Regulation 32*, 122-130.

Kato T, Kobayashi M, Sasaki N, Kitahara Y and Takahashi N. 1978. The coumarin heraclenol as a growth inhibitor in parsley seeds. *Phytochemistry 17*, 158-159.

Kato T, Tsunakawa M, Sasaki N, Aizawa H, Fujita K, Kitahara Y and Takahashi N. 1977. Growth and germination inhibitors in rice husks. *Phytochemistry 16*, 45-48.

Kearns CA and Inouye DW. 1993. Techniques for pollination biologists. *University Press of Colorado, Colorado* p. 583.

Keçpczyński J and Keçpczyńska E. 1997. Ethylene in seed dormancy and germination. *Physiologia Plantarum 101*, 720-726.

Keeley JE. 1993. Smoke-induced flowering in the fire-lily *Cyrtanthus ventricosus*. *South African Journal of Botany 59*, 638.

Keeley JE and Fotheringham CJ. 1998. Smoke-induced seed germination in California chaparral. *Ecology 79*, 2320-2336.

Keeley JE and Fotheringham CJ. 2000. Role of fire in regeneration from seed. In: Seeds: The ecology of regeneration in plant communities. Fenner M (ed) *CABI Publishing, Wallingford* pp. 311-330.

Keeley JE and Zedler PH. 1978. Reproduction of chaparral shrubs after fire: A comparison of sprouting and seeding strategies. *American Midland Naturalist 99*, 142-161.

Keromnes J and Thouvenot D. 1985. Role of penicillic acid in the phytotoxicity of *Penicillium cyclopium* and *Penicillium canescens* to the germination of corn seeds. *Applied and Environmental Microbiology 49*, 660-663.

Khatun S and Flowers TJ. 1995. The estimation of pollen viability in rice. *Journal of Experimental Botany 46*, 151-154.

Knudson L. 1922. Nonsymbiotic germination of orchid seeds. *Botanical Gazette 73*, 1-25.

Kruger FJ. 1977. Ecology of Cape fynbos in relation to fire. Symposium of environmental consequences of fire and management in mediterranean ecosystems, United States department of Agriculture and Forest Service General Technical Report WO, 391-396.

Kruger FJ. 1978. Some aspects of the demography of *Watsonia pyramidata* (Andr.) Stapf in relation of fire. Joint SAAB and GSSA congress, Bloemfontein.

Kruger FJ and Bigalke RC. 1984. Fire in Fynbos. In: Ecological Effects of Fire in South African Ecosystems. Booysen PV and Tainton NM (eds). *Springer Berlin Heidelberg* pp. 67-114.

Kulkarni MG, Amoo SO, Kandari LS and Van Staden J. 2014. Seed germination and phytochemical evaluation in seedlings of *Aloe arborescens* Mill. *Plant Biosystems 148*, 460-466.

Kulkarni MG, Ascough GD and Van Staden J. 2008. Smoke-water and a smoke-isolated butenolide improve growth and yield of tomatoes under greenhouse conditions. *HortTechnology 18,* 449-454.

Kulkarni MG, Ascough GD, Verschaeve L, Baeten K, Arruda MP and Van Staden J. 2010. Effect of smoke-water and a smoke-isolated butenolide on the growth and genotoxicity of commercial onion. *Scientia Horticulturae 124*, 434-439.

Kulkarni MG, Light ME and Van Staden J. 2011. Plant-derived smoke: Old technology with possibilities for economic applications in agriculture and horticulture. *South African Journal of Botany 77*, 972-979.

Kulkarni MG, Sparg SG, Light ME and Van Staden J. 2006a. Stimulation of rice (*Oryza sativa* L.) seedling vigour by smoke-water and butenolide. *Journal of Agronomy and Crop Science 192*, 395-398.

Kulkarni MG, Sparg SG and Van Staden J. 2006b. Dark conditioning, cold stratification and a smoke-derived compound enhance the germination of *Eucomis autumnalis* subsp. *autumnalis* seeds. *South African Journal of Botany 72*, 157-162.

Kumari A, Komal R, Rajesh R and Pandey AK. 2009. *In vitro* pollen germination, pollen tube growth and pollen viability in *Trichosanthes dioica* Roxb. (Cucurbitaceae). *The International Journal of Plant Reproductive Biology 1*, 147-151.

Lamont BB, Swanborough PW and Ward D. 2000. Plant size and season of burn affect flowering and fruiting of the grasstree *Xanthorrhoea preissii. Austral Ecology 25*, 268-287.

Lavie D, Levy EC, Cohen A, Evenari M and Guttermann Y. 1974. New germination inhibitor from *Aegilops ovata* L. *Nature 249*, 388-388.

Le Maitre DC. 1984. A short note on seed predation in *Watsonia pyramidata* (Andr.) Stapf in relation to season of burn. *South African Journal of Botany 50*, 407-415.

Le Maitre DC and Brown PJ. 1992. Life cycles and fire-stimulated flowering in geophytes. In: Fire in South African Mountain Fynbos. Van Wilgen BW, Richardson DM, Kruger FJ and Hensbergen HJ (eds). *Springer Berlin Heidelberg* pp. 145-160.

Lerner HR, Mayer AM and Evenari M. 1959. The nature of the germination inhibitors present in dispersal units of *Zygophyllum dumosum* and *Trigonella arabica. Physiologia Plantarum 12*, 245-250.

Lichtenthaler HK. 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. In: Methods in Enzymology. R. D and L. P (eds). *Academic Press, New York* pp. 350-382.

Light ME. 2006. The role of smoke as a germination cue. Ph.D. School of Biological and Conservation Sciences. University of KwaZulu-Natal.

Light ME, Burger BV, Staerk D, Kohout L and Van Staden J. 2010. Butenolides from plant-derived smoke: natural plant-growth regulators with antagonistic actions on seed germination. *Journal of Natural Products 73*, 267-269.

Light ME, Burger BV and Van Staden J. 2005. Formation of a seed germination promoter from carbohydrates and amino acids. *Journal of Agricultural and Food Chemistry 53*, 5936-5942.

Light ME, Daws MI and Van Staden J. 2009. Smoke-derived butenolide: towards understanding its biological effects. *South African Journal of Botany 75*, 1-7.

Light ME, Gardner MJ, Jäger AK and Van Staden J. 2002. Dual regulation of seed germination by smoke solutions. *Plant Growth Regulation 37*, 135-141.

Light ME and Van Staden J. 2004. The potential of smoke in seed technology. *South African Journal of Botany 70*, 97-101.

Linskens HF. 1967. Pollen. In: Handbuch der Pflanzenphysiologie. Ruhland W (ed) *Berlin and New York* pp. 368-406.

Lloyd MV, Dixon KW and Sivasithamparam K. 2000. Comparative effects of different smoke treatments on germination of Australian native plants. *Austral Ecology 25*, 610-615.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry 193*, 265-275.

Lyra DH, Sampaio LS, Pereira DA, Silva AP and Amaral CLF. 2011. Pollen viability and germination in *Jatropha ribifolia* and *Jatropha mollissima* (Euphorbiaceae): Species with potential for biofuel production. *African Journal of Biotechnology 10*, 368-374.

Maga JA. 1988. Smoke in food processing. *CRC Press, Boca Raton* p. 160.

Marais C, Van Wilgen BW and Stevens D. 2004. The clearing of invasive alien plants in South Africa: a preliminary assessment of costs and progress: working for water. *South African Journal of Science 100*, 97-103.

Martins DJ. 2009. Pollination and facultative ant-association in the African leopard orchid *Ansellia africana*. *Journal of East African Natural History 98*, 67-77.

Matilla AJ. 2000. Ethylene in seed formation and germination. *Seed Science Research 10*, 111-126.

Mavi K, Light ME, Demir I, van Staden J and Yasar F. 2010. Positive effect of smoke-derived butenolide priming on melon seedling emergence and growth. *New Zealand Journal of Crop and Horticultural Science 38*, 147-155.

Mayak S, Tirosh T and Glick BR. 2004. Plant growth-promoting bacteria that confer resistance to water stress in tomatoes and peppers. *Plant Science 166*, 525-530.

Mayer AM and Evenari M. 1952. The relation between the structure of coumarin and its derivatives, and their activity as germination inhibitors. *Journal of Experimental Botany 3*, 246-252.

Miller DD, Callaham DA, Gross DJ and Hepler PK. 1992. Free Ca^{2+} gradient in growing pollen tubes of *Lillium*. *Journal of Cell Science 101*, 7-12.

Miransari M. 2013. Soil microbes and the availability of soil nutrients. *Acta Physiologiae Plantarum 35*, 3075-3084.

Mondal S and Ghanta R. 2012. Effect of sucrose and boric acid on *in vitro* pollen germination of *Solanum macranthum* Dunal. *Indian Journal of Fundamental and Applied Life Sciences 2*, 202-206.

Munzuroǧlu and Gür N. 2000. The effects of heavy metals on the pollen germination and pollen tube growth of apples (*Malus sylvestris* Miller cv. Golden). *Turkish Journal of Biology 24*, 677-684.

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

Nagase R, Katayama M, Mura H, Matsuo N and Tanabe Y. 2008. Synthesis of the seed germination stimulant 3-methyl-2*H*-furo[2,3-*c*]pyran-2-ones utilizing direct and regioselective Ti-crossed aldol addition. *Tetrahedron Letters 49*, 4509-4512.

Nair JJ, Pošta M, Papenfus HB, Munroc OQ, Beier P and Van Staden J. 2014. Synthesis, X-ray structure determination and germination studies of some smoke-derived karrikins. *South African Journal of Botany 91*, 53-57.

Nawamaki K and Kuroyanagi M. 1996. Sesquiterpenoids from *Acorus calamus* as germination inhibitors. *Phytochemistry 43*, 1175-1182.

Negi YK, Prabha D, Garg SK and Kumar J. 2011. Genetic diversity among cold-tolerant fluorescent *Pseudomonas* isolates from Indian Himalayas and their characterization for biocontrol and plant growth-promoting activities. *Journal of Plant Growth Regulation 30*, 128-143.

Nel JL, Richardson DM, Rouget M, Mgidi TN, Mdzeke N, Le Maitre DC, Van Wilgen BW, Schonegevel L, Henderson L and Neser S. 2004. Proposed classification of invasive alien plant species in South Africa: towards prioritizing species and areas for management action. *South African Journal of Science 100*, 53-64.

Nelson DC, Flematti GR, Riseborough J-A, Ghisalberti EL, Dixon KW and Smith SM. 2010. Karrikins enhance light responses during germination and seedling development in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences 107*, 7095-7100.

Nelson DC, Riseborough J-A, Flematti GR, Stevens J, Ghisalberti EL, Dixon KW and Smith SM. 2009. Karrikins discovered in smoke trigger *Arabidopsis* seed germination by a mechanism requiring gibberellic acid synthesis and light. *Plant Physiology 149*, 863-873.

Nelson DC, Scaffidi A, Dun EA, Waters MT, Flematti GR, Dixon KW, Beveridge CA, Ghisalberti EL and Smith SM. 2011. F-box protein MAX2 has dual roles in karrikin and strigolactone signaling in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences 108*, 8897-8902.

Niederwieser JG, Terblanche M and Spreeth MH. 2002. Potential of South African members of the Amaryllidaceae for new crop development. *Acta Horticulturae 570*, 359-365.

Nikolaeva MG. 1977. Factors controlling the seed dormancy pattern. In: Physiology and Biochemistry of Seed dormancy and Germination. Khan AA (ed) *Elsevier, Amsterdam* pp. 51-74.

Nutile GE. 1945. Inducing dormancy in lettuce seed with coumarin. *Plant Physiology 20*, 433.

Olivier W and Werner W. 1980. The genus *Cyrtanthus* Ait. *Veld and Flora 66*, 78-81.

Papenfus HB, Kumari A, Kulkarni MG, Finnie JF and Van Staden J. 2014. Smoke-water enhances *in vitro* pollen germination and tube elongation of three species of Amaryllidaceae. *South African Journal of Botany 90*, 87-92.

Pierce SM, Esler K and Cowling RM. 1995. Smoke-induced germination of succulents (Mesembryanthemaceae) from fire-prone and fire-free habitats in South Africa. *Oecologia 102*, 520-522.

Pooley B. 1998. A field guide to wild flowers of KwaZulu-Natal and the eastern Region. *Natal Flora Publications Trust, Durban* p. 630.

Pošta M, Light ME, Papenfus HB, Van Staden J and Kohout L. 2013. Structure–activity relationships of analogs of 3,4,5-trimethylfuran-2(5*H*)-one with germination inhibitory activities. *Journal of Plant Physiology 170*, 1235-1242.

Preston CA and Baldwin IT. 1999. Positive and negative signals regulate germination in the post-fire annual, *Nicotiana attenuata. Ecology 80*, 481-494.

Quinn RD. 1994. Animals, fire, and vertebrate herbivory in Californian chaparral and other Mediterranean-type ecosystems. In: The role of fire in Mediterranean-type ecosystems. *Springer, New York* pp. 46-78.

Reynolds T. 1978. Comparative effects of aromatic compounds on inhibition of lettuce fruit germination. *Annals of Botany 42*, 419-427.

Roche S, Dixon KW and Pate JS. 1994. Smoke–a new process for germinating Australian plants. *Australian Horticulture 91*, 46-48.

Roche S, Dixon KW and Pate JS. 1997a. Seed ageing and smoke: partner cues in the amelioration of seed dormancy in selected Australian native species. *Australian Journal of Botany 45*, 783-815.

Roche S, Koch JM and Dixon KW. 1997b. Smoke enhanced seed germination for mine rehabilitation in the southwest of Western Australia. *Restoration Ecology 5*, 191-203.

Rundel PW. 1981. Fire as an ecological factor. In: Physiological Plant Ecology I. Lange OL, Nobel PS, Osmond CB and Ziegler H (eds). *Springer, Berlin Heidelberg* pp. 501-538.

Sabiiti EN and Wein RW. 1987. Fire and *Acacia* seeds: a hypothesis of colonization success. *The Journal of Ecology* 937-946.

Sabrine H, Afif H, Mohamed B, Hamadi B and Maria H. 2010. Effects of cadmium and copper on pollen germination and fruit set in pea (*Pisum sativum* L.). *Scientia Horticulturae 125*, 551-555.

Sadasivam S and Manickam A. 1996. Biochemical methods. *New Age International Publishers, New Delhi* p. 257.

Salomon MV, Bottini R, De Souza Filho GA, Cohen AC, Moreno D, Gil M and Piccoli P. 2014. Bacteria isolated from roots and rhizosphere of *Vitis vinifera* retard water losses, induce abscisic acid accumulation and synthesis of defense-related terpenes in *in vitro* cultured grapevine. *Physiologia Plantarum 151*, 359-374.

Sarma RK and Saikia R. 2014. Alleviation of drought stress in mung bean by strain *Pseudomonas aeruginosa* GGRJ21. *Plant and Soil 377*, 111-126.

Sato S, Katoh N, Iwai S and Hagimori M. 1998. Establishment of reliable methods of *in vitro* pollen germination and pollen preservation of *Brassica rapa* (syn. *B. campestris*). *Euphytica 103*, 29-33.

Scaffidi A, Flematti GR, Nelson DC, Dixon KW, Smith SM and Ghisalberti EL. 2011. The synthesis and biological evaluation of labelled karrikinolides for the elucidation of the mode of action of the seed germination stimulant. *Tetrahedron 67*, 152-157.

Scaffidi A, Waters MT, Bond CS, Dixon KW, Smith SM, Ghisalberti EL and Flematti GR. 2012. Exploring the molecular mechanism of karrikins and strigolactones. *Bioorganic & Medicinal Chemistry Letters 22*, 3743-3746.

Shen W, Lin X, Shi W, Min J, Gao N, Zhang H, Yin R and He X. 2010. Higher rates of nitrogen fertilization decrease soil enzyme activities, microbial functional diversity and nitrification capacity in a Chinese polytunnel greenhouse vegetable land. *Plant and Soil 337*, 137-150.

Shivanna KR and Heslop-Harrison J. 1981. Membrane state and pollen viability. *Annals of Botany 47*, 759-770.

Shivanna KR and Rangaswamy NS. 1992. Pollen biology: A laboratory manual. *Springer-Verlag, Berlin* p. 192.

Singh DP, Jermakow AM and Swain SM. 2002. Gibberellins are required for seed development and pollen tube growth in Arabidopsis. *The Plant Cell 14*, 3133-3147.

Singh S, Kulkarni MG and Van Staden J. 2014. Biochemical changes associated with gibberellic acid-like activity of smoke-water, karrikinolide and vermicompost leachate during seedling development of *Phaseolus vulgaris* L. *Seed Science Research 24*, 63-70.

Smith PF. 1942. Studies of the growth of pollen with respect to temperature, auxins, colchicine and vitamin B1. *American Journal of Botany* 56-66.

Soós V, Sebestyen E, Juhasz A, Light ME, Kohout L, Szalai G, Tandori J, Van Staden J and Balazs E. 2010. Transcriptome analysis of germinating maize kernels exposed to smoke-water and the active compound KAR_1. *BMC Plant Biology 10*, 236-251.

Soós V, Sebestyén E, Pošta M, Kohout L, Light ME, Staden J and Balázs E. 2012. Molecular aspects of the antagonistic interaction of smoke-derived butenolides on the germination process of Grand Rapids lettuce (*Lactuca sativa*) achenes. *New Phytologist 196*, 1060-1073.

Sorkheh K, Shiran B, Rouhi V, Khodambashi M, Wolukau JN and Ercisli S. 2011. Response of *in vitro* pollen germination and pollen tube growth of almond (*Prunus dulcis* Mill.) to temperature, polyamines and polyamine synthesis inhibitor. *Biochemical Systematics and Ecology 39*, 749-757.

Sparg SG, Kulkarni MG, Light ME and Van Staden J. 2005. Improving seedling vigour of indigenous medicinal plants with smoke. *Bioresource Technology 96*, 1323-1330.

Sparg SG, Kulkarni MG and Van Staden J. 2006. Aerosol smoke and smoke-water stimulation of seedling vigor of a commercial maize cultivar. *Crop Science 46*, 1336-1340.

Spencer GF, England RE and Wolf RB. 1984. (−)-Cryptocaryalactone and (−)-deacetylcryptocaryalactone-germination inhibitors from *Cryptocarya moschata* seeds. *Phytochemistry 23*, 2499-2500.

Stanley RG and Linskens HF. 1974. Pollen: Biology, Biochemistry and Management. *Springer, Berlin, Heidelberg and New York* p. 374.

Steer MW and Steer JM. 1989. Pollen tube tip growth. *New Phytologist 111*, 323-358.

Stevens JC, Merritt DJ, Flematti GR, Ghisalberti EL and Dixon KW. 2007. Seed germination of agricultural weeds is promoted by the butenolide 3-methyl-2*H*-furo[2, 3-*c*]pyran-2-one under laboratory and field conditions. *Plant and Soil 298*, 113-124.

Stewart-Jones A and Poppy GM. 2006. Comparison of glass vessels and plastic bags for enclosing living plant parts for headspace analysis. *Journal of Chemical Ecology 32*, 845-864.

Sun K, Chen Y, Wagerle T, Linnstaedt D, Currie M, Chmura P, Song Y and Xu M. 2008. Synthesis of butenolides as seed germination stimulants. *Tetrahedron Letters 49*, 2922-2925.

Surmont R, Verniest G and De Kimpe N. 2010. Short synthesis of the seed germination inhibitor 3,4,5-trimethyl-2(5*H*)-furanone. *The Journal of Organic Chemistry 75*, 5750-5753.

Taylor JLS and Van Staden J. 1998. Plant-derived smoke solutions stimulate the growth of *Lycopersicon esculentum* roots *in vitro. Plant Growth Regulation 26*, 77-83.

Taylorson RB and Hendricks SB. 1973. Promotion of seed germination by cyanide. *Plant Physiology 52*, 23-27.

Thomas TH and Van Staden J. 1995. Dormancy break of celery (*Apium graveolens* L.) seeds by plant derived smoke extract. *Plant Growth Regulation 17*, 195-198.

Trinh C, Gevaert L, Kohout L, Van Staden J and Verschaeve L. 2010. Genotoxicity evaluation of two kinds of smoke-water and 3,7-dimethyl-2*H*-furo[2,3-*c*]pyran-2-one. *Journal of Applied Toxicology 30*, 596-602.

Tuinstra MR and Wedel J. 2000. Estimation of pollen viability in grain sorghum. *Crop Science 40*, 968-970.

Tuna AL, Burun B, Yokas I and Coban E. 2002. The effects of heavy metals on pollen germination and pollen tube length in the tobacco plant. *Turkish Journal of Biology 26*, 109-113.

Van Staden J, Brown NAC, Jäger AK and Johnson TA. 2000. Smoke as a germination cue. *Plant Species Biology 15*, 167-178.

Van Staden J, Jäger AK, Light ME and Burger BV. 2004. Isolation of the major germination cue from plant-derived smoke. *South African Journal of Botany 70*, 654-659.

Van Staden J, Sparg SG, Kulkarni MG and Light ME. 2006. Post-germination effects of the smoke-derived compound 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one, and its potential as a preconditioning agent. *Field Crops Research 98*, 98-105.

Vasudevan R and Van Staden J. 2010. *In vitro* asymbiotic seed germination and seedling growth of *Ansellia africana* Lindl. *Scientia Horticulturae 123*, 496-504.

Vasudevan R and Van Staden J. 2011. Cytokinin and explant types influence *in vitro* plant regeneration of Leopard Orchid (*Ansellia africana* Lindl.). *Plant Cell, Tissue and Organ Culture 107*, 123-129.

Verschaeve L, Maes J, Light ME and Van Staden J. 2006. Genetic toxicity testing of 3-methyl-2*H*-furo[2,3-*c*]pyran-2-one, an important biologically active compound from plant-derived smoke. *Mutation Research-Genetic Toxicology and Environmental Mutagenesis 611*, 89-95.

Voyiatzis DG and Paraskevopoulou-Paroussi G. 2000. The effect of photoperiod and gibberellic acid on strawberry pollen germination and stamen growth. IV International Strawberry Symposium 567, 257-260.

Wang Y, Zhang N, Qiang W, Xiong Z and Du G. 2006. Effects of reduced, ambient, and enhanced UV-B radiation on pollen germination and pollen tube growth of six alpine meadow annual species. *Environmental and Experimental Botany 57*, 296-302.

Wang Z-Y, Ge Y, Scott M and Spangenberg G. 2004. Viability and longevity of pollen from transgenic and nontransgenic tall fescue (*Festuca arundinacea*)(Poaceae) plants. *American Journal of Botany 91*, 523-530.

Wicklow DT. 1988. Parallels in the development of post-fire fungal and herb communities. *Fungi and Ecological Disturbance. Proceedings of the Royal Society of Edinburgh 94B*, 87-95.

Williams PM, Ross JD and Bradbeer JW. 1973. Studies in seed dormancy. VII. The abscisic acid content of seeds and fruits of *Corylus avellana* L. *Planta 110*, 303-310.

Wolters JHB and Martens MJM. 1987. Effects of air pollutants on pollen. *The Botanical Review 53*, 372-414.

Wyse DL. 1992. Future of weed science research. *Weed Technology 6*, 162-165.

Zimdahl RL. 1988. The concept and application of the critical weed-free period. In: Weed management in agroecosystems: ecological approaches. Altieri MA and Liebman M (eds). *CRC, Boca Raton* pp. 145-155.

Zobolo AM. 2010. Effect of temperature, light intensity and growth regulators on propagation of *Ansellia africana* from cuttings. *African Journal of Biotechnology 9*, 5566-5574.