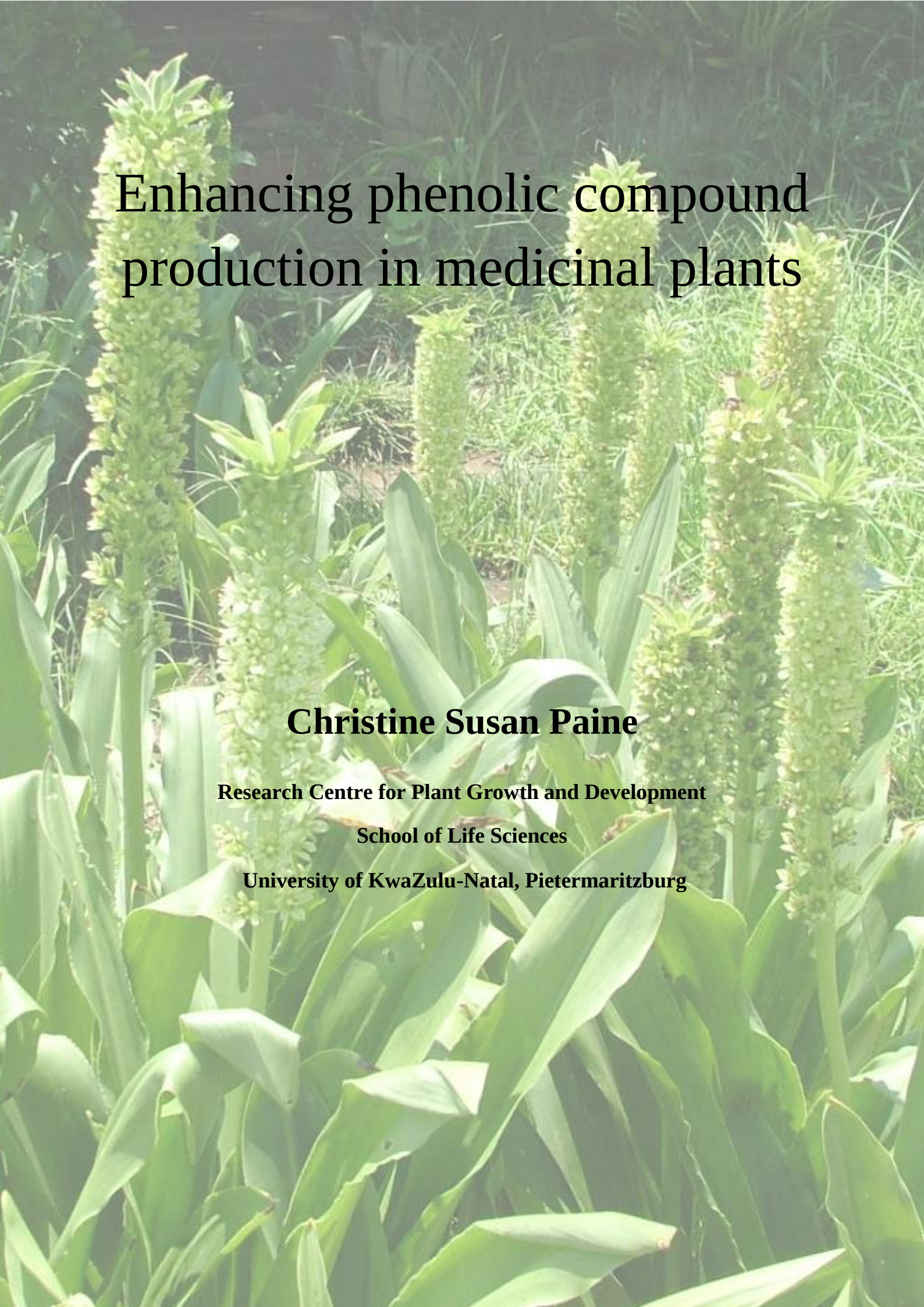

Enhancing phenolic compound production in medicinal plants

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Research Centre for Plant Growth and Development

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University of KwaZulu-Natal, Pietermaritzburg

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By

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Submitted in fulfilment of the requirements for the degree of

Master of Science

Research Centre for Plant Growth and Development

School of Life Sciences

University of KwaZulu-Natal

Pietermaritzburg

December, 2017

STUDENT DECLARATION

Enhancing phenolic compound production in medicinal plants

I, Christine Susan Paine, student number: 209535131 declare that:

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

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DECLARATION BY SUPERVISORS

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

Students Full Name: Christine Susan Paine

Student Number: 209535131

Thesis Title: Enhancing phenolic compound production in medicinal plants

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

SUPERVISOR PROFESSOR J.F. FINNIE

CO-SUPERVISOR PROFESSOR J. VAN STADEN

COLLEGE OF AGRICULTURE, ENGINEERING AND SCIENCE

DECLARATION 1 - PLAGIARISM

I, Christine Susan Paine, student number: 209535131, declare that

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CONFERENCE CONTRIBUTIONS

C. Paine, J.F. Finnie, J. van Staden., 2017. Elicitation of phenolic compounds of medicinal value in plants. 43rd Annual Conference of the South African Association of Botanists (SAAB), University of the Western Cape, Bellville, Cape Town, South Africa (8-11 January 2017). Oral Presentation.

ACKNOWLEDGEMENTS

I would like to thank:

- Prof. J. F. Finnie, my supervisor. For helping me narrow down on a project when all I knew was that I wanted to research something in the line of plant biotechnology. For looking out for my mental state when everything seemed to be falling apart. For the advice given throughout my project, and ultimately, your enthusiasm for plant biotechnology research, the reason why I furthered my studies beyond an undergraduate degree, and why I found a field of research that I enjoy and thrive in.
- Prof. J. Van Staden, my co-supervisor. For allowing me to work in the Research Centre for Plant Growth and Development, which has not only provided the equipment that I required, but also exposed me to the world of scientific research. I am grateful for the opportunity to present my research at the RCPGD conferences. I thank you for constantly motivating and guiding my research, and for aiding my growth in the world of biological research.
- Dr Devashan Naidoo. The person who helped me with just about every aspect of my work. From calming me down during my numerous panic attacks, to laughing with me as I danced around each time my project moved in the right direction. Teaching me how to do the assays, helping me with my write up, reminding me of deadlines, and helping me with my stats late into the night, are just a few ways you helped me. I am forever grateful and in your debt for everything you have done.
- To my UKZN friends, for encouraging me throughout, and Mrs Alison Young, Chief Horticulturalist of the University Botanical Gardens, for the *Eucomis autumnalis* photos, and for all her help in the gardens.
- The National Research Foundation, Pretoria, for the funding they provided though the duration of my Masters studies.
- To my matric biology teacher, Ms Ellis for bringing biology to life. It has been said that teaching is a calling and I am eternally grateful to have had a teacher who inspired so much joy for biological sciences.
- Finally, to my friends and family for the constant support and understanding throughout. To my father, Anthony Paine, for insisting that I studied at a tertiary level. To my mother, Sharon Paine, words could not describe how much you have done for me; and finally to my sister and brother, Sandra and Jaco Knies, for the support and advice.

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LIST OF ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxy acetic acid
ANOVA	Analysis of variance
ATP	Adenosine tri-phosphate
BA	Benzyl adenine
CNB	Carbon / nitrogen balance hypothesis
COX	Cyclo-oxygenase
DNA	Deoxyribonucleic acid
DMRT	Duncan's multiple range tests
Folin-C	Folin-Ciocalteu
GAE	Gallic acid equivalents
GDBH	Growth-differentiation balance hypothesis
Hr	Hour
HSP	Heat-shock protein
IAA	Indole-3-acetic acid
IUCN	International Union for Conservation of Nature
KIN	Kinetin
LED	Light emitting diodes
Min	Minutes
MS	Murashige and Skoog
NAA	α-Naphthaleneacetic acid
ODT	Optimal defence theory
PAL	Phenylalanine ammonia lyase
PAR	Photosynthetically active radiation
PGR	Plant growth regulator
PIC	Picloram (4-Amino-3,5,6-trichloro-2-pyridinecarboxylic acid)
PPFD	Photosynthetic photon flux density
RCPGD	Research Centre for Plant Growth and Development
ROS	Reactive oxygen species
RPM	Revolutions per minute
SATM	South African Traditional Medicine
SD	Standard deviation

SE	Standard error
Sec	Seconds
SOD	Superoxide dismutase
UKZN	University of KwaZulu-Natal
UV	Ultraviolet
VOC	Volatile organic compounds
WHO	World Health Organization

ABSTRACT

Beyond the provision of nourishment, one of the most significant applications of plant physiological properties to human existence is their use in the production of medicinal compounds, with more than 50% of synthetically produced medications derived either directly or indirectly from these plant metabolites. The plants that produce these metabolites provide the readily available and inexpensive remedies utilized by South African traditional healers. Harvesting from wild populations lack legislative regulation, causing several plants to become threatened or endangered, thus it is essential that *ex-situ* methods of conservation be employed to ensure the sustainability of South African Traditional Medicine (SATM). *In vitro* cultivation under aseptic environmental conditions enables the mass production of genetically identical plantlets which are devoid of pathogens, developmental deformities, or physiological irregularities. Though it is not economically feasible to conserve all SATM plants by micro-propagation, these *in vitro* methods have extended our understanding of the physical, physiological, biochemical, and genetic components involved in the production of medicinal metabolites. Defined by their low levels throughout the plant, these metabolites accumulate at high concentrations in reserved cells or organs. The three distinct secondary metabolite groups differ in their chemical formation and biosynthetic pathway, yet their functions within the plant may overlap. Furthermore, the production of these metabolites in response to environmental stress is highly co-ordinated relative to the plants developmental phase, and the availability of limited resources.

Phenolic compounds are predominant in all higher plants, with the preservation of phenolic compounds through natural selection due to the versatility of their chemical formation and functional nature. These metabolites are prevalent in various plants of SATM, including *Eucomis autumnalis*, due to their anti-microbial, anti-inflammatory, antioxidant, and anti-carcinogenic capacity. *In vitro* micro-propagation of *E. autumnalis* has provided for the sustainable conservation of this over exploited Hyacinthaceae species. The intricate nature of phenolic compound chemical composition and biosynthetic pathways has curbed the synthetic production of these metabolites for commercial gain. Therefore, the induction of phenolic compound production by *in vitro* methods has been extensively studied.

Following photosynthesis, the shikimic acid pathway induces a deviation from the primary metabolism of carbon, resulting in the production of aromatic amino acids. Phenylalanine ammonia-lyase (PAL) exclusion of the ammonia results in the production of a variety of

phenolic compound derivatives, categorized as phenyl-propanoids. Thus, PAL serves as the breaching point between the primary and secondary metabolism, and is the determinant enzyme of the phenyl-propanoid pathway. The responsiveness of PAL activity to the effect of environmental alteration has promoted the development of various biotechnological methods which aim to optimize the production of these desired metabolites. Therefore, the aim of this dissertation was to enhance the production of phenolic compounds in response to environmental change, using *E. autumnalis* as a model for the potential application of these methods to other medicinally important plant species.

Provided that secondary metabolite production may be induced *in vitro* by the metabolic processes involved in cellular differentiation and specialization, *E. autumnalis* callus cultures were established under *in vitro* conditions. Solitary specialized plant cells produce a mass of undifferentiated plant tissue devoid of any organization. These simplified homogeneous callus cell formations develop in response to the reduction in function specific, morphological and developmental specialization of plant tissues. Callogenesis is dependent on plant growth regulators (PGR's) and light exposure. However, variation in auxin-to-cytokinin concentrations and the intensity of light exposure influence the rate of callus growth. Callus cultured on MS media supplemented with 2,4-Dichlorophenoxy acetic acid (2,4-D) produced greater volumes of callus over the eighty-four-day growth period. However, exposure to the high light intensity of 1.53 µmol m^{-2} s^{-1} resulted in the necrosis of callus cells within the eighty-four-day growth period. Furthermore, the addition of kinetin (KIN) increased the callus volume of 2,4-D cultures. On the other hand, the addition of KIN to picloram (PIC) cultures reduced the callus growth rate and volume. Overall, the optimum callus volume was obtained from cultures exposed to the lower light intensity of 0.75 µmol m^{-2} s^{-1}, though cultures exposed to 0.00 µmol m^{-2} s^{-1} had fewer incidences of callus culture browning. Though 2,4-D cultures produced greater volumes of callus, these cultures were mostly compact in their formation which restricted the inner cells from accessing oxygen, resulting in cell death. Furthermore, PIC cultures produced friable callus which is most advantageous for the proliferation of callus cultures.

These variations in the callus culture environment also influenced the production of phenolic compounds by *E. autumnalis* callus cultures. Subsequent to the eighty-four-day callus growth period, the phenolic content of the above mentioned callus cultures was determined using a modified Folin-C assay. In callus cultures which were exposed to 0.00 µmol m^{-2} s^{-1} light, PIC cultures produced a greater phenolic content than 2,4-D cultures with the concentration of

2,4-D having no significant effect on the phenolic content. Alternatively, the PIC concentration of 15 µM produced a significantly greater phenolic content than 10 µM and 20 µM PIC cultures, however the additional supplementation of 2,5 µM KIN reduced this influence of PIC concentration on the production of phenolic compounds. Furthermore, exposure of callus cultures to variations in the light intensity resulted in three main effects. First, an increase in light intensity resulted in a corresponding rise in phenolic production, where raised light intensities speed up the rate of carbon fixation by photosynthesis, providing ample quantities of carbon resources for phenolic production. Furthermore, the increase in light intensity implies that these cultures are exposed to higher quantities of UV radiation, thus enhancing the production of phenolic compounds which absorb these harmful UV wavelengths. However, the second main effect observed in these callus cultures implies that phenolic compounds are not the only secondary metabolites which are produced in response to elevated UV exposure. Several callus cultures produced a significantly greater phenolic content when exposed to the lower light intensity of 0.75 µmol m^{-2} s^{-1}, than cultures exposed to the higher 1.53 µmol m^{-2} s^{-1} light intensity. This is potentially due to the preferential production of alkaloid metabolites which absorb all wavelengths of the light spectrum, resulting in the limitation of carbon resources for phenolic production. Finally, the third main effect observed in *E. autumnalis* callus cultures was caused by an interaction between PGR and light intensity. The phenolic content of callus cultured on 15 µM PIC + 2,5 µM KIN and 20 µM PIC + 2,5 µM KIN supplemented MS media, which were exposed to light intensities of 0.00 µmol m^{-2} s^{-1} and 1.53 µmol m^{-2} s^{-1} produced greater phenolic contents than cultures exposed to 0.75 µmol m^{-2} s^{-1}. This is potentially due to the photo-degradation potential of synthetically produced auxin-like PGR's, as both 2,4-D and PIC should theoretically inhibit the production of secondary metabolites. Where PIC cultures exposed to the lower light intensity of 0.75 µmol m^{-2} s^{-1} produced a reduced phenolic content which was substantially increased by the photo-degradation of PIC at the higher light intensity of 1.53 µmol m^{-2} s^{-1}.

Though phenolic production is influenced by the environmental conditions of *in vitro* cultures, the induction of phenyl-propanoid pathway derived phenolic production may be enhanced by the acceleration of PAL activity in response to thermal stress. These phenolic compounds provide a short-term mechanism of thermo-tolerance. Thermal incline in plant cells has an adverse effect on the chloroplasts and mitochondrial enzymes which regulate the production and removal of reactive oxygen species (ROS). Metabolic processes accelerate

under thermal stress conditions, which result in the accumulation of ROS among other metabolites. ROS accumulation in the cell cytoplasm function as heat stress signals provoking the acceleration of PAL activity, which stimulates the production of scavenging enzymes. This amplification of PAL activity induces the production of phenyl-propanoid pathway derived phenolic compounds, which are quintessential to the acclimation of plant cells exposed to thermal stress, as these phenolic compounds increase the antioxidant capacity of plant cells. Callus samples and *in vitro* cultured plantlets were sub-cultured into suspension media supplemented with their respective PGR combinations. These suspension cultures and *in vivo* plants were exposed to thermal stress simulations of 30 ºC, 35 ºC, and 40 ºC. The accumulation of phenolic content in each sample was determined over a 20 hr period. Thermal incline enhanced the production of phenolic compounds in callus cultures and *in vitro* cultured plantlets, however once the thermal threshold of these cultures was breached this production of phenolic compounds was reduced. Furthermore, abiotic heat stress is determined by both thermal incline and the duration of exposure to the elevated temperature. Thermal incline induces the rapid production of phenolic compounds, subsequently reducing over time. Due to the ephemeral nature of this thermal stress mechanism, the production of phenolic compounds by *in vitro* cultured plantlet leaves becomes completely diminished following the initial surge in response to thermal incline. Alternatively, the phenolic content of callus cultures is reduced following the initial surge in production, however a low phenolic content is retained throughout the 20 hr exposure period, providing a mechanism of sustained thermo-tolerance. The level of thermo-tolerant phenolic production, and the rate at which this thermo-tolerance level is achieved varied between PGR combinations. However, both thermal incline beyond the cultures thermal threshold, and the additional supplementation of KIN resulted in reduced thermo-tolerance levels which were obtained rapidly. Callus cultures maintained a relatively constant production of phenolic compounds in response to thermal stress, while the phenolic content produced by the leaves of *in vitro* cultured plantlets duplicated that of the callus cultures. Finally, the leaves of whole *in vivo* plants demonstrated a lack of significant correlation between phenolic compound production and thermal stress.

CHAPTER 1: LITERATURE REVIEW

1.1. Medicinal properties of plants

Plants serve as the primary producers of the hierarchical food chain, offering all life forms a seemingly infinite supply of nourishment (**WALLACE *et al.*, 1996a**), in the form of food and drink (**SCHRIPSEMA *et al.*, 1996**). However, humans have relied on plants for centuries beyond the scope of their provision as primary producers (**SIDHU, 2010**). In addition to the use of plant fibres within human and animal diets, the physiological components of some plants have resulted in their use for the production of various materials, including their incorporation into construction supplies (**SCHRIPSEMA *et al.*, 1996**). Industries have also explored the pigments and chemical elements of plants for their aroma, taste, and potential for harm against both humans and agricultural pests (**SCHRIPSEMA *et al.*, 1996**). One of the most significant applications of plant physiological properties to human existence, other than the provision of nourishment, is their use in the production of medicinal compounds (**SCHRIPSEMA *et al.*, 1996**; **SIDHU, 2010**). The first use of medicinal plants by humans is estimated to have been approximately 60 000 years ago according to palaeontological studies (**ELGORASHI *et al.*, 2003**). Plants are essential donors toward global health (**CALIXTO, 2000**) accommodating the primary healthcare of almost 80% of the global populace (**AFOLAYAN and ADEBOLA, 2004**). Despite the majority of western or modern medicinal drugs being produced synthetically in the laboratory (**CALIXTO, 2000**), over 50% of these are either derived or pure natural commodities (**ELDEEN *et al.*, 2005**). Furthermore, approximately 25% of these drugs are derived either directly or indirectly from natural plant products (**CALIXTO, 2000**; **ELDEEN *et al.*, 2005**). Though synthetic chemistry, combinatorial, and molecular modelling to improve synthetic medication development has been extensively researched lately; the use of plant medicinal metabolites for human health care remains invaluable (**NCUBE *et al.*, 2012a**). Furthermore, research regarding medicinal plants has surpassed that of traditional medicines since the 1980's, during which time the harvesting of metabolites, of pharmaceutical importance, has promoted numerous innovations in production technology of medicinal significance (**SIDHU, 2010**).

1.2. South African Traditional Medicine

In South Africa medicinal plants provide the needs for approximately twenty-seven million people (**FENNELL *et al.*, 2004b**; **STREET *et al.*, 2008b**). Believed to be the origin of modern

man, southern Africa boasts an extensive account of how plants have been utilized by humans (**NIGRO *et al.*, 2004**), which influenced various aspects of the African culture and tradition significantly (**FENNELL *et al.*, 2004b**). In addition to the cultural importance of traditional remedies, these plants aid in providing readily available and inexpensive treatments (**FENNELL *et al.*, 2004a**; **STREET *et al.*, 2008b**). Though these traditional methods may be the only affordable or available remedies found in the rural sectors of southern Africa (**JÄGER and VAN STADEN, 2000**), approximately 60% of the South African population (**ELGORASHI *et al.*, 2003**; **NCUBE *et al.*, 2011**; **TAYLOR *et al.*, 2003**), and 80% of urban residing African people, particularly people of Zulu heritage, will confer with a traditional healer following a consultation with a medical physician of modern or western training (**ELGORASHI *et al.*, 2003**; **JÄGER *et al.*, 1996**). This has resulted in an estimated 350 000 people employed in traditional healing practices greatly exceeding the 250 000 people employed by western medicinal practices (**JÄGER and VAN STADEN, 2005**). The World Health Organisation (WHO) determined that traditional medicine methods are extensively dependent on the passing of information (**RUKANGIRA, 2001**), gathered over many years (**TAYLOR *et al.*, 2003**), onto the next generation (**RUKANGIRA, 2001**), ensuring that tradition remains and thus being of greater cultural and spiritual significance (**TAYLOR *et al.*, 2003**). Given that such a large portion of the South African population favour traditional healing methods over modern medications (**FENNELL *et al.*, 2004b**; **STREET *et al.*, 2008b**) the growing concern for the conservation of these plants used by traditional healers is not surprising. Per annum, South Africans harvest approximately 19 500 tonnes of medicinal plants from their natural habitats (**STREET *et al.*, 2008a**), resulting in these natural medicinal sources becoming severely reduced due to their extensive exploitation (**AFOLAYAN and ADEBOLA, 2004**; **FENNELL *et al.*, 2004a**). Rural communities of southern Africa benefit greatly from traditional healing methods beyond the facility of medicinal and spiritual therapy, as the gathering of medicinal plants among other natural resources for medicinal markets also provides employment for many (**AFOLAYAN and ADEBOLA, 2004**). The absence of co-ordination and constraint in the collection of medicinal plants (**AFOLAYAN and ADEBOLA, 2004**; **MOYO *et al.*, 2011**) has resulted in the haphazard gathering of plants from their natural habitats (**AFOLAYAN and ADEBOLA, 2004**) with little effort put toward sustaining these wild populations (**MOYO *et al.*, 2011**).

1.3. The significance of medicinal plants

The extinction of a plant species is subject to a combination of ecological, environmental and anthropogenic pressures (**NCUBE *et al.*, 2015**). Given the majority of South African medicinal plants are harvested (**NIGRO *et al.*, 2004**), devoid of regulation or restraint (**MOYO *et al.*, 2011**), from wild populations (**NIGRO *et al.*, 2004**) it is essential that methods of conservation be employed to alleviate this reliance on naturally occurring populations (**AFOLAYAN and ADEBOLA, 2004**). *In-situ* methods of conservation are based on maintaining the species within its natural habitat (**KASAGANA and KARUMURI, 2011**). However, the implementation of current legislation regarding the control of wild population harvesting in South Africa remains deficient (**AFOLAYAN and ADEBOLA, 2004**; **MOYO *et al.*, 2011**). Regardless of these anthropogenic pressures, limitations of the transformed ecosystem are enforced, resulting in the tedious attempt of habitat restoration (**WOCHOK, 1981**). Thus, *in-situ* methods are only suitable for plant species that are threatened but not yet in danger of extinction. Alternatively, *ex-situ* conservation is preferable for endangered species, as this incorporates various methods of preserving the genetic diversity of the species and / or the population independent of its natural environment (**KASAGANA and KARUMURI, 2011**). In addition to the establishment of botanical gardens, and gene banks, these methods involve the storage of DNA, pollen, and seeds (**KASAGANA and KARUMURI, 2011**; **NIGRO *et al.*, 2004**). However, micro-propagation in terms of *in vitro* plantlet establishment, is paramount to the *ex-situ* conservation of plants (**AFOLAYAN and ADEBOLA, 2004**; **MOYO *et al.*, 2011**; **NCUBE *et al.*, 2015**; **NIGRO *et al.*, 2004**).

In vitro plant tissue propagation is the establishment of plantlets within an antiseptic environment (**KASAGANA and KARUMURI, 2011**). Successful cellular proliferation (**SIDHU, 2010**) is dependent on the regulation of various environmental factors (**KOZAI *et al.*, 1997**), including temperature, pH, and the quantity of nutrients (**SIDHU, 2010**). Culturing under aseptic conditions enables mass production of genetically identical plantlets that are devoid of pathogens and do not bear any developmental or physiological abnormalities (**HUSSAIN *et al.*, 2012**; **KASAGANA and KARUMURI, 2011**; **KOZAI *et al.*, 1997**). Cultivation in sterilized vessels diminishes the risk of contamination, further permitting their transportation and storage (**KASAGANA and KARUMURI, 2011**). Additionally, the rapid growth rate and acclimatization of *in vitro* cultures (**KASAGANA and KARUMURI, 2011**; **KOZAI *et al.*, 1997**) have provided an efficient method for the conservation and growth of various plant species (**NCUBE *et al.*, 2015**).

The benefits of *in vitro* plant tissue culture surpass those of traditional vegetative reproduction (**KOZAI *et al.*, 1997**). Micro-propagation enables the conservation of plant populations that face certain constraints which vegetative reproduction methods could not accommodate (**KASAGANA and KARUMURI, 2011**). These include plant populations where the probability of seed germination and growth is negligible (**ABOEL-NIL, 1997**; **KASAGANA and KARUMURI, 2011**), where there is a deficiency of seeds (**KASAGANA and KARUMURI, 2011**; **WOCHOK, 1981**), or where there is a lack of appropriate pollinator availability for the species (**KASAGANA and KARUMURI, 2011**).

Plant tissue culture has been implemented within various industries including forestry, ornamental markets, horticulture, and agriculture (**WOCHOK, 1981**), paying particular attention to crops of commercial value (**ABOEL-NIL, 1997**). However, given that private industries have undertaken the majority of these efforts (**WOCHOK, 1981**), research toward medicinal plant micro-propagation has lagged behind that of plant species which are more economically valuable to these businesses (**ABOEL-NIL, 1997**). Globally, these industries have focused primarily on the use of micro-propagation techniques for the mass production of whole plantlets (**HUSSAIN *et al.*, 2012**; **NIGRO *et al.*, 2004**), providing the foundation for further plant biotechnological research (**MATKOWSKI, 2008**; **NIGRO *et al.*, 2004**; **ROUT *et al.*, 2000**).

The study and use of micro-propagation methods for the conservation of economically significant plants in South Africa has been extensive (**MOYO *et al.*, 2011**), relative to improving medicinal plant market values in addition to *ex-situ* preservation of the plants (**NIGRO *et al.*, 2004**). It is believed, that *in vitro* mass production of medicinal plants in South Africa could alleviate the detrimental harvesting of entire natural populations by providing an alternative and sustainable source of these plants for traditional medicine markets (**AFOLAYAN and ADEBOLA, 2004**). However, the feasibility of implementing such methods of conservation to all South African medicinal plants remains limited (**KOZAI *et al.*, 1997**). In 2009, the IUCN Red List assessment determined that roughly 10% (2 062 plant species) of South African indigenous vascular plants are used by traditional healers (**WILLIAMS *et al.*, 2013**). Slow plantlet growth rates and high acclimatization mortality, in addition to workforce expenses discourage the use of *in vitro* methods for the mass production of South African medicinal plants (**KOZAI *et al.*, 1997**). Thus conservation efforts need to concentrate on indigenous species that are under threat of extinction, of which eighty-two species are of high conservation concern, ninety-eight species occurring in their natural habitats, and two species

that have been eliminated from their natural habitats (**WILLIAMS *et al.*, 2013**). Provided that *in-situ* methods of conservation cannot be applied to all of these threatened species, conservation efforts need to be established *ex-situ* (**KASAGANA and KARUMURI, 2011**). Bulk production of plantlets by *in vitro* methods may not be economically viable for all species (**KOZAI *et al.*, 1997**), though an effective establishment of micro-propagation procedures have enabled further investigations into the plants physical, physiological, biochemical and genetic constituents (**NCUBE *et al.*, 2015**).

Further investigation of these physical, physiological, biochemical and genetic elements has led to the discovery and continued study of the metabolites produced (**MOYO *et al.*, 2011**; **NCUBE *et al.*, 2015**; **NIGRO *et al.*, 2004**; **ROUT *et al.*, 2000**). These may be derived from the metabolic processes involved in cellular differentiation and specialization *in vitro* (**NCUBE *et al.*, 2015**), though their production is not limited to *in vitro* conditions, as they are also synthesized *in vivo* in response to environmental or ecological strain (**TAIZ and ZEIGER, 2010**). In South African research, plant biotechnological tools have focused on conservation *in vitro*, and the production of medicinally valuable metabolites (**MOYO *et al.*, 2011**), particularly from the medicinally utilized Hyacinthaceae (**AFOLAYAN and ADEBOLA, 2004**; **STREET *et al.*, 2007**).

1.4. *Eucomis autumnalis* in South African Traditional Medicine

Some of the most frequently utilized medicinal plants of southern Africa include the bulbous plants (**ZSCHOCKE *et al.*, 2000**) of the family Hyacinthaceae (**CHEESMAN *et al.*, 2010**; **STREET *et al.*, 2007**). Providing an array of bioactive metabolites (**LOUW *et al.*, 2002**) approximately 14% of plants sold in South African medicinal plant markets are of the Hyacinthaceae family (**AFOLAYAN and ADEBOLA, 2004**). Fourteen species of the Hyacinthaceae family have been identified to contain biological properties pertaining to the treatment of urinary or venereal diseases, gastro-intestinal diseases, respiratory infections, headaches and fever, swellings or growths and joint pains, and skin, bruises, sprains and fractures (**LOUW *et al.*, 2002**). Though the medicinal potential of various Hyacinthaceae species still requires further investigation (**LOUW *et al.*, 2002**), species of the *Eucomis* genus have been investigated extensively with regard to their medicinal metabolites (**AULT, 1995**; **CHEESMAN *et al.*, 2010**; **MOYO *et al.*, 2011**).

Previously belonging to the family Liliaceae (**LOUW *et al.*, 2002**), the categorisation of various Hyacinthaceae species overlap in their taxonomy (**SPARG *et al.*, 2002**). For example,

the *Eucomis* genus has been classified within the Hyacinthaceae family (**AFOLAYAN and ADEBOLA, 2004**; **MOYO *et al.*, 2011**) however, the species *Eucomis autumnalis* falls under the family Asparagaceae (**NDHLALA *et al.*, 2012**). The majority of *Eucomis* species that have been documented originate from southern Africa (**LOUW *et al.*, 2002**; **TAYLOR and VAN STADEN, 2001a**, **2001b**), including the subspecies, *Eucomis autumnalis autumnalis*, *E. autumnalis amaryllidifolia*, and *E. autumnalis clavata* (**TAYLOR and VAN STADEN, 2001b**) which are all exploited in South African traditional medicine relative to their local availability (**TAYLOR and VAN STADEN, 2001c**).

The vertical raceme of compact yellow-green, pale-green (**Figure 1.1 C**), or white flower arrangement (**CHEESMAN *et al.*, 2010**; **LOUW *et al.*, 2002**; **TAYLOR and VAN STADEN, 2001b**, **2001c**, **2001d**) capped with green bracts mirror the appearance of pineapples, resulting in *Eucomis* plants being frequently referred to as Pineapple lilies (**LOUW *et al.*, 2002**; **NDHLALA *et al.*, 2012**; **TAYLOR and VAN STADEN, 2001b**, **2001d**). Additionally, the Greek origins of the genus' label, *Eucomis*, describes the crown of bracts atop the inflorescence (**LOUW *et al.*, 2002**; **TAYLOR and VAN STADEN, 2001b**). These inflorescence stalks (**Figure 1.1 B**) are protruding from a rosette of elongated leaves (**NDHLALA *et al.*, 2012**; **TAYLOR and VAN STADEN, 2001d**) which are both generated by concentrically layered (**Figure 1.1 A**), egg-shaped bulbs (**CHEESMAN *et al.*, 2010**; **NDHLALA *et al.*, 2012**; **TAYLOR and VAN STADEN, 2001b**), located below the soil surface (**TAYLOR and VAN STADEN, 2001b**).

Figure 1.1: The morphology of *Eucomis autumnalis* plant features, (**A**) concentrically layered bulb, (**B**) inflorescence stalk protruding from a rosette of elongated leaves, (**C**) vertical raceme of white-green flowers topped by a crown of green bracts. (Photos **B** and **C** were provided by Mrs Alison Young, Chief Horticulturalist of the University of KwaZulu-Natal botanical gardens).

These deciduous geophytes (**AFOLAYAN and ADEBOLA, 2004**; **AULT, 1995**; **LOUW *et al.*, 2002**; **NDHLALA *et al.*, 2012**; **TAYLOR and VAN STADEN, 2001b**, **2001d**), remain dormant during the winter months following their summer season growth phase (**AULT, 1995**; **TAYLOR and VAN STADEN, 2001b**, **2001c**, **2001d**). Requiring nutrient rich soils and either

complete or partial exposure to solar radiation (**TAYLOR and VAN STADEN, 2001c**) *Eucomis* species thrive in the moderately moist grasslands of southern Africa (**NDHLALA *et al.*, 2012**; **TAYLOR and VAN STADEN, 2001b**). However, excessive harvesting of these plants has restricted their availability (**TAYLOR and VAN STADEN, 2001b**, **2001d**; **ZSCHOCKE *et al.*, 2000**) to isolated grassland regions of higher elevations of the Drakensberg along South Africa's north-eastern coast line (**TAYLOR and VAN STADEN, 2001b**). Though South African legislature aims to secure the sustainability of all Liliaceae (**TAYLOR and VAN STADEN, 2001c**), various *Eucomis* species rank as threatened (**TAYLOR and VAN STADEN, 2001d**). Given that *Eucomis autumnalis* (subspecies *autumnalis*) is targeted the most by South African traditional healers (**CHEESMAN *et al.*, 2010**; **TAYLOR and VAN STADEN, 2001a**; **ZSCHOCKE *et al.*, 2000**), unregulated and uncontrolled harvesting of wild populations has resulted in their declining status of conservation (**DZEREFOS and WITKOWSKI, 2001**; **NDHLALA *et al.*, 2012**; **TAYLOR and VAN STADEN, 2001c**). The collection of entire plants or solely the bulbs (**AFOLAYAN and ADEBOLA, 2004**; **DZEREFOS and WITKOWSKI, 2001**; **ZSCHOCKE *et al.*, 2000**) provide approximately seventy-three tonnes of *Eucomis autumnalis* material to "muthi" markets annually (**STREET *et al.*, 2007**).

Various attempts have been made by subsistence farmers to grow these plants as a substitute source for South African "muthi" markets (**TAYLOR and VAN STADEN, 2001d**). However, cultivation from seeds is a tedious process requiring a minimum of three years for the plants to achieve maturity, thus *in vitro* methods provide a more rapid means of bulk cultivation (**TAYLOR and VAN STADEN, 2001c**). Beyond the mass production of vulnerable plant species, plant biotechnological tools can be exercised for the production of desired medicinal metabolites (**ROUT *et al.*, 2000**).

1.5. Secondary metabolites in plants

These compounds of pharmaceutical value are derived from the plants secondary metabolic pathways (**GIULIETTI and ERTOLA, 1999**; **ROUT *et al.*, 2000**). Plants produce an assortment of organic compounds that do not appear to serve toward the plant's primary functions (**NCUBE *et al.*, 2012b**; **TAIZ and ZEIGER, 2010**), such as the division and elongation of cells, storage, respiration, and propagation (**BOURGAUD *et al.*, 2001**).

The definition of secondary metabolites has been investigated and altered over the years (**VERPOORTE, 2000**). Initially known as metabolites which oppose the primary metabolism, they were later thought to be the 'end-products' of nitrogen metabolism (**BOURGAUD *et al.*,**

2001; **GIULIETTI and ERTOLA, 1999**). However, it is currently understood that not all secondary metabolites are derived from nitrogen based metabolic pathways (**TAIZ and ZEIGER, 2010**). More recently, these metabolites were distinguished by their features (**BOURGAUD *et al.*, 2001**). These compounds are highly concentrated (**WINK, 1999**) yet they generally have diminished abundances (**BOURGAUD *et al.*, 2001**; **WINK, 1999**). Believed to be waste products for a period of time (**VERPOORTE, 2000**; **WINK, 1999**) it would be anticipated for these metabolites to be stored in senescent organs or cells, given that plants lack the ability to excrete waste products (**WINK, 1999**). However, these metabolites are produced and stored in reserved organs or cells (**BOURGAUD *et al.*, 2001**; **GIULIETTI and ERTOLA, 1999**). Furthermore, by definition waste products are only synthesized following primary metabolic processes (**WINK, 1999**), however, these metabolites are synthesized, undergo transformation and are accumulated throughout various developmental stages (**GIULIETTI and ERTOLA, 1999**). These metabolites were later defined as products or intermediates derived from various primary metabolites (**NCUBE *et al.*, 2012b**; **VERPOORTE, 2000**) by a greater array of pathways that deviate from primary metabolism (**VERPOORTE, 2000**), which entails the biosynthesis of proteins, lipids, and carbohydrates (**TAIZ and ZEIGER, 2010**).

Where primary metabolism remains constant throughout the plant kingdom, secondary metabolites differ among plant genera, and occasionally between species (**TAIZ and ZEIGER, 2010**; **VERPOORTE, 2000**). Biosynthesis of these metabolites was initially thought to be inadvertent (**GIULIETTI and ERTOLA, 1999**) however, these metabolic processes that deviate from the plants primary metabolism (**NCUBE *et al.*, 2012b**; **TAIZ and ZEIGER, 2010**) are substantially co-ordinated relative to the plants development (**NCUBE *et al.*, 2012b**). These secondary metabolites are classified, based on their chemical formation, into three categories consisting of terpenes, nitrogen-containing compounds and phenolic compounds (**TAIZ and ZEIGER, 2010**). Beyond the fundamental aspects of plant growth and reproduction (**TAIZ and ZEIGER, 2010**) these compounds function toward protecting the plant, and attracting pollinators (**TAIZ and ZEIGER, 2010**; **WINK, 1999**). Sexual reproduction in flowering plants requires pollination and dispersal by wind or more commonly by insect pollinators (**WALLACE *et al.*, 1996b**). Various secondary compounds serve as visual cues for pollinators in the form of pigments (**BOURGAUD *et al.*, 2001**) and ultraviolet (UV) guides (**WALLACE *et al.*, 1996b**). Several secondary metabolites possess elements capable of retaining UV radiation (**BOURGAUD *et al.*, 2001**) serving a dual functionality in protecting the plant against the harmful effects of UV radiation (**WINK, 1999**) and in attracting pollinators such as bees

(**WALLACE *et al.*, 1996b**). Though the three categories of secondary metabolites differ in their chemistry, each category is not limited in their function (**TAIZ and ZEIGER, 2010**). For example, terpene compounds of *Arabidopsis thaliana* flowers serve toward pollinator attraction, however these terpenes also function in anti-herbivory protection by means of volatile mixtures (**KLIEBENSTEIN, 2004**). Additionally, the defensive capacity of these metabolites provide protection against further damage by herbivores and microbial organisms (**TAIZ and ZEIGER, 2010**; **WINK, 1999**).

1.5.1. Secondary metabolite response to stress

Based on the study of secondary metabolite composition and function, these metabolites have been further defined as those that secure an organisms survival, serving an interactive function between the organism and the surrounding environment (**NCUBE *et al.*, 2012b**; **VERPOORTE, 2000**). The stationary nature of plants prevents them from fleeing when environmental influences become less than favourable for their primary function of plant growth (**NCUBE *et al.*, 2013a**). Thus, secondary metabolites are believed to be essential to the plants ability to adjust to their ever changing environment (**MOYO *et al.*, 2011**; **NCUBE *et al.*, 2012b**). Secondary metabolites function in plant defence against biotic (**WINK, 1999**) and abiotic environmental stresses (**NCUBE *et al.*, 2012b**).

Chemical defence mechanisms have been inherited by plants (**MOYO *et al.*, 2011**; **TAIZ and ZEIGER, 2010**), subject to natural selection and evolutionary processes (**TAIZ and ZEIGER, 2010**). This evolutionary 'arms race' persists due to the diverse variation in the secondary metabolite profiles (**WINK, 1999**). Production of these defensive secondary metabolites put a high demand on the plants limited resources (**CRONIN and HAY, 1996**), thus allocation of resources is substantially co-ordinated, relative to when and where these chemical defenders are synthesized and stored within the plant (**WINK, 1999**). For example, cyanogenic glycosides discourage insect and herbivore feeding by producing toxic hydrogen cyanide gas when broken down (**TAIZ and ZEIGER, 2010**). However, cyanogenesis is substantially demanding on nitrogen resources, therefore when soil nutrition is limited, plants alternatively allocate carbon, rather than nitrogen resources, to secondary defence metabolite production (**NCUBE *et al.*, 2012b**). The two leading concepts of plant chemical defence are the optimal defence theory (ODT) and the growth-differentiation balance hypothesis (GDBH) (**CRONIN and HAY, 1996**). Given that the primary objective is to defend tissues that are vulnerable to herbivory or microbial infection (**WINK, 1999**), ODT motivates for the preferential protection

of developing tissues over that of the older specialized tissues, that are favoured by the GDBH (**CRONIN and HAY, 1996**). Though juvenile tissues contribute significantly to plant fitness (**WINK, 1999**), the biosynthesis of these defence chemicals is limited to dedicated cells of differentiated tissues (**GIULIETTI and ERTOLA, 1999**). Furthermore, despite the greater abundance of secondary metabolites often occurring in juvenile developing plant tissues, as suggested by ODT, this does not oppose the GDBH (**CRONIN and HAY, 1996**), as these metabolites are often synthesized within specialized cells and transferred via the phloem throughout the plant (**CRONIN and HAY, 1996**; **WINK, 1999**). However, translocation and concentration of these defensive metabolites in juvenile tissues becomes detrimental to the plants growth and development when environmental stress is lacking (**CRONIN and HAY, 1996**). The transport of these metabolites through the phloem (**WINK, 1999**), provides a mode of metabolite transfer between specialized organs (**CRONIN and HAY, 1996**), subsequently guarding these plants from the harmful effects of sucking insects (**WINK, 1999**).

Plant defence against biotic stress consists of three key mechanisms that are prompted by tissue wounding by herbivores, and / or microbial infection (**WINK, 1999**). The first consists of boosting the production and storage of defensive metabolites that are present (**WINK, 1999**). These are often located in external organs of the plant, and provide instant release of deterrents (**WINK, 1999**), though some of these potentially prevent wounding or infection by means of advertising their toxicity in glandular hairs (**TAIZ and ZEIGER, 2010**). The second is predominantly induced by microbial infection, giving rise to novel metabolites that possess anti-microbial properties (**WINK, 1999**), many of which are iso-flavonoids (**TAIZ and ZEIGER, 2010**), however the efficacy of these novel metabolites is not limited to microbial deterrence (**WINK, 1999**). The third entails the combination of hydrolysing enzymes and pre-fabricated allelo-chemicals by means of the wound induced breakdown of specialized tissues (**WINK, 1999**). This activation of allelo-chemicals, often results in the release of plant volatiles that either attract natural enemies of the intruder, or signal response mechanisms in surrounding plants (**WINK, 1999**).

Allelo-chemicals also participate in response to various abiotic environmental stresses relative to competition between plants (**TAIZ and ZEIGER, 2010**). Several phenolic compounds, may be released into the soil (**TAIZ and ZEIGER, 2010**) in attempt to impede the germination and development of seeds (**BOURGAUD *et al.*, 2001**; **WINK, 1999**), or alternatively to poison another plant (**BOURGAUD *et al.*, 2001**). This allelopathic behaviour in

plants is essential to their evolutionary fitness in the plants battle for access to limited light, nutrient, and water resources (**TAIZ and ZEIGER, 2010**).

The ability to sense, signal, and respond to abiotic stress (**DE KLERK, 2007**; **NCUBE *et al.*, 2013b**) is exhibited throughout the plant kingdom, however the susceptibility and mode of response to climatic pressures differ between plant species (**NCUBE *et al.*, 2013b**). Stress intensity in conjunction with the plants genotype and growth phases are the primary components that determine the manner in which the plant identifies and responds to environmental stress (**AGARWAL and ZHU, 2005**). Various anatomical and physiological alterations in plants have evolved, including the adaptation of life cycles and the production of seeds and buds that are capable of dormancy, to facilitate the plants survival during anticipated periods of unfavourable environmental conditions, such as drought or frigid winters (**DE KLERK, 2007**). Alternatively, cross-linked pathways stimulate physiological (**NCUBE *et al.*, 2013b**) and biochemical reactions in plants that are subjected to sudden abiotic pressures (**DE KLERK, 2007**), resulting in the generation of defensive secondary metabolites (**DE KLERK, 2007**; **NCUBE *et al.*, 2013b**). Distinct abiotic stress factors, stimulate explicit modification of metabolic processes that are pertinent in the plants response to the distinct abiotic stress (**AGARWAL and ZHU, 2005**). Climatic or abiotic environmental stress response mechanisms, primarily affect the quantity and quality of the secondary metabolites that are produced (**NCUBE *et al.*, 2012b**).

Though various secondary metabolites are produced in response to each of these environmental stress factors (**NCUBE *et al.*, 2012b**), these different forms of abiotic stress are frequently inflicted on plants in a concurrent or simultaneous manner (**AGARWAL and ZHU, 2005**). Thus, plants have developed intricate stress signalling pathway complexes that intersect within the stem nodes, potentially affecting the plants abiotic stress perception capability (**AGARWAL and ZHU, 2005**). Though the various above mentioned abiotic stress factors may induce the production of factor specific secondary metabolites, each of these stress factors are known to result in the production of reactive oxygen species (ROS) (**NCUBE *et al.*, 2012b**). However, in addition to this accumulation of ROS, the secondary metabolites produced in response to these abiotic stress factors function in the scavenging of ROS (**DE KLERK, 2007**).

Given that the fixation of carbon during photosynthesis is essential to the livelihood of plants, the ability to detect various ranges of the light spectrum was acquired by plants (**NCUBE *et***

***al.*, 2012b**). However, various wavelengths of UV radiation are harmful to the plants photosynthetic processes (**BOURGAUD *et al.*, 2001**), thus the biosynthesis of several secondary metabolites have been established to deter the effects of destructive UV wavelengths (**NCUBE *et al.*, 2012b**; **WINK, 1999**). Exposure to severe light intensities and/or UV radiation (**NCUBE *et al.*, 2012b**; **TAIZ and ZEIGER, 2010**), influences the production of various secondary metabolites (**NCUBE *et al.*, 2012b**). These metabolites absorb both harmful UV and functional wavelengths (**NCUBE *et al.*, 2012b**; **TAIZ and ZEIGER, 2010**). For example; selected tetra-terpene compounds are produced in cells capable of photosynthesis as auxiliary pigments (**TAIZ and ZEIGER, 2010**) that prevent photo-oxidation (**SINHA, 2004**; **TAIZ and ZEIGER, 2010**) in chloroplasts by absorbing surplus radiation (**SINHA, 2004**). Furthermore, the production of several phenolic compounds in epidermal cells corresponds with amplified exposure to UV-B radiation, indicating that biosynthesis of these metabolites is induced by light stress (**TAIZ and ZEIGER, 2010**). These compounds include the furocoumarins (**BOURGAUD *et al.*, 2001**) and flavonoids of the plant stems and leaves (**TAIZ and ZEIGER, 2010**). In addition to this stress imposed on the plants (**NCUBE *et al.*, 2012b**), UV radiation is involved in the formation of ozone (O_3) (**WALLACE *et al.*, 1996c**). Exposure to high quantities of ozone has opposing effects on the different groups of secondary metabolites, where phenolic content is reduced, terpene is amplified (**NCUBE *et al.*, 2012b**).

Soil stress involves several interacting properties (**RAMÍREZ-RODRÍGUEZ *et al.*, 2005**), with the levels of chemical precursors in the soil controlling secondary metabolite production (**NCUBE *et al.*, 2012b**; **RAMÍREZ-RODRÍGUEZ *et al.*, 2005**). Certain metabolites are expelled into the rhizosphere, influencing the selective absorption of nutrients in addition to improving the nutrients ability to dissolve (**RAMÍREZ-RODRÍGUEZ *et al.*, 2005**). These metabolites prevent the uptake of phyto-toxic heavy metals (**DE KLERK, 2007**; **RAMÍREZ-RODRÍGUEZ *et al.*, 2005**), while improving the solubility of deficient nutrients (**RAMÍREZ-RODRÍGUEZ *et al.*, 2005**). For example, variations in soil nitrogen content have demonstrated different secondary metabolite responses (**MOYO *et al.*, 2011**). Provided primary metabolic functions of growth is prioritized above secondary metabolic processes, the concentration of certain metabolites regulates secondary metabolite biosynthesis (**NCUBE *et al.*, 2012b**).

Environments devoid of moisture impose multiple stress factors on plants, commonly associated with salinity stress (**NCUBE *et al.*, 2013b**), and generally triggered by thermal pressures, where elevated temperatures may result in drought (**NCUBE *et al.*, 2012b**). Furthermore salinity stress induced by osmotic stress promotes ROS production thus

inflicting oxidative stress (**BOTELLA *et al.*, 2005**; **MOYO *et al.*, 2011**). Plants incorporate multiple metabolic response mechanisms protecting against UV radiation damage and desiccation (**NCUBE *et al.*, 2012b**), predominantly by phenolic compounds (**DE KLERK, 2007**; **NCUBE *et al.*, 2012b**, **2013b**).

Abiotic heat stress in plants has a significant negative effect on agricultural and natural environments (**WANG *et al.*, 2003**), with marked consequences for the global production of crops (**HALL, 2001**) and medicinal plants used by South African traditional healers. Heat tolerance can be defined by a plants ability to produce developmental and economic yield under high temperatures; however heat stress is determined by the intensity, temporal extent, and the rate at which the temperature increases, as a multifaceted event (**WAHID *et al.*, 2007**). Plant heat stress tolerance mechanisms fluctuate relative to the tissue structure and consist of long-term adaptations of an evolutionary base, and short-term avoidance or temporary adaptations (**WAHID *et al.*, 2007**). Chronic exposure of plants to heat stress provokes various morphological, anatomical, biochemical, phenological, physiological, and molecular responses (**NCUBE *et al.*, 2012b**; **WAHID *et al.*, 2007**). Morphological amendments prompted by high temperatures directly influence the prevailing physiological processes, indirectly transforming the direction of developmental processes (**WAHID *et al.*, 2007**). This is caused by the inhibition of cellular elongation, the localization of cellular division stimulation, and the adjustment to cellular differentiation status (**POTTERS *et al.*, 2007**). Crop yield is diminished due to the discernible effect of pro-longed heat stress on reproductive activities, though the extent of such modifications may vary with the phenological phase and plant species (**WAHID *et al.*, 2007**). The gravity of potential damage to crops (**WAHID *et al.*, 2007**), and medicinal plants may be determined by the phenological phase at which the plant is exposed to heat stress (**WAHID *et al.*, 2007**). The sessile nature of plants restricts heat stress responses, thus heat stress tolerance is dependent on systems of cellular and physiological acclimation and defence (**WAHID *et al.*, 2007**). The restoration of damaged tissue, protection and homeostasis; post heat stress event; are resultant of signalling processes and transcription controls which are activated by means of primary stress signals (**WAHID *et al.*, 2007**). Heat stress manipulates energy distribution and carbon metabolism enzyme activities, reducing the photosynthetic competence of C_3 plants more than C_4 plants (**WAHID *et al.*, 2007**). Due to chlorophyll *a* and *b* degradation in the chloroplast stroma, carbon metabolism is reduced more in young leaves than fully developed leaves (**WAHID *et al.*, 2007**).

Declining thermal conditions stimulate phenolic production and integration into the plant cell walls (**NCUBE *et al.*, 2012b**; **RIVERO *et al.*, 2001**; **WAHID *et al.*, 2007**) as a mechanism of acclimation (**RIVERO *et al.*, 2001**). Conversely, thermal incline prompts the generation of ROS (**DE KLERK, 2007**; **NCUBE *et al.*, 2012b**) inducing atmospheric photochemical reactions of volatile organic compounds (VOC's), which stimulate the activity of enzymes involved in ROS scavenging processes (**NCUBE *et al.*, 2012b**). These VOC's include isoprenoids which are discharged from the plant leaves, protecting photosynthetic processes and organelles from the harmful effects of ROS (**WAHID *et al.*, 2007**). For instance, the vulnerability of thylakoid membranous lipids to ROS injury is diminished by photo-protective carotenoids that enable the compaction of membranous lipids to prevent absorption of harmful compounds into the thylakoids (**WAHID *et al.*, 2007**).

Secondary metabolites are categorized by their biosynthetic pathways (**BOURGAUD *et al.*, 2001**), due to the deviation of particular enzymes from the primary metabolism of carbon (**BALASUNDRAM *et al.*, 2006**; **TAIZ and ZEIGER, 2010**). These chemical compounds can be divided into three main functional groups (**BOURGAUD *et al.*, 2001**; **NCUBE *et al.*, 2012b**; **TAIZ and ZEIGER, 2010**). The three principle groups are terpenes, phenolic compounds and nitrogen-containing compounds, which include alkaloids (**TAIZ and ZEIGER, 2010**). Each group is derived from primary carbon metabolism (**TAIZ and ZEIGER, 2010**) and their biosynthesis can be easily provoked (**BOURGAUD *et al.*, 2001**) however the type of secondary compound produced is extensively dependent on, the expression of their tissue-, organ-, or developmental- pathways and the specific biosynthetic enzyme that induces their synthesis (**WINK, 1999**).

1.6. Phenolic compounds in medicinal plants

Terpenes, phenolic compounds and nitrogen-containing compounds each exhibit distinct pharmaceutical potential (**GIULIETTI and ERTOLA, 1999**; **TAIZ and ZEIGER, 2010**), in addition to their operation within the plant relative to chemical protection and signalling (**WINK, 1999**). The versatile nature of these secondary metabolites motivated their preservation through natural selection (**WINK, 1999**), however the most prevalent of these, throughout vascular plants, are the phenolic compounds (**BOURGAUD *et al.*, 2001**). These compounds produce lignin (**BOURGAUD *et al.*, 2001**) which provide stems and vascular tissues with structural rigidity (**TAIZ and ZEIGER, 2010**). Additionally, these metabolites are prevalent in the secondary metabolism and exhibit medicinal potential (**DIAS *et al.*, 2016**).

1.6.1. Medicinal capacity of phenolic compounds

Heterogeneous in nature, this collection of secondary metabolites consists of approximately 1000 chemically distinct compounds (**TAIZ and ZEIGER, 2010**). The diversity of these metabolites accounts for their various functions within the plants primary and secondary metabolism (**VALAVANIDIS and VLACHOGIANNI, 2013**) though great attention has recently been attributed to the broad scope of phenolic pharmacological qualities (**AL-NUMAIR *et al.*, 2012**). Phenolic compounds are prevalent in numerous sunscreen products, given that the aromatic rings absorb potentially harmful UV-B radiation (**VERMERRIS and NICHOLSON, 2008**). Additionally, the insoluble nature of certain phenolic compounds (**TAIZ and ZEIGER, 2010**), including octyl-methylcinnamate, are incorporated into various water-proof sunscreen products (**VERMERRIS and NICHOLSON, 2008**). However, current water-proof sunscreens favour alternative compounds that reflect rather than absorb harmful radiation, that potentially induce epidermal irritation (**VERMERRIS and NICHOLSON, 2008**). In addition to their prominent bioactivity (**DIAS *et al.*, 2016**), flavonoids provide a significant portion of the phenolic compound group (**TAIZ and ZEIGER, 2010**). These metabolites provide multiple bioactive agents (**NCUBE *et al.*, 2012b**), that are metabolically and physiologically advantageous (**FARINAZZI-MACHADO *et al.*, 2012**). However, pharmaceutical research concentrates on their anti-microbial, anti-inflammatory, antioxidant, and anti-carcinogenic potential (**VALAVANIDIS and VLACHOGIANNI, 2013**).

Anti-microbial bioactivity of phenolic compounds entails defence against viral, bacterial, and fungal infection. Phenol metabolites were initially utilized for their anti-microbial potential in antiseptic treatments (**VERMERRIS and NICHOLSON, 2008**). However, given that phenolic compounds denature proteins (**ANYANWU, 2012**), the presence of high phenol concentrations in anti-septic treatments had an adverse impact on live tissues (**VERMERRIS and NICHOLSON, 2008**). *E. autumnalis* has been found to produce these anti-microbial metabolites, particularly with regard to bacterial infection of the leaves, roots and bulbs (**LOUW *et al.*, 2002**).

Alternatively, low phenol concentrations in throat lozenges provide an oral anaesthetic for pain related to inflammation (**VERMERRIS and NICHOLSON, 2008**). Following damage or microbial contamination of human tissues, membranous phospholipids release arachidonic acids that provide a substrate for cyclo-oxygenase (COX) enzymes (**RICCIOTTI and FITZGERALD, 2011**). COX-1 enzymes are present at all times in small amounts, which aid in the production of cyto-protective prostaglandins (**FAWOLE *et al.*, 2009**; **GAIDAMASHVILI and**

VAN STADEN, 2006), particularly in terms of gastro-intestinal ailments (**FAWOLE *et al.*, 2009**; **RICCIOTTI and FITZGERALD, 2011**), for which *E. autumnalis* decoctions are utilized by South African traditional healers (**LOUW *et al.*, 2002**). This anti-inflammatory activity could be achieved through the inhibition of COX enzymes by phenolic compounds found in *E. autumnalis* (**JÄGER and VAN STADEN, 2005**). These phenolic compounds result in a post transcriptional regulatory mechanism that leads to the expression of specific genes that down-regulate inflammatory gene expression (**TAIZ and ZEIGER, 2010**). COX enzymes functioning as inflammatory intermediaries are supressed by several flavonoid variants, including condensed tannins and gallotannins (**FAWOLE *et al.*, 2010**).

However, these anti-inflammatory procedures become diminished in the presence of ROS, where these COX inhibitors also serve as antioxidants by means of radical scavenging (**FAWOLE *et al.*, 2010**). Glycosylation of these flavonoids reduces their reactive potential by donating electrons or hydrogen elements to free radical species (**RICE-EVANS *et al.*, 1997**), impeding the peroxidation of membranous lipids (**FENNELL *et al.*, 2004b**); in addition to increasing their solubility, further enabling the absorption of free radicals into the vacuoles (**RICE-EVANS *et al.*, 1997**). Though these flavonoid groups are paramount to anti-oxidative functions, the majority of cinnamic and / or benzoic polyphenols also retain anti-oxidative properties (**MATKOWSKI, 2008**; **VALAVANIDIS and VLACHOGIANNI, 2013**), reducing DNA damage caused by free radicals and ROS (**VALAVANIDIS and VLACHOGIANNI, 2013**). This defence against oxidative activities by the various phenolic compounds deter the progression of degenerative diseases (**MONAJJEMI *et al.*, 2012**).

The anti-inflammatory and antioxidant qualities of phenolic intermediaries in medicinal plants have been extensively studied relative to their anti-tumour potential and the development of novel anti-carcinogenic treatments (**VALAVANIDIS and VLACHOGIANNI, 2013**). Where COX enzymes result in the formation of inflammatory prostaglandins, COX-2 inhibitors function to reducing inflammation, with the additional potential for limiting the growth and dispersal of carcinogen initiating proliferative diseases (**RICCIOTTI and FITZGERALD, 2011**). These investigations have provided epidemiological confirmation that phenolic compounds guard against vascular diseases, cardiac diseases, and certain carcinogenic forms, in addition to alleviating the frequency of chronic diseases, specifically neurodegenerative diseases (**VALAVANIDIS and VLACHOGIANNI, 2013**).

1.6.2. Biosynthesis of phenolic compounds in plants

Given the intricate nature of phenolic metabolite structures and their biosynthetic pathways (**BALASUNDRAM *et al.*, 2006**; **TAIZ and ZEIGER, 2010**), the synthetic production of these compounds for commercial profit has not yet been achieved (**GIULIETTI and ERTOLA, 1999**; **ROUT *et al.*, 2000**). Thus, various biotechnological methods have been developed to optimize the production of these desired metabolites (**MOYO *et al.*, 2011**; **NIGRO *et al.*, 2004**).

Phenolic compounds are derived from the primary metabolism of carbon that follows photosynthesis (**TAIZ and ZEIGER, 2010**). Though there are several variations in the biochemical pathways that result in their formation (**BALASUNDRAM *et al.*, 2006**; **TAIZ and ZEIGER, 2010**), the phenolic compounds which this study is focused on, are derived from the shikimic acid pathway (**TAIZ and ZEIGER, 2010**). This shikimic acid pathway is provoked by the presence of erythrose-4-phosphates that are derived from primary carbon metabolism by means of the pentose phosphate pathway (**BALASUNDRAM *et al.*, 2006**; **TAIZ and ZEIGER, 2010**). This shikimic acid pathway results in the formation of aromatic gallic amino acids, which produce hydrolysable tannins (**TAIZ and ZEIGER, 2010**); and aromatic phenylalanine amino acids (**BALASUNDRAM *et al.*, 2006**; **TAIZ and ZEIGER, 2010**). The more complex production of simple phenolic cinnamic acids, coumarins and benzoic acid derivatives involves the removal of ammonia from the phenylalanine by means of the phenylalanine ammonia-lyase (PAL) enzyme that branches between primary and secondary metabolism (**TAIZ and ZEIGER, 2010**). PAL is the determinant enzyme of the phenyl-propanoid pathway (**RIVERO *et al.*, 2001**), that leads to the production of flavonoids and ultimately anthocyanins (**TAIZ and ZEIGER, 2010**). Following the PAL induced exclusion of ammonia from phenylalanine; *trans*-cinnamic acid binds with a hydroxyl group to form *p*-coumaric acid that binds with acetyl co-enzyme A to produce *p*-coumaroyl-CoA (**TAIZ and ZEIGER, 2010**). The development of these simple phenolic compounds (**TAIZ and ZEIGER, 2010**) is better known as the phenyl-propanoid pathway (**RIVERO *et al.*, 2001**) as deviation from each simple phenolic compound results in the production of a variety of phenyl-propanoids (**TAIZ and ZEIGER, 2010**).

Of the phenyl-propanoid metabolites, the binding of *p*-coumaroyl-CoA and malonyl-CoA molecules results in the fabrication of flavonoids (**HASSAN and MATHESIUS, 2012**; **TAIZ and ZEIGER, 2010**). The malonyl-CoA molecules consist of carboxylated acetyl-CoA (**DOWNEY *et al.*, 2006**). This is followed by chalcone synthase to produce chalcones that later lose

hydrogen to form flavanones (**TAIZ and ZEIGER, 2010**). Further elaboration of these flavonoid compounds result in the biosynthesis of pro-anthocyanins (**BOGS *et al.*, 2005**) as the final product of the phenyl-propanoid pathway (**DOWNEY *et al.*, 2006**; **TAIZ and ZEIGER, 2010**). The binding of a hydroxyl group to flavanones produce di-hydro-flavonols, after which the binding of more hydroxyl groups determine the exact form of the anthocyanin (**TAIZ and ZEIGER, 2010**).

1.6.3. Phenolic compound production as a response mechanism to stress

Given that the quantity of desired metabolites that are initially produced is minimal (**DIAS *et al.*, 2016**), the economic feasibility of *in vitro* secondary metabolite generation has been questioned (**GIULIETTI and ERTOLA, 1999**; **MOYO *et al.*, 2011**). However, advances in biotechnological devices have enabled structured genetic (**MOYO *et al.*, 2011**) and environmental modification to optimize the production of desired metabolites (**NIGRO *et al.*, 2004**). *In vitro* methods of phenolic production have been established, based on the understanding of their biosynthetic pathways (**NCUBE *et al.*, 2013a**; **NIGRO *et al.*, 2004**). Pharmaceutically valuable phenolic compounds are produced following the phenyl-propanoid pathway (**DIAS *et al.*, 2016**) in which PAL activity is readily influenced by various culture environment conditions (**TAIZ and ZEIGER, 2010**). Though PAL is frequently activated by biotic contamination (**DIAS *et al.*, 2016**; **TAIZ and ZEIGER, 2010**), this activity also responds to a variety of chemical and physical abiotic pressures (**DIAS *et al.*, 2016**).

Various plant pathogens have been shown to stimulate the production of furocoumarins (**BOURGAUD *et al.*, 2001**), phenolic compounds derived from the *p*-coumaric acids through the addition of a furan ring (**TAIZ and ZEIGER, 2010**). Pathogenic infection or herbivory by invertebrates have prompted furocoumarin production in the roots, stalks and leaves of several plants relative to the incident location on the plant (**BOURGAUD *et al.*, 2001**; **DIAS *et al.*, 2016**). Primarily designed to guard against infection and herbivory (**DIAS *et al.*, 2016**; **TAIZ and ZEIGER, 2010**), these phenolic derivatives become highly phototoxic once exposed to UV-A radiation (**TAIZ and ZEIGER, 2010**). However, *in vitro* methods of cultivation eliminate stress caused by microbes and herbivory, as the sample explants and the culture apparatus are decontaminated and sterilized prior to cultivation (**SIDHU, 2010**).

The biotechnological methods used to chemically induce phenolic production in plants incorporate the manipulation of plant growth regulators (PGR's) (**DIAS *et al.*, 2016**), which are integrated into the Murashige and Skoog (MS) nutrient growth medium used to propagate

plants *in vitro* (**ROUT *et al.*, 2000**; **SAAD and ELSHAHED, 2012**). Within the primary metabolism, auxins prompt the initiation of shoot and root development, callogenesis, and somatic embryogenesis (**SAAD and ELSHAHED, 2012**). Though organic indole-3-acetic acid (IAA) is prevalent in natural habitats, these auxins are reactive to light and heat (**SAAD and ELSHAHED, 2012**), thus synthetically developed 2,4-Dichlorophenoxy-acetic acid (2,4-D), naphthalene-acetic acid (NAA), and picloram (PIC) are better suited to *in vitro* initiation of callogenesis and non-zygotic embryogenesis (**BEYL *et al.*, 2015**; **SAAD and ELSHAHED, 2012**). Auxins generally restrain secondary metabolite production due to a lack of cellular specialization (**LUCZKIEWICZ *et al.*, 2014**). NAA has been demonstrated to enhance alkaloid production rather than phenolic production in *Tabernaemontana divaricate* (**VERPOORTE *et al.*, 1999**). On the other hand, the production of anthocyanins and antraquinones in *Daucus carota* and *Morinda citrifolia* is prevented entirely by 2,4-D (**LUCZKIEWICZ *et al.*, 2014**). The impact of synthetically produced PIC, on phenolic production is not yet fully understood, though PIC promotes the accumulation of alkaloids in *Leucojum aestivum* callus (**PTAK *et al.*, 2013**). Yet iso-flavone generation by *Genista tinctoria* is promoted by these auxins when in conjunction with cytokinin supplementation (**LUCZKIEWICZ *et al.*, 2014**). The promotion of phenolic metabolite generation appears to be influenced more by the presence of both cytokinins and auxins, as demonstrated in the roots and leaves of *Merwilla plumbea* (**DIAS *et al.*, 2016**).

Nutrient limitation induces an increase in PAL activity (**TAIZ and ZEIGER, 2010**) where the combination and concentration of available nutrients regulate the rate of phenolic production (**NCUBE *et al.*, 2012b**). The carbon / nitrogen balance hypothesis (CNB) indicates that a limitation of nitrogen availability corresponds with a surplus storage of carbon molecules (**NCUBE *et al.*, 2012b**). Furthermore, the allocation of nitrogen and carbon resources to the primary and secondary metabolite production remains balanced (**DIAS *et al.*, 2016**). Where restricted nitrogen levels correspond with a surplus availability of carbon, resulting in carbon allocation to secondary metabolite production and nitrogen allocation to primary metabolism use (**NCUBE *et al.*, 2012b**). This allocation of surplus carbon to secondary metabolism leads to abundant phenolic production and storage (**NCUBE *et al.*, 2012b**).

The last form of phenolic metabolite production stimulation consists of physical provocation, which occasionally combine with certain chemical factors, but never incorporates biotic elements of elicitation (**DIAS *et al.*, 2016**). For example, the limitation of nitrogen resources, and inclined heavy metal levels promote ROS formation, sparking the production and storage

of various medicinally valuable phenolic compounds, particularly relevant to their anti-oxidative bioactivity (**KOVÁČIK *et al.*, 2007**). These responses differ relative to the plant species, and the chemical structure of the heavy metal causing the reaction (**DIAS *et al.*, 2016**). For instance; *Camellia sinensis* callus induces lignin and flavonoid production when exposed to cadmium; *Vitis vinifera* tissues amplify synthesis and storage of anthocyanin compounds in response to magnesium (**DIAS *et al.*, 2016**). Furthermore, copper induces phenolic and particularly flavonoid production in *Apium graveolens* leaves, *Psoralea cinerea* fruits (**BOURGAUD *et al.*, 2001**) and *Panax ginseng* root cultures (**DIAS *et al.*, 2016**).

Finally, the two leading forces of abiotic physical stress in plants, that promote phenolic compound production, are light intensity and temperature (**DIAS *et al.*, 2016**). Light induced phenolic biosynthesis has been demonstrated by the leaves and stems of *Eucalyptus camaldulensis*, where a sixteen hour photoperiod amplified the concentration of phenolic compounds exceeding those acquired from plants cultured in complete darkness (**DIAS *et al.*, 2016**). A lack of light exposure diminishes flavonoid quantity, however this is due to the reduced fixation of carbon which limits the production of all carbon based metabolites (**DOWNEY *et al.*, 2006**). Photo-receptive cells detect variations in solar radiation wavelengths (**NCUBE *et al.*, 2012b**), inducing the production of several phenolic compounds in response to increased UV radiation exposure (**DIAS *et al.*, 2016**; **SEIBERT *et al.*, 1975**). These range from the simple phenolic metabolites, furocoumarins (**BOURGAUD *et al.*, 2001**), to the complex polyphenol derivatives, flavonoids including anthocyanins (**DIAS *et al.*, 2016**; **DOWNEY *et al.*, 2006**; **NCUBE *et al.*, 2012b**). UV radiation exposure increases furocoumarin content produced by the leaves, roots, and stalks of *Ruta graveolens*, *Glehnia littoralis*, and *Apium graveolens* respectively (**BOURGAUD *et al.*, 2001**). Though these furocoumarins among other simple phenolic compounds are produced in response to UV-A exposure (**DIAS *et al.*, 2016**), UV-B radiation provokes the synthesis of more complex polyphenols (**NCUBE *et al.*, 2012b**; **TAIZ and ZEIGER, 2010**). UV-B radiation has a crippling effect on photosynthetic processes, and genetic transcription and translation (**NCUBE *et al.*, 2012b**). Phyto-receptive cryptochrome proteins (**DIAS *et al.*, 2016**) located in the leafs upper epidermal cells (**NCUBE *et al.*, 2012b**; **TAIZ and ZEIGER, 2010**) induce an assortment of morpho-anatomical responses to UV solar irradiation, including the amplified generation of flavonoids and their derivatives (**DIAS *et al.*, 2016**). Flavonoids and their derivatives are present and serve toward vascular plant primary metabolism (**DOWNEY *et al.*, 2006**). For example anthocyanins, employ the shorter red, pink, purple, and blue wavelengths as pollinator attractants, where flavones and flavonols retain

UV-B radiation as nectar guides for pollinators (**TAIZ and ZEIGER, 2010**). These anthocyanins provide the coloration of grapes, however these anthocyanins are dependent on light for their biosynthesis (**DOWNEY *et al.*, 2006**), thus a raised flavonoid content corresponds with an amplified exposure to light (**CORDELL, 2014**; **DOWNEY *et al.*, 2006**). In their secondary metabolic capacity, these phenyl-propanoid derivatives (**NCUBE *et al.*, 2012b**) permit photosynthetically active visible wavelengths to permeate, while absorbing harmful UV-B wavelengths, accumulating in stem and leaf epidermal cells (**NCUBE *et al.*, 2012b**; **TAIZ and ZEIGER, 2010**). These flavones and flavonols have exhibited a magnified synthesis in response to a heightened UV-B detection (**TAIZ and ZEIGER, 2010**).

Though reduced exposure to solar radiation may limit flavonoid generation due to the lack of carbon fixation, flavonoid production could be induced relative to the raised humidity caused by shading (**DOWNEY *et al.*, 2006**). Shading may induce the production of anthocyanins stored in the leaves (**DOWNEY *et al.*, 2006**) however, their production is reduced in red apple reproductive organs when exposed to heat (**WAHID *et al.*, 2007**). These anthocyanins diminish the osmotic capacity of the leaves (**WAHID *et al.*, 2007**) resulting in high osmotic stress, relative to salinity stress (**BOTELLA *et al.*, 2005**; **MOYO *et al.*, 2011**) which follow heat-induced drought conditions (**NCUBE *et al.*, 2012b**). However, this osmotic stress could be alleviated by shading, given that shading increases humidity (**DOWNEY *et al.*, 2006**). Thermal incline results in the acceleration of metabolic procedures which correspond with metabolic accumulation, however breach of a plants thermal threshold would either diminish or block these metabolic processes (**DOWNEY *et al.*, 2006**). This heat stress induces the generation of phenolic hypericin and hyperforin in response to the elevated activity of peroxidase (**NCUBE *et al.*, 2012b**). The amplified action of PAL is believed to be the primary mechanism employed in response to thermal stress, stimulating the biosynthesis of phenolic compounds while simultaneously reducing their oxidative potential (**RIVERO *et al.*, 2001**; **WAHID *et al.*, 2007**), by means of diminishing the activity of polyphenol-lyase and peroxidase (**WAHID *et al.*, 2007**).

However, this initiation of phenolic compound production is not limited to the elevation of thermal conditions but also in the case of reduced temperatures (**NCUBE *et al.*, 2012b**). The influence of thermal incline correlates with high light intensities (**TAYLOR and VAN STADEN, 2001d**), as is evident in grapes, where shading reduced the fruit temperature by approximately 10 ºC (**DOWNEY *et al.*, 2006**). In this instance, phenolic production is induced by the increased activation of PAL, resulting in the integration of these compounds, including

anthocyanins, into the cellular walls (**NCUBE *et al.*, 2012b**) of sugarcane and rose leaves (**WAHID *et al.*, 2007**). The variation in effects of thermal abiotic heat stress may be observed diurnally, with plants that cool each evening producing a much greater anthocyanin accumulation in comparison to those that retain their heat throughout the evening (**DOWNEY *et al.*, 2006**). Overall, the lack of optimal biomass accumulation in plants results in the production of secondary metabolites (**DOWNEY *et al.*, 2006**; **RIVERO *et al.*, 2001**).

1.7. Aims and objectives

The stationary nature of plants prevents them from fleeing when environmental influences become less than favourable for their primary function of growth (**NCUBE *et al.*, 2013a**). Regular diurnal and seasonal alterations to environmental conditions may result in minor changes in the plant's primary metabolism (**NCUBE *et al.*, 2013a**). However, once environmental conditions push beyond the parameters of the plant's routine metabolic fluctuations, the metabolic procedures may become influenced by cross-linked stress response pathways (**NCUBE *et al.*, 2013a**). This is based on the presumption that the ability to recognise, indicate, and respond to the presence of environmental stress factors, is encoded in all plants (**NCUBE *et al.*, 2013b**). However, though primary metabolism remains constant throughout the plant kingdom, the production of secondary metabolites (**TAIZ and ZEIGER, 2010**; **VERPOORTE, 2000**) is dependent on the parameters of the stress mechanisms imposed on the plant, and may vary among plant species (**NCUBE *et al.*, 2013a**).

Tissue culture methods provide a controlled environment (**SAAD and ELSHAHED, 2012**) that may restrict or boost metabolic pressures (**NIGRO *et al.*, 2004**). Biotic stress incursion is eliminated through various biotechnological strategies, including decontamination and sterilization of the explant samples and culture apparatus (**SIDHU, 2010**). Nutrient resource variation across cultures is reduced due to the fundamental MS basal growth medium, which provides all mineral ions required for explant culture success (**ROUT *et al.*, 2000**; **SAAD and ELSHAHED, 2012**). Prior to solidification of the growth medium, the pH is adjusted to 5.8, followed by a final media sterilization by high pressure and temperature in an autoclave (**TAYLOR and VAN STADEN, 2001c**). Culture rooms are maintained at 25 ºC ± 3 ºC, with a sixteen hour light to eight hour dark cycle. This extensive regulation of environmental factors has made it possible to investigate the influence of specific environmental stress factors on a plants response mechanisms.

The aim of this study was to get a plant to produce phenolic compounds, the secondary metabolite group that is most prevalent in vascular plants, in response to stress, using the medicinal plant species *Eucomis autumnalis*, as a model for potential application of these methods to numerous endangered medicinal plants.

The objectives of this study were to:

- Establish *in vitro* tissue culture and callogenesis of *E. autumnalis*, the model species, under standard culture environmental conditions. Observing how variations in the PGR concentrations and combinations influence the rate of callus growth under three distinct light intensity settings;
- Determine how these PGR and light intensity variation influence the quantity of total phenolic compounds produced by callus of the model species; and
- Investigate how variations in the extent and the duration of thermal stress, may influence the total phenolic content of *E. autumnalis* callus, *in vitro* cultured tissue samples, and the leaves of intact plants *in vivo*s.

CHAPTER 2: PROPAGATION METHODOLOGY

2.1. Introduction

Humans have relied on plants for centuries beyond the scope of their provision as primary producers, particularly in terms of their chemistry related to their medicinal properties (**SIDHU, 2010**). Plants are essential donors toward global health (**CALIXTO, 2000**) accommodating the primary healthcare of almost 80% of the global populace (**AFOLAYAN and ADEBOLA, 2004**). Despite the majority of western / modern medicinal drugs being produced synthetically in the laboratory; approximately a quarter of these drugs are derived either directly or indirectly from natural plant products (**CALIXTO, 2000**). Medicinal plants possess a number of biological properties in addition to their ornamental and horticultural allure (**NAIR *et al.*, 2013**). In South Africa these medicinal plants provide for approximately twenty-seven million people (**FENNELL *et al.*, 2004a**; **STREET *et al.*, 2008b**). In addition to the cultural importance of traditional remedies, these plants aid in providing readily available and inexpensive treatments (**FENNELL *et al.*, 2004b**; **STREET *et al.*, 2008b**). However, the extensive exploitation of these natural medicinal sources has resulted in the drastically reduced abundance of these plants (**FENNELL *et al.*, 2004a**).

The natural sources of medicinal plants used by South African traditional healers have been extensively exploited (**AFOLAYAN and ADEBOLA, 2004**; **FENNELL *et al.*, 2004a**) due to the lack of regulation and restraint (**MOYO *et al.*, 2011**) of harvesting from wild populations (**NIGRO *et al.*, 2004**). Given the limitations associated with the restoration of exploited habitats (**WOCHOK, 1981**) and the lack of harvesting regulation (**AFOLAYAN and ADEBOLA, 2004**; **MOYO *et al.*, 2011**), the maintenance of a species within its natural habitat by *in-situ* methods (**KASAGANA and KARUMURI, 2011**) does not provide a sustainable means of medicinal plant conservation in South Africa. *Ex-situ* conservation methods preserve a species and / or population's genetic diversity, independent of the natural habitat (**KASAGANA and KARUMURI, 2011**). The *in vitro* establishment of plantlets by micro-propagation is the dominant method of *ex-situ* conservation (**AFOLAYAN and ADEBOLA, 2004**; **MOYO *et al.*, 2011**; **NCUBE *et al.*, 2015**; **NIGRO *et al.*, 2004**).

2.1.1. *In vitro* tissue propagation

Plant cultivation *in vitro* is the "cloning" of plant organs, tissues, or cells (**HUSSAIN *et al.*, 2012**) within an aseptic environment (**KASAGANA and KARUMURI, 2011**). This aseptic

environment is vital in the successful establishment of tissue cultures *in vitro* as a diverse range of micro-organisms infect the exterior and interior of plant samples collected from the natural environment (**ROUT *et al.*, 2000**). Though *in vitro* methods could theoretically be utilized for the cultivation of any plant species (**DIAS *et al.*, 2016**), sterilization procedures differ at the plant species and organ levels. *Eucomis autumnalis* was selected as the model species for this study given that the sterilization protocols and successful micro-propagations of this species had already been established (**TAYLOR and VAN STADEN, 2001e**).

2.1.2. *In vitro* propagation of callus

The first "true" botanical tissue culture that was established between 1934 and 1935 resulted in the rapid production of callus cultures (**DIAS *et al.*, 2016**). Defined as the disorganized accumulation of undifferentiated plant tissue (**HUSSAIN *et al.*, 2012**; **IKEUCHI *et al.*, 2013**; **SIDHU, 2010**; **TAYLOR and VAN STADEN, 2001e**) callus is produced by solitary specialized plant cells (**IKEUCHI *et al.*, 2013**; **KRISHNAMURTHY, 2015**) that have either been exposed to some form of biological infection, or have suffered injury (**IKEUCHI *et al.*, 2013**; **TAYLOR and VAN STADEN, 2001e**). Essentially, function specific morphological and developmental specialization becomes reduced, resulting in a simplified homogeneous callus cell formation (**SIDHU, 2010**). These callus cells are often totipotent (**IKEUCHI *et al.*, 2013**) which enables organogenesis (**HUSSAIN *et al.*, 2012**) with the callus structure presenting partial shoot, root, or embryonic cellular specialization (**IKEUCHI *et al.*, 2013**). However, *in vitro* production of callus does not necessarily result in organ generation, but may develop either a compact or friable callus structure, completely devoid of cellular specialization (**IKEUCHI *et al.*, 2013**). This friable callus can be effortlessly fragmented, yet compact callus maintains a firm structure due to their high lignin content (**TAYLOR and VAN STADEN, 2001e**). The *in vitro* production of callus is subject to several environmental conditions (**HUSSAIN *et al.*, 2012**), that influence the structure and rate of callus development.

2.1.3. Culture environment

Increased regulation of culture environmental conditions have reduced the prevalence of physiological, pathological and morphological issues that were associated with conventional *in vitro* propagation environments (**KOZAI *et al.*, 1997**). These micro-environment controls promote cellular multiplication and vegetative growth in an attempt to achieve an optimal production rate (**SIDHU, 2010**). Elements of the cultivation environment that influence

propagation include biological contaminants, ventilation, light intensity (**KOZAI *et al.*, 1997**), pH, thermal radiation, and mineral concentration (**KOZAI *et al.*, 1997**; **SIDHU, 2010**).

Micro-organism contamination is eradicated by decontaminating and sterilizing explant samples and equipment (**SIDHU, 2010**). Potential biological contamination is further limited as culture vessel ventilation is reduced. An optimal pH of 5.8 has been established for *in vitro* plant tissue propagation (**TAYLOR and VAN STADEN, 2001c**). Thermal radiation is retained at 25 ºC ± 3 ºC in special culture growth rooms (**VAN STADEN *et al.*, 1991**). A Murashige and Skoog (MS) nutritional growth medium (**MURASHIGE and SKOOG, 1962**) used for *in vitro* plant tissue culture consists of various, functionally distinct, chemical compounds (**ROUT *et al.*, 2000**; **SAAD and ELSHAHED, 2012**) that are required for the explant to thrive. The *in vitro* propagation of callus cultures however requires modification of these environmental controls.

The plant growth regulator (PGR) groups, auxin and cytokinin, have been broadly investigated and utilized for the *in vitro* induction of callogenesis (**IKEUCHI *et al.*, 2013**; **TAYLOR and VAN STADEN, 2001e**). Furthermore, these plant hormones regulate organogenesis and the production of specialized plant cells by undifferentiated callus cultures (**HAMIDEH *et al.*, 2012**; **HUSSAIN *et al.*, 2012**; **IKEUCHI *et al.*, 2013**), with endogenous auxin-to-cytokinin ratios influencing the type of organogenesis that occurs (**HUSSAIN *et al.*, 2012**; **IKEUCHI *et al.*, 2013**).

Initiation of *E. autumnalis* callogenesis required a high auxin concentration (**BALDI *et al.*, 2009**; **TAYLOR and VAN STADEN, 2001e**). Synthetically produced naphthalene-acetic acid (NAA), and 2,4-Dichlorophenoxy-acetic acid (2,4-D), and the naturally occurring indole-3-acetic acid (IAA) are the three primary auxin variants used in plant tissue culture (**SAAD AND ELSHAHED, 2012**). All three function to stimulate the enlargement and division of cells within tissue and cell suspension cultures (**BEYL *et al.*, 2015**; **SAAD and ELSHAHED, 2012**). However, the physical and chemical progression of the metabolism, and the displacement through plant tissue differs among the variants (**SAAD and ELSHAHED, 2012**). When incorporated into *in vitro* plant culture media, IAA regulates the specialization of cells that produce xylem and phloem, and the quantity of sieve and tracheid elements (**ALONI, 1980**), thus the IAA-equivalent compounds, 2,4-D and picloram (PIC) (**LUCZKIEWICZ *et al.*, 2014**) are better suited for the propagation of *E. autumnalis* callus. Both 2,4-D and PIC have demonstrated effective production of totipotent callus cultures (**FITCH and MOORE, 1990**). Furthermore, addition of a low cytokinin, kinetin (KIN), concentration has demonstrated

improved callus growth in various plant species (**LUCZKIEWICZ *et al.*, 2014**; **STANLY *et al.*, 2011**; **TAYLOR and VAN STADEN, 2001e**). Synthetically manufactured KIN stimulate the division of cells (**HUSSAIN *et al.*, 2012**), aiding in the accumulation of callus cell cultures.

The second culture environment element believed to influence callus development is light intensity. In their natural environment plants are dependent on solar radiation for various essential metabolic and morphological processes (**ECONOMOU and READ, 1987**; **STANLY *et al.*, 2011**). The effects of light exposure on callus culture biomass differ relative to the intensity and quality of the light source, as well as the duration of exposure (**ECONOMOU and READ, 1987**). The spectrum of wavelengths emitted by light determines the quality of the light source (**ECONOMOU and READ, 1987**) with modern light emitting diodes (LED) enabling a cost effective means of manipulating photo-morphogenesis by exposing cultures to specific wavelengths (**KOZAI *et al.*, 1997**). However, fluorescent tube lighting remains the most common form of lighting in growth rooms, exposing cultures to a broad array of wavelengths (**ECONOMOU and READ, 1987**; **KOZAI *et al.*, 1997**) which accommodate *in vitro* propagation requirements (**KOZAI *et al.*, 1997**). Furthermore, the duration of exposure to these wavelengths also influence culture photo-morphogenesis, and differ greatly between species (**ECONOMOU and READ, 1987**; **KOZAI *et al.*, 1997**). For example, the propagation and preservation of *Zingiber zerumbet* callus cultures is dependent on their uninterrupted exposure to light (**STANLY *et al.*, 2011**), alternatively *Falcaria vulgaris* callus thrived in complete darkness (**HAMIDEH *et al.*, 2012**). A ratio of sixteen hours light to eight hours dark exposure has been maintained for conventional propagation of plant species (**KOZAI *et al.*, 1997**). However, manipulations of this light-to-dark ratio are known to alter the development of particular organs (**ECONOMOU and READ, 1987**; **KOZAI *et al.*, 1997**). Finally, light intensity, previously referred to as the illuminance of light (**ECONOMOU and READ, 1987**) or the photosynthetic photon flux density (PPFD) (**KOZAI *et al.*, 1997**), is measured as the quantity of photosynthetically active radiation (PAR). The source, quality, and intensity of light exposure stimulate species-specific responses, which often differ between the organs and the type of tissue cultured (**ECONOMOU and READ, 1987**). In this chapter, the quality and duration of light exposure were not manipulated, retaining the conventional use of fluorescent tube lights that were set on a sixteen hour light to eight hour dark cycle, with the light intensity being the only component of light exposure that was altered.

2.2.Materials and Methods

Whole plants of *Eucomis autumnalis* were potted and placed in the botanical garden greenhouse (G12) located at the University of KwaZulu-Natal (UKZN) Agricultural campus, Pietermaritzburg, South Africa (29º 62' 47" S, 30º 40' 40" E).

2.2.1. *In vitro* tissue propagation

Following the sterilization protocol set out by **TAYLOR and VAN STADEN (2001e)**, leaf samples, devoid of wilting or disease damage, were excised from greenhouse plants and rinsed under running tap water. Following a 5 min, 70% ethanol immersion samples were placed in 0.2% Benlate® ($C_{23}H_{26}Cl_3N_5O_5S$) solution for 10 min. Finally, a 20 min submersion in 1.75% sodium hypochlorite (NaClO) was used to sterilize the leaf samples followed by a repeated distilled water rinse. These leaves were then cut into 1 cm^2 which were placed onto full strength MS (**MURASHIGE and SKOOG, 1962**) growth medium, supplemented with 30 $g\ell^{-1}$ sucrose and 0.1 g ℓ^{-1} myo-inositol ($C_6H_{12}O_6$). These cultures were left to grow in culture rooms of a 1.53 µmol m^{-2} s^{-1} light intensity set for a sixteen hour light to eight hour dark cycle, maintaining a constant temperature of 25 ºC ± 3 ºC.

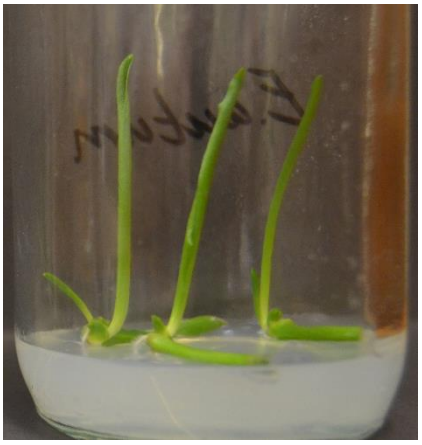

Figure 2.1: Sixteen-week-old *Eucomis autumnalis* plantlets cultured on Murashige and Skoog (MS) growth media devoid of plant growth regulator (PGR) supplementation.

2.2.2. Propagation of the callus

The leaves of plantlets grown *in vitro* (as seen in **Figure 2.1**) on PGR free MS media were cut into 1 cm^2 samples of leaf tissue. The upper epidermal layer of 1 cm^2 leaf samples were

then placed on fresh MS media supplemented with the various PGR concentrations and combinations (**Table 2.1**).

2.2.2.1. Source of plant growth regulators

2,4-Dichlorophenoxy acetic acid (2,4-D) was purchased from BDH Biochemicals Ltd. (Poole, England), while picloram (PIC) and kinetin (KIN) were purchased from Sigma-Aldrich (Steinheim, Germany).

Table 2.1: Combinations and concentrations of plant growth regulators (PGR's) supplemented Murashige and Skoog (MS) media used for the initiation of *Eucomis autumnalis* callogenesis.

PGR concentration		
2,4-D (µM)	**PIC (µM)**	**KIN (µM)**
10	0	0
15	0	0
20	0	0
10	0	2,5
15	0	2,5
20	0	2,5
0	10	0
0	15	0
0	20	0
0	10	2,5
0	15	2,5
0	20	2,5

Twelve replicates of each PGR treatment were equally divided between the three separate tissue culture growth rooms of differing light intensity, each maintaining a constant 25 ºC (± 3 ºC) temperature. The three light intensity treatments were (i) complete darkness at 0.00 µmol m^{-2} s^{-1}, (ii) low light intensity at 0.75 µmol m^{-2} s^{-1}, and (iii) high light intensity at 1.53 µmol m^{-2} s^{-1} maintaining a sixteen hour light to eight hour dark cycle. For twelve weeks the volume of callus growth was calculated for each treatment replicate, with the mean volume per PGR and light intensity treatment calculated in fourteen-day increments. The volume was calculated by multiplying the length, width, and height of the largest callus segment, using a

standard ruler. Growth curves for each PGR combination and concentration exposed to each light intensity treatment were compiled, displaying the volume of callus growth measured in fourteen day increments. The callus growth rate was measured as the volume (cm^3) of callus, not including the leaf tissue from which the callus originated. However, these measurements would only account for the quantity of callus cultured, disregarding the callus type and quality. Observed alterations in the callus morphology, such as browning, and callus type were noted.

2.2.3. Data Analysis

The R^2 and the slope values of each growth curve (**Table 2.2**) were determined using linear regression analyses of log transformed data (**HUSSEIN *et al.*, 2016**). The R^2 value indicates how close the data is to the fitted regression curve. Variations between growth curve slope values were then compared using a two-way analysis of variance (ANOVA), with a significant effect having a *p-value* < 0.05. Furthermore, the total volume of callus cultured over the eighty-four-day growth period was then compared between PGR and light intensity treatments using a two-way ANOVA, with a significant variation indicated at $p = 0.05$. A Duncan's Multiple Range test was then used to separate treatment means that were significantly different. All statistical analyses were processed using IBM SPSS version 24, and all growth curves were compiled using GraphPad Prism® version 5.02.

2.3. Results and Discussion

2.3.1. Influence of culture environment on callus growth curves

The growth of *E. autumnalis* callus presented in **Figure 2.2** and **Figure 2.3**, follows a sigmoidal growth curve design. In mathematical terms this curvilinear growth consists of logarithmic, linear and senescence phases (**KRISHNAMURTHY, 2015**) however, these three phases are broken down further to better describe the different phases of plant callus growth. The initial lag phase (**HUSSEIN *et al.*, 2016**), is characterized by the marginal increase in the callus volume (**HUSSEIN *et al.*, 2016**; **KHANPOUR-ARDESTANI *et al.*, 2015**; **TAN *et al.*, 2010**), commencing with a zero callus volume (**HUSSEIN *et al.*, 2016**) at day zero. *In vitro* propagated leaf tissue is sub-cultured onto MS media supplemented with the various PGR's required to induce the production of callus. During this lag phase, plant cells reduce their cellular specialization to form simplified homogeneous callus cells (**SIDHU, 2010**), making provision for the multiplication and division of callus cells (**CHAWLA, 2002**; **GASPAR *et al.*, 1996**). The second phase of growth is exponential, resulting in a pattern of logarithmic callus

growth (**KRISHNAMURTHY, 2015**; **SOOMRO and MEMON, 2007**). During this exponential growth phase, callus cell proliferation and multiplication rapidly increases (**CHAWLA, 2002**; **KHANPOUR-ARDESTANI *et al.*, 2015**; **TAN *et al.*, 2010**) until the maximum rate of cellular division is achieved (**CHAWLA, 2002**). The third phase of growth is linear (**KRISHNAMURTHY, 2015**), maintaining a relatively constant rate of callus growth (**KHANPOUR-ARDESTANI *et al.*, 2015**; **SOOMRO and MEMON, 2007**), however cellular multiplication becomes reduced with the increasing callus growth resultant of cellular enlargement rather than cellular division (**CHAWLA, 2002**). The fourth phase of callus growth models a progressive deceleration of cell division and elongation (**CHAWLA, 2002**), until all forms of callus growth are terminated during the fifth and final stationary growth phase (**CHAWLA, 2002**; **KHANPOUR-ARDESTANI *et al.*, 2015**). This stationary phase illustrates the asymptote of callus growth, during which the rate of growth is zero (**HUSSEIN *et al.*, 2016**) yet the abundance and size of callus cells remain unaltered (**CHAWLA, 2002**). These deceleration and stationary stages of callus growth mark the initiation of cellular senescence (**KRISHNAMURTHY, 2015**), ultimately resulting in cellular death unless the callus is sub-cultured onto fresh growth medium. The lag phase of *E. autumnalis* callus growth was observed within the first fourteen days of exposure to the new culture environment (**Figure 2.2** and **Figure 2.3**). Leaf explants became bloated and produced soft white hairy structures, similar to that of *Centella asiatica* (**TAN *et al.*, 2010**), following which callus formation was initiated. These callus cells acclimated to their modified culture environment, resulting in the stunted rate of callus growth observed (**Figure 2.2** and **Figure 2.3**). Furthermore, the subsequent exponential, linear, decelerating, and stationary phases of callus growth are moulded by the culture environment. The influence of culture environment on growth phase progression was evident in 2,4-D, and 2,4-D + KIN cultures (**Figure 2.2**).

The impact of culture environment on callus growth curves was determined by the comparison of R^2 and slope values (**Table 2.2**) calculated by regression analyses. Light intensity was determined to have a significant ($p = 0.0001$) effect on slope values across PGR combinations, where callus slope values of 1.53 µmol m^{-2} s^{-1} < 0.75 µmol m^{-2} s^{-1} > 0.00 µmol m^{-2} s^{-1} (**Table 2.2**). However, the initial growth of samples exposed to a light intensity of 1.53 µmol m^{-2} s^{-1} was greater than samples exposed to 0.75 µmol m^{-2} s^{-1} (**Figure 2.2**). This increase in growth rate corresponded with an accelerated progression of growth phases. Callus growth curves overall (**Figure 2.2 and Figure 2.3**) reflected the lag, exponential, linear, decelerating, and stationary phases of callus development, within the

eighty-four day growth period. However, 2,4-D, and 2,4-D + KIN cultures exposed to a light intensity of 1.53 µmol m^{-2} s^{-1} (**Figure 2.2**) progressed beyond the stationary growth phase, displaying cellular necrosis after seventy days, with the exception of 15 µM 2,4-D + 2.5 µM KIN (**Figure 2.2 D**) where cellular decay was first observed on the fifty-sixth day. The combined growth phase progression and slope values of 1.53 µmol m^{-2} s^{-1} exposed 15 µM 2,4-D + 2.5 µM KIN, and 20 µM 2,4-D + 2.5 µM KIN cultures, resulted in the moderate deviation of observed growth curves from fitted regression curves (**Table 2.2**).

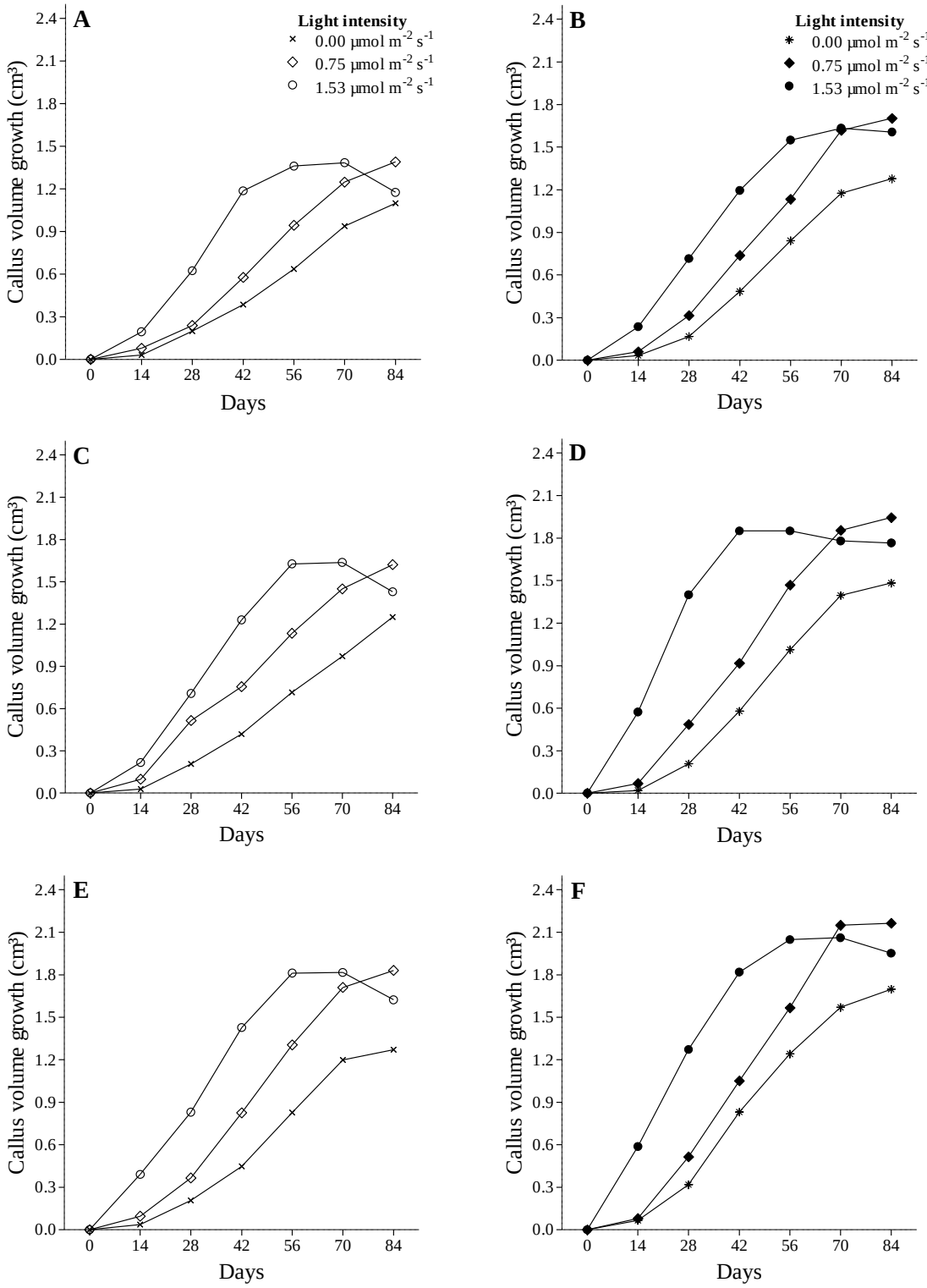

Figure 2.2: The influence of light intensity on the growth rate of *E. autumnalis* callus cultured on MS media supplemented with (**A**) 10 µM 2,4-D, (**B**) 10 µM 2,4-D + 2,5 µM KIN, (**C**) 15 µM 2,4-D, (**D**) 15 µM 2,4-D + 2,5 µM KIN, (**E**) 20 µM 2,4-D, and (**F**) 20 µM 2,4-D + 2,5 µM KIN.

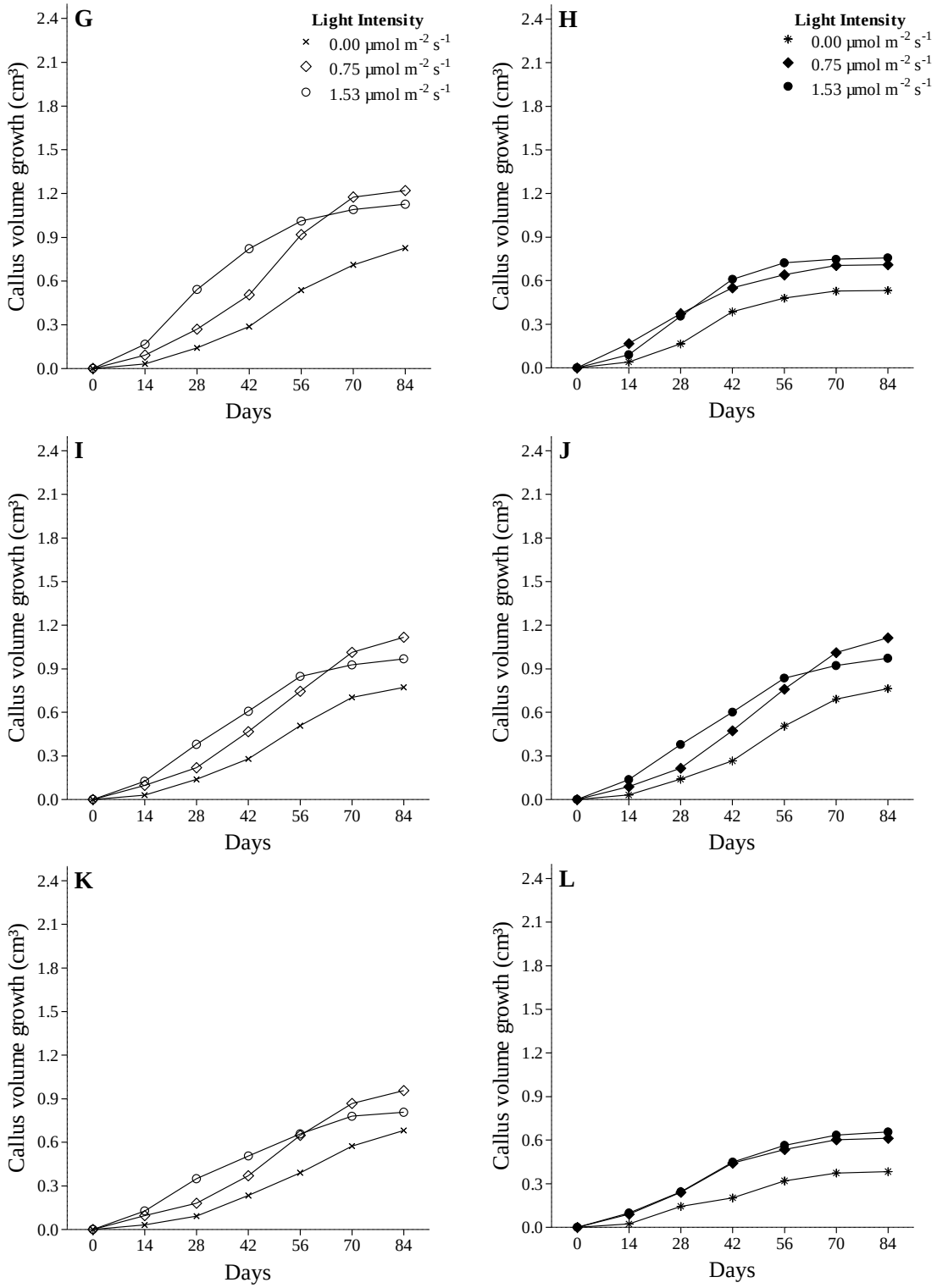

Figure 2.3: The influence of light intensity on the growth rate of *E. autumnalis* callus cultured on MS media supplemented with (**G**) 10 µM PIC, (**H**) 10 µM PIC + 2,5 µM KIN, (**I**) 15 µM PIC, (**J**) 15 µM PIC + 2,5 µM KIN, (**K**) 20 µM PIC, and (**L**) 20 µM PIC + 2,5 µM KIN.

Table 2.2: The R^2 and slope values for growth curves of callus cultured on MS media supplemented with various PGR combinations and concentrations and cultured under the three light intensity treatments.

	0.00 µmol m^{-2} s^{-1}		**0.75 µmol m^{-2} s^{-1}**		**1.53 µmol m^{-2} s^{-1}**	
PGR	**R^2**	**Slope**	**R^2**	**Slope**	**R^2**	**Slope**
10 µM 2,4-D	0.972 (2.8)	1.982 (±0.084)	0.927 (2.8)	1.683 (±0.071)	0.836 (16.4)	1.071 (±0.118)
15 µM 2,4-D	0.948 (5.2)	2.108 (±0.124)	0.945 (5.5)	1.533 (±0.092)	0.865 (13.5)	1.121 (±0.111)
20 µM 2,4-D	0.968 (3.2)	2.045 (±0.093)	0.981 (1.9)	1.708 (±0.059)	0.892 (10.8)	0.884 (±0.077)
10 µM 2,4-D + 2,5 µM KIN	0.955 (4.5)	2.181 (±0.118)	0.954 (4.6)	1.946 (±0.107)	0.930 (7.0)	1.106 (±0.076)
15 µM 2,4-D + 2,5 µM KIN	0.957 (4.3)	2.455 (±0.130)	0.950 (5.0)	1.876 (±0.108)	**0.725 (27.5)**	0.629 (±0.097)
20 µM 2,4-D + 2,5 µM KIN	0.909 (9.1)	1.958 (±0.155)	0.956 (4.4)	1.861 (±0.100)	**0.565 (43.5)**	0.877 (±0.192)
10 µM PIC	0.934 (6.6)	1.889 (±0.125)	0.913 (8.7)	1.519 (±0.117)	0.904 (9.6)	1.077 (±0.088)
15 µM PIC	0.755 (24.5)	2.116 (±0.301)	0.986 (1.4)	1.442 (±0.042)	0.955 (4.5)	1.162 (±0.063)
20 µM PIC	0.974 (2.6)	1.802 (±0.074)	0.963 (3.7)	1.394 (±0.069)	0.966 (3.4)	1.040 (±0.049)
10 µM PIC + 2,5 µM KIN	0.904 (9.6)	1.511 (±0.123)	0.942 (5.8)	0.822 (±0.051)	0.892 (10.8)	1.191 (±0.104)
15 µM PIC + 2,5 µM KIN	0.988 (1.1)	1.821 (±0.051)	0.988 (1.1)	1.495 (±0.042)	0.967 (3.3)	1.117 (±0.051)
20 µM PIC + 2,5 µM KIN	0.922 (7.8)	1.566 (±0.133)	0.946 (5.4)	1.104 (±0.066)	0.962 (3.8)	1.102 (±0.055)

R^2 values represent the deviation of more than 25% from the fitted regression curve (% deviation), n = 18. Slope values represent the rate of callus growth over the eighty-four-day growth period (± Std. Error).

Callus cultured on PIC + KIN supplemented MS media exhibited significantly lower (p = 0.061) slope values than 2,4-D; 2,4-D + KIN; with the exception of cultures exposed to a 1.53 µmol m^{-2} s^{-1} light intensity. Furthermore, the addition of KIN to PIC cultures resulted in considerably lower final callus volumes (**Figure 2.4**).

2.3.2. Influence of auxin-to-cytokinin ratio on callus

It has been well established that the exogenous supplementation (**BEYL *et al.*, 2015**) of plant growth regulators is essential for *in vitro* callus induction (**TAN *et al.*, 2010**). High auxin concentrations (**TAYLOR and VAN STADEN, 2001e**), stimulate cellular enlargement and division, in addition to the configuration of meristems, regulating the development of either specialized organs or callus (**GASPAR *et al.*, 1996**). Naturally derived plant growth hormones, including IAA, exhibit less stable effects in *in vitro* environments (**SAAD and ELSHAHED, 2012**) than synthetically produced 2,4-D and PIC herbicides (**FITCH and MOORE, 1990**; **GASPAR *et al.*, 1996**). The comparable auxin-like properties (**FITCH and MOORE, 1990**) of these PGR's, substitute for the growth hormones produced naturally in plants (**BEYL *et al.*, 2015**).

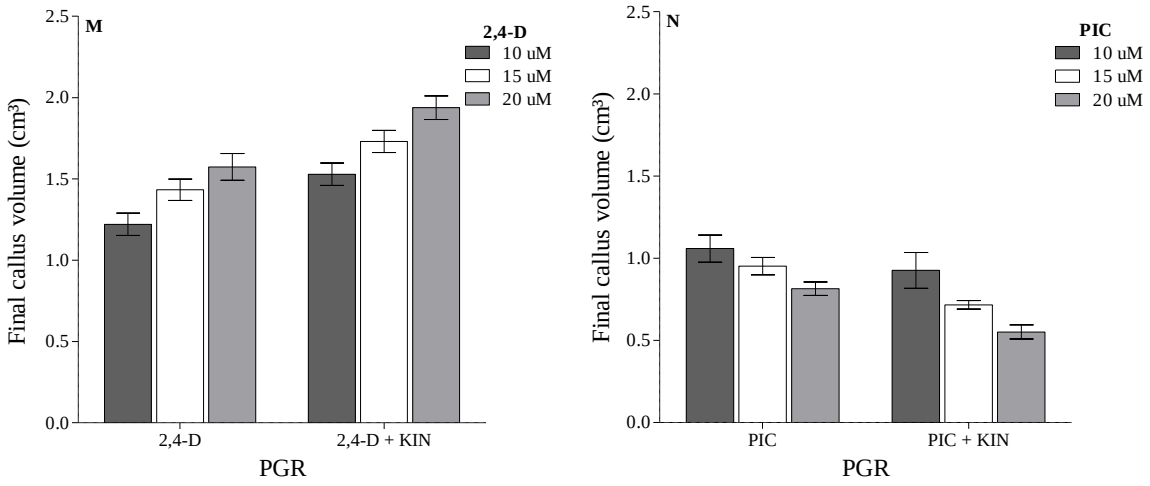

Figure 2.4: The effect of auxin concentration and the addition of KIN on *E. autumnalis* callus growth. The final callus volume (mean ± SE) cultured on (**M**) 2,4-D, and (**N**) PIC supplemented MS media.

2,4-D cultures produced significantly greater final callus volumes (p = 0.001) than PIC cultures (**Figure 2.4**). Remarkably, *Merwilla plumbea* callus cultured on PIC supplemented MS media produced a greater quantity of friable embryogenic callus than 2,4-D cultures

(**BASKARAN and VAN STADEN, 2012**). Though 2,4-D is predominantly used for the *in vitro* induction of callogenesis (**HAMIDEH *et al.*, 2012**) the regeneration of totipotent *Saccharum* callus cells was better maintained by PIC cultures, and not by 2,4-D cultures (**FITCH and MOORE, 1990**). This effect was influenced further by auxin concentration, displaying a positive correlation effect between 2,4-D concentration and final callus volume (**Figure 2.4 M**), and a negative correlation effect between PIC concentration and final callus volume (**Figure 2.4 N**). High concentration 2,4-D and PIC supplemented MS media have been used to produce friable embryonic callus of *Drimia robusta* (**BASKARAN and VAN STADEN, 2014**), and *Merwilla plumbea* (Lindl.) Speta (**BASKARAN and VAN STADEN, 2012**). Though the propagation of somatic embryos is advantageous to various fields of plant biotechnology research (**BASKARAN and VAN STADEN, 2014**), friable callus lacking organ regeneration is preferable for the repetitive proliferation of callus cultures (**IKEUCHI *et al.*, 2013**). 20 µM 2,4-D supplemented *E. autumnalis* cultures produced an off-white velvety compact callus (**Figure 2.5 P**), devoid of any organ regeneration (**IKEUCHI *et al.*, 2013**), with more compact callus formations developing at lower concentrations (**Figure 2.5 Q**). These compact callus formations darken with the increased level of compaction as inner cells lack access to oxygen, resulting in callus browning, due to cellular necrosis. However, the accelerated growth rate achieved by 2,4-D cultures (**Figure 2.2**) exposed to a 1.53 µmol m^{-2} s^{-1} light intensity expressed an inclination toward the production of specialized cells (**Figure 2.5 R**) similar to that of *Leucojum aestivum* callus, cultured on 2,4-D supplemented MS media and exposed to high light intensities, which resulted in the induction of somatic embryogenesis (**PTAK *et al.*, 2013**). On the other hand, 10 µM PIC supplemented MS media developed a yellow friable callus structure (**Figure 2.5 S**) with the increase in PIC concentration producing a lower volume of yellowed callus (**Figure 2.5 T**).

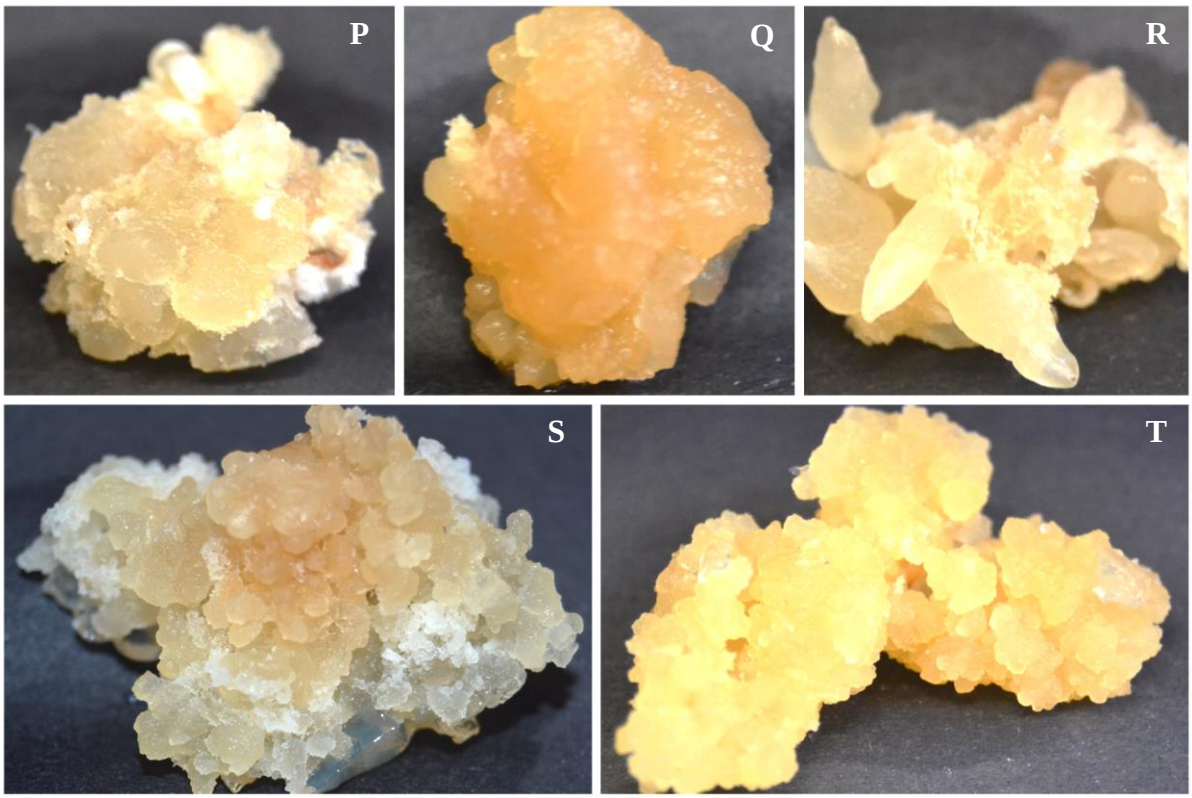

Figure 2.5: Forty-eight-day-old *Eucomis autumnalis* callus cultured on PGR supplemented MS media displaying formations of (**P**) velvety partially compact callus (20 µM 2,4-D, exposed to 0.75 µmol m^{-2} s^{-1}), (**Q**) compact callus (10 – 15 µM 2,4-D, exposed to 0.75 µmol m^{-2} s^{-1}), (**R**) nodular callus (10 – 20 µM 2,4-D, exposed to 1.53 µmol m^{-2} s^{-1}), (**S**) yellowing friable callus (10 µM PIC, exposed to 0.75 µmol m^{-2} s^{-1}), and (**T**) yellowed friable callus (15 – 20 µM PIC, exposed to 0.75 µmol m^{-2} s^{-1}).

The discoloration of these callus cultures was reduced by the additional supplementation of a low concentration cytokinin. Benzyl adenine (BA) is the most prominent synthetic cytokinin used in plant biotechnology, however the undesirable remnant effects associated with acclimatization (**MASONDO *et al.*, 2014**) resulted in the use of synthetically produced KIN instead. These cytokinins regulate cellular division in conjunction with the supplemented auxin (**GASPAR *et al.*, 1996**; **HUSSAIN *et al.*, 2012**). This interaction allows for the *in vitro* manipulation of plant morphology, with a balanced cytokinin-to-auxin ratio resulting in the production of callus (**HUSSAIN *et al.*, 2012**). The addition of KIN to 2,4-D supplemented

cultures produced significantly greater quantities of callus, maintaining the positive correlation between 2,4-D concentrations and the final volume of callus produced (**Figure 2.4 M**). Furthermore, incline in 2,4-D concentration resulted in reduced callus browning, with cultures exposed to 1.53 µmol m^{-2} s^{-1} light intensity presenting lower levels of compaction (**Figure 2.5 R**) and nodular formations of callus (**SOOMRO and MEMON, 2007**). PIC supplemented cultures produced a reduced final callus volume (**Figure 2.4 N**), with a marginal effect of the additional KIN supplementation on callus formation. The reduced final volume of callus occurs when cytokinin concentrations are low relative to the auxin concentration, yet high cytokinin and low auxin concentrations boost the production of callus (**LUCZKIEWICZ *et al*., 2014**).

2.4. Conclusions

This chapter aimed to determine how variation in light intensity and auxin-to-cytokinin ratios would influence the production of *E. autumnalis* callus from leaf explants. Ensuring that all elements of the culture environment remained constant, *E. autumnalis* callus cultures were initiated on MS media supplemented with various auxin-to-cytokinin ratios, and were exposed to three light intensity treatments.

High light intensity (1.53 µmol m^{-2} s^{-1}) exposure resulted in rapid exponential and linear growth of 2,4-D and 2,4-D + KIN cultures (**Figure 2.2**), though this rate of growth was greatly reduced in PIC and PIC + KIN cultures (**Figure 2.3**). The accelerated growth rate achieved by 2,4-D cultures (**Figure 2.2**) exposed to a light intensity of 1.53 µmol m^{-2} s^{-1} would appear to be advantageous, however these cultures rapidly progressed beyond the stationary growth phase resulting in cell necrosis. On the other hand, exposure to the lower light intensity (0.75 µmol m^{-2} s^{-1}) and the zero light exposure (0.00 µmol m^{-2} s^{-1}) growth curves progressed through exponential, linear, and decelerating growth phases within the eighty-four-day growth period, absent of cellular decay.

Optimum callus volumes were obtained from cultures exposed to a 0.75 µmol m^{-2} s^{-1} light intensity (p = 0.0001). Though 0.00 µmol m^{-2} s^{-1} cultures produced lower volumes of callus, the absence of light exposure reduced callus browning, providing a suitable environment for the preservation of friable callus (**TAYLOR and VAN STADEN, 2001e**).

CHAPTER 3: INFLUENCE OF CULTURE ENVIRONMENT ON PHENOLIC PRODUCTION

3.1. Introduction

The application of biotechnological methodologies, particularly those regarding the *in vitro* propagation of medicinal plants utilized by South African traditional healers could serve toward resolving the issue of their declining abundance (**AFOLAYAN and ADEBOLA, 2004**). However, beyond the mass production of vulnerable plant species (**HUSSAIN *et al.*, 2012**; **NIGRO *et al.*, 2004**), plant biotechnological tools can be used to promote the production of desired medicinal metabolites (**NIGRO *et al.*, 2004**; **ROUT *et al.*, 2000**).

Though *in vitro* methods for botanical conservation are expensive (**KOZAI *et al.*, 1997**), these methods allowed biotechnologists to study and better understand the physical, physiological, biochemical and genetic components of these plants (**NCUBE *et al.*, 2015**). Investigation of these botanical elements enables the study of plant metabolites (**MOYO *et al.*, 2011**; **NCUBE *et al.*, 2015**; **NIGRO *et al.*, 2004**; **ROUT *et al.*, 2000**) produced in response to various environmental stimuli (**TAIZ and ZEIGER, 2010**). Studies under *in vitro* conditions have determined that these metabolites are produced by cellular differentiation and specialization processes derived from the plants primary metabolism (**NCUBE *et al.*, 2015**).

3.1.1. Plant secondary metabolites

Primary metabolic functions and pathways are standard in all plants (**TAIZ and ZEIGER, 2010**), with secondary metabolites produced as either intermediaries or final products, derived from the primary metabolites (**NCUBE *et al.*, 2012b**; **VERPOORTE, 2000**) which are products of protein, lipid, and carbohydrate biosynthesis (**TAIZ and ZEIGER, 2010**). Given that these deviations from the primary metabolism are diverse (**VERPOORTE, 2000**) the presence and biosynthesis of these secondary metabolites vary among plant genera and species (**TAIZ and ZEIGER, 2010**). Furthermore, the production and accumulation of these metabolites often differ between a plants organs and cells (**BOURGAUD *et al.*, 2001**; **GIULIETTI and ERTOLA, 1999**), demonstrating a substantial co-ordination of metabolic deviation from a plants primary metabolism (**NCUBE *et al.*, 2012b**; **TAIZ and ZEIGER, 2010**) relative to the different phases of the plants development (**GIULIETTI and ERTOLA, 1999**; **NCUBE *et al.*, 2012b**).

These variations among plant genera, species (**TAIZ and ZEIGER, 2010**), organs, and cells (**BOURGAUD *et al.*, 2001**; **GIULIETTI and ERTOLA, 1999**) are determined by the metabolites function, relative to the surrounding environment (**NCUBE *et al.*, 2012b**; **VERPOORTE, 2000**). These functions of the secondary metabolism include the attraction of pollinators (**TAIZ and ZEIGER, 2010**; **WALLACE *et al.*, 1996b**; **WINK, 1999**) by pigmented secondary metabolites (**BOURGAUD *et al.*, 2001**), though the majority of secondary metabolites provide a defensive mechanism against abiotic (**NCUBE *et al.*, 2012b**) and biotic stress (**WINK, 1999**). However, secondary metabolite production depletes the plants limited resources (**CRONIN and HAY, 1996**), resulting in the allocation of resources dependent on when and where these defensive metabolites are required (**WINK, 1999**). Thus the synthesis and collection of these secondary metabolites vary within a plants organs and cells (**BOURGAUD *et al.*, 2001**; **GIULIETTI and ERTOLA, 1999**).

Characterised by their diminished abundance throughout the plant (**BOURGAUD *et al.*, 2001**; **WINK, 1999**) these metabolites are accumulated in reserve cells or organs (**BOURGAUD *et al.*, 2001**; (**BOURGAUD *et al.*, 2001**; **GIULIETTI and ERTOLA, 1999**) at high concentrations (**WINK, 1999**). These organic compounds are further classified as terpenes, nitrogen-containing compounds, or phenolic compounds based on their chemical structure (**TAIZ and ZEIGER, 2010**). Of these three groups, phenolic compounds are predominant in vascular plants (**BOURGAUD *et al.*, 2001**; **TAIZ and ZEIGER, 2010**), producing numerous bioactive agents with medicinal capacity (**DIAS *et al.*, 2016**).

3.1.2. Biosynthesis of phenolic compounds

Phenolic compounds consist of an aromatic ring and one or more hydroxyl groups, and range from simple to highly polymerized compounds (**TAIZ and ZEIGER, 2010**). These phytochemicals are produced by means of several pathways which deviate from primary carbon metabolism following photosynthesis (**BALASUNDRAM *et al.*, 2006**; **NCUBE *et al.*, 2012b**; **TAIZ and ZEIGER, 2010**; **VERPOORTE, 2000**). Following the first pathway, respiration converts glucose ($C_6H_{12}O_6$) into pyruvate (CH_3COCOO), where carbon dioxide (CO_2) is then released in the mitochondrial matrix by pyruvate decarboxylation, producing acetyl co-enzyme A ($C_{23}H_{38}N_7O_{17}P_3S$), from which miscellaneous phenolic compounds are derived following the malonic acid pathway (**DOWNEY *et al.*, 2006**; **TAIZ and ZEIGER, 2010**). However, this process is of minimal significance to the production of phenolic compounds in higher plants as this pathway is typical of bacterial and fungi (**TAIZ and ZEIGER, 2010**). In

higher plants however, two deviations from primary metabolism of carbon occur (**BALASUNDRAM *et al.*, 2006**; **NCUBE *et al.*, 2012b**; **TAIZ and ZEIGER, 2010**; **VERPOORTE, 2000**), resulting in the production of phenolic compounds following the shikimic acid pathway (**BALASUNDRAM *et al.*, 2006**; **TAIZ and ZEIGER, 2010**). The first involves the glycolytic conversion of glucose ($C_6H_{12}O_6$) into phosphoenol-pyruvate ($C_3H_5O_6P$) (**TAIZ and ZEIGER, 2010**). The second involves the pentose phosphate pathway formation of erythrose-4-phosphate ($C_2H_9O_7P$) (**BALASUNDRAM *et al.*, 2006**; **TAIZ and ZEIGER, 2010**). These simple carbohydrate precursors ($C_3H_5O_6P$ and $C_2H_9O_7P$) produce the aromatic amino acid, phenylalanine ($C_9H_{11}NO_2$), following the shikimic acid pathway (**BALASUNDRAM *et al.*, 2006**; **TAIZ and ZEIGER, 2010**). Furthermore, an ammonia (NH_3) molecule is removed by phenylalanine ammonia-lyase (PAL) to yield cinnamic acids ($C_9H_8O_2$) (**TAIZ and ZEIGER, 2010**). Thus, production of phenolic compounds in higher plant is induced by PAL (**TAIZ and ZEIGER, 2010**).

3.1.3. Factors influencing the production of phenolic compounds

Doubt has been cast over the economic feasibility of extracting phenolic compounds produced by plants (**GIULIETTI and ERTOLA, 1999**; **MOYO *et al.*, 2011**) due to the initial production of these desired metabolites yielding negligible quantities (**DIAS *et al.*, 2016**). Furthermore, deviation from the primary metabolism of carbon is not unique to the production of phenolic compounds (**TAIZ and ZEIGER, 2010**). The biosynthesis of each secondary metabolite group is dependent on the developmental, organ, or tissue phase at which a specific biosynthetic enzyme triggers the primary metabolism deviation (**WINK, 1999**). For example, phenolic compound production is dependent on the PAL enzyme induced deviation from the primary carbon metabolism, following photosynthesis (**BALASUNDRAM *et al.*, 2006**; **NCUBE *et al.*, 2012b**; **TAIZ and ZEIGER, 2010**; **VERPOORTE, 2000**). Understanding of these biosynthetic pathways has enabled enhanced *in vitro* production of phenolic compounds (**NCUBE *et al.*, 2013a**; **NIGRO *et al.*, 2004**), by means of modifying the culture environment (**NIGRO *et al.*, 2004**).

The biosynthesis of phenolic compounds is effortlessly triggered (**BOURGAUD *et al.*, 2001**) as the condition of the culture environment induces an instant effect on the activity of PAL (**TAIZ and ZEIGER, 2010**). Though biotic infection is often used to stimulate PAL activity (**DIAS *et al.*, 2016**; **TAIZ and ZEIGER, 2010**), the simulation of various physical and / or

chemical abiotic stressors have also demonstrated a significant effect on PAL activity (**DIAS *et al.*, 2016**).

In vitro cultivation systems enable the optimal regulation of PAL activity stimuli within the culture environment (**SIDHU, 2010**). Decontamination of the culture equipment and explant samples prior to cultivation (**SIDHU, 2010**) regulate the amplification of PAL activity by biotic contamination (**DIAS *et al.*, 2016**; **TAIZ and ZEIGER, 2010**). PAL activity stimulation by the limited availability of nutrients (**TAIZ and ZEIGER, 2010**) was standardized by the use of a constant strength MS media (**ROUT *et al.*, 2000**) with the standardization of culture room temperature preventing the stimulation of PAL activity, based on the production of phenolic compounds in response to thermal stress (**RIVERO *et al.*, 2001**; **WAHID *et al.*, 2007**).

This chapter aimed to determine how variation in the combination and concentration of PGR's, and light intensity influenced the phenolic content of *E. autumnalis* callus. Light is an integral component of the plants primary metabolism, providing the energy required for carbon fixation during photosynthesis (**ECONOMOU and READ, 1987**; **NCUBE *et al.*, 2012b**; **STANLY *et al.*, 2011**). However, the effect of light exposure on plant metabolism is dependent on the duration, intensity, and quality, or range of wavelengths that the plant is exposed to (**ECONOMOU and READ, 1987**). Exposure to different wavelengths of the light spectrum enables the manipulation of plant photo-morphogenesis (**KOZAI *et al.*, 1997**) in addition to influencing the plants metabolic responses. Wavelengths ranging from 400-700 nm are absorbed by photosynthetic organelles to form ATP (**TAIZ *et al.*, 2014**) for the photosynthetic fixation of carbon (**ECONOMOU and READ, 1987**; **NCUBE *et al.*, 2012b**; **STANLY *et al.*, 2011**; **TAIZ *et al.*, 2014**). However, cultures are exposed to a wider range of wavelengths emitted from white light fluorescent tubes (**ECONOMOU and READ, 1987**; **KOZAI *et al.*, 1997**). Exposure to wavelengths of less than 400 nm has a deleterious effect on photosynthetic systems (**BOURGAUD *et al.*, 2001**) thus defensive secondary metabolite production is induced (**NCUBE *et al.*, 2012b**; **WINK, 1999**). Given that plants are exposed to the full spectrum of solar radiation, the ability to detect certain ranges of the light spectrum has developed in plants (**NCUBE *et al.*, 2012b**). This chapter examined how phenolic compound production was influenced by the intensity of light exposure in conjunction with the effect of PGR variation. Auxins generally promote primary metabolic growth (**SAAD and ELSHAHED, 2012**) while simultaneously inhibiting the production of secondary metabolites (**LUCZKIEWICZ *et al.*, 2014**). However, this inhibition of secondary metabolite production by auxins is reversed by

the additional supplementation of cytokinins promoting secondary metabolite production (**LUCZKIEWICZ *et al.*, 2014**).

3.2. Materials and Methods

Following a four month period in which the primary callus growth rate was determined (as described in **section 2.2**), modified Folin-Ciocalteu (Folin-C) assays were used to establish the quantity of phenolic compounds which were produced by *Eucomis autumnalis* callus that was cultured on MS media supplemented with various PGR concentrations and combinations, and exposed to light intensities of 0.00 µmol m^{-2} s^{-1}, 0.75 µmol m^{-2} s^{-1}, and 1.53 µmol m^{-2} s^{-1} (described in **section 2.2.2**). Three callus samples per PGR*light intensity were randomly selected, with the phenolic content determined for three replicates of each sample. Thus, nine replicates per PGR*light intensity treatment were assessed for phenolic content. An average phenolic content was determined for each sample, providing three mean values of phenolic content for each PGR*light intensity treatment. This method of sampling reduced errors based on sampling bias and errors encountered during the phenolic assays.

3.2.1. Assessing phenolic content

Samples were placed in pill vials, frozen in liquid nitrogen and put in a freezer set at - 20 ºC for 24 hrs. These samples were then dried in a Virtis Freeze-drier and crushed into a powder which was then dissolved in 50% methanol at a ratio of 10 mℓ (50% methanol) for every 50 mg crushed callus sample. Extraction entailed sonication for 20 min on ice, followed by filtration through Whatman No. 1 filter paper, under vacuum. The quantity of phenolic compounds was then assessed by means of the modified Folin-Ciocalteu (Folin-C) assay.

50 µℓ extract was dissolved in 950 µℓ distilled water, followed with 500 µℓ of 50% Folin-C reagent, and 2,5 mℓ of 2% sodium carbonate (Na_2CO_3) was added in a dark room. Following a 30 min incubation in a dark cupboard under ambient room temperature of 25 ºC (± 3 ºC), absorbance was read at 725 nm (**NCUBE *et al.*, 2013a**), using a Genova Plus Spectrophotometer. These values were then compared to a standard 1 mℓ solution of (5.8782 µM) gallic acid, 950 µℓ distilled water, 500 µℓ Folin-C reagent, and 2,5 mℓ of 2% Na_2CO_3 that was also incubated at room temperature in a dark cupboard for 30 min (**NCUBE *et al.*, 2013a**). Absolute concentrations of phenolic compounds are expressed as gallic acid equivalents (GAE) (**FAWOLE *et al.*, 2010**; **NCUBE *et al.*, 2013a**) per gram dry weight (**NCUBE *et al.*, 2013a**).

3.2.2. Data Analysis

Callus samples were randomly selected from replicates of each PGR and light intensity treatment. A two-way ANOVA was used to compare the quantity of phenolic compounds produced by callus cultured under the various PGR combinations and the three light intensity treatments. Given that the data was positively skewed, a log transformation was used to obtain a normal distribution of the residuals. A Duncan's Multiple Range Test (DMRT) was used to separate the means of homogeneous subset groups which differed at $p = 0.05$. All analyses were performed using IBM SPSS Statistics for Windows, version 24; and all graphs were compiled using GraphPad Prism® version 5.02.

3.3. Results and Discussion

3.3.1. Effect of light exposure on the phenolic content

Light intensity was found to have a significant overall effect ($p = 0.0001$) on the production of phenolic compounds, with exposure to light intensities of both 0.75 µmol m^{-2} s^{-1}, and 1.53 µmol m^{-2} s^{-1} demonstrating an amplified production and accumulation of phenolic compounds by most cultures (**Figure 3.1**). However, the addition of 2,5 µM KIN to 15 µM and 20 µM PIC cultures resulted in a reduced phenolic production and accumulation when exposed to 0.75 µmol m^{-2} s^{-1} (**Figure 3.1D**).

Disregarding the intensity of light exposure, the observed increase in phenolic content for cultures exposed to light (**Figure 3.1**) was anticipated, given the number of species that have also expressed increased phenolic accumulation when exposed to light (**DIAS *et al.*, 2016**; **NCUBE *et al.*, 2012b**). Phenolic compound biosynthesis is dependent on a PAL induced deviation from primary carbon metabolism, following photosynthesis (**BALASUNDRAM *et al.*, 2006**; **NCUBE *et al.*, 2012b**; **TAIZ and ZEIGER, 2010**; **VERPOORTE, 2000**). Furthermore, the production of these secondary phenolic metabolites is limited by the allocation of available carbon resources (**WINK, 1999**), obtained from the fixation of carbon by photosynthetic processes, which require exposure to light (**CORDELL, 2014**; **DOWNEY *et al.*, 2006**; **NCUBE *et al.*, 2012b**). Therefore, cultures exposed to 0.00 µmol m^{-2} s^{-1} light suffer a reduced availability of carbon resources derived from photosynthesis (**DOWNEY *et al.*, 2006**). Sucrose derived carbon resources, provided by the MS growth medium (**SAAD and ELSHAHED, 2012**), enabled the limited production of phenolic compounds by cultures devoid of light exposure

(**Figure 3.1**). However, the phenolic content of 0.00 µmol m^{-2} s^{-1} light intensity cultures did not demonstrate consistency between PGR treatments (**Figure 3.1**).

3.3.2. Influence of auxin-to-cytokinin ratios on phenolic content

The effect of PGR manipulation on *E. autumnalis* callus growth (described in **section 2.3**), determined that 2,4-D, PIC, and KIN influenced the morphology (**LUCZKIEWICZ *et al.*, 2014**; **SIDHU, 2010**) of *E. autumnalis* callus (**Figure 2.5**, of **section 2.3**). However, these synthetically produced plant growth hormones (**BEYL *et al.*, 2015**) have demonstrated a regulatory effect on the production of secondary metabolites (**DIAS *et al.*, 2016**; **LUCZKIEWICZ *et al.*, 2014**; **PTAK *et al.*, 2013**) derived from the phenyl-propanoid pathway (**LUCZKIEWICZ *et al.*, 2014**), including phenolic compounds. The influence of PGR concentration and combination on phenolic production and accumulation was established by callus cultures that were exposed to a 0.00 µmol m^{-2} s^{-1} light intensity (**Figure 3.1**).

Both 2,4-D and PIC are synthetically produced herbicides which imitate the properties of naturally occurring auxins (**BEYL *et al.*, 2015**; **LUCZKIEWICZ *et al.*, 2014**; **TU *et al.*, 2001**). However, the effect of these auxin-like PGR's on phenolic production differs greatly, with PIC cultures producing a substantially higher phenolic content than 2,4-D cultures (**Figure 3.1**). This reduced phenolic content of 2,4-D cultures (**Figure 3.1 A** and **B**) is not exclusive to the model species (*E. autumnalis*), as 2,4-D appeared to terminate the production of secondary metabolites (**SIDHU, 2010**) in *Daucus carota*, *Morinda citrifolia* (**LUCZKIEWICZ *et al.*, 2014**), *Leucojum aestivum* (**PTAK *et al.*, 2013**), and *Cathranthus roseus* (**SIDHU, 2010**). Alternatively, the phenolic content of *Leucojum aestivum* did not significantly differ between 2,4-D and PIC cultures (**PTAK *et al.*, 2013**), as opposed to the substantially greater phenolic content obtained from PIC supplemented *E. autumnalis* callus cultures over 2,4-D cultures (**Figure 3.1**).

The addition of cytokinin to the roots and aerial segments of *Merwilla plumbea*, and callus cultures of *Vitis vinifera* resulted in an increased production of phenolic compounds (**DIAS *et al.*, 2016**). However, in *E. autumnalis* this increase in phenolic content was only observed when 2,5 µM KIN was added to 10 µM and 20 µM PIC callus cultures which were exposed to a light intensity of 0.00 µmol m^{-2} s^{-1} (**Figure 3.1 C** and **D**). This cytokinin induced increase in phenolic content was only achieved by *Cathranthus roseus* callus cultures that were initiated on an auxin rich medium which was later replaced by an auxin free medium containing a low cytokinin concentration (**SIDHU, 2010**). The addition of 2,5 µM KIN to

callus cultured under a 0.00 µmol m^{-2} s^{-1} light intensity, resulted in a slight yet significant (p = 0.0001) reduction in the phenolic content of 2,4-D (**Figure 3.1 A** and **B**), and 15 µM PIC (**Figure 3.1 C** and **D**) supplemented cultures. The phenolic content of 10 µM PIC cultures exposed to a light intensity of 0.00 µmol m^{-2} s^{-1} was raised slightly by the addition of 2,5 µM KIN, however a significant (p = 0.0001) increase in phenolic content was only achieved by the addition of 2,5 µM KIN to 20 µM PIC cultures under 0.00 µmol m^{-2} s^{-1} (**Figure 3.1 C** and **D**).

In the absence of exposure to light, variations in the concentration of 2,4-D failed to influence phenolic production (p = 0.172) significantly (**Figure 3.1 A** and **B**). The addition of 2,5 µM KIN significantly (p = 0.0001) reduced the phenolic content of 2,4-D cultures, with the concentration of 2,4-D having no significant (p = 0.229) effect (**Figure 3.1 A** and **B**). On the other hand, the concentration of PIC cultures exhibited a significant (p = 0.0001) effect on the phenolic content, with the greatest phenolic content (9.4715 GAE ± 0.2068) obtained from 15 µM PIC callus cultures (**Figure 3.1 C** and **D**). However, this significant effect of PIC concentration on phenolic content was not maintained when 2,5 µM KIN (p = 0.078) was added (**Figure 3.1 C** and **D**). Though the effect of auxin concentration did not influence phenolic content in cultures exposed to a 0.00 µmol m^{-2} s^{-1} light intensity (**Figure 3.1**), however the effect of auxin concentration on phenolic content is substantially influenced by the intensity of light exposure (**Figure 3.1**).

3.3.3. Effect of PGR and light intensity variation on phenolic content

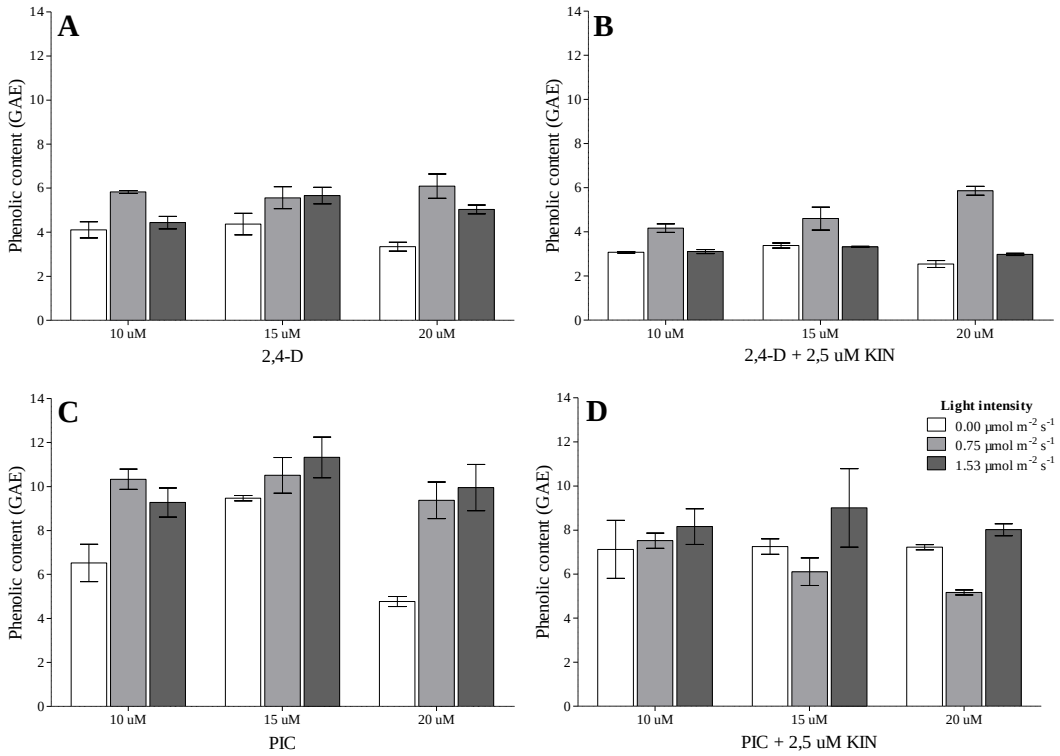

Figure 3.1: The influence of light intensity on the (mean ± SE) phenolic content produced by *E. autumnalis* callus cultured on (**A**) 2,4-D, (**B**) 2,4-D + 2,5 µM KIN, (**C**) PIC, (**D**) PIC + 2,5 µM KIN supplemented MS media. Phenolic content is measured as gallic acid equivalents (GAE).

Exposure to light resulted in an overall increase in phenolic content (**Figure 3.1**), suggesting that photo-receptive cryptochrome proteins in callus cells identified an increased exposure to harmful UV radiation (**DIAS *et al.*, 2016**; **LILLO *et al.*, 2008**; **NCUBE *et al.*, 2012b**; **TAIZ and ZEIGER, 2010**). Though cultures only require light wavelengths of 400-700 nm for photosynthesis (**ECONOMOU and READ, 1987**; **NCUBE *et al.*, 2012b**; **STANLY *et al.*, 2011**; **TAIZ *et al.*, 2014**; **TAIZ and ZEIGER, 2010**), the white light fluorescent tubes used in culture rooms emit a broader range of the light spectrum (**ECONOMOU and READ, 1987**; **KOZAI *et al.*, 1997**), thus exposing cultures to 280-400 nm wavelengths of UV radiation (**DIAS *et al.*, 2016**; **NCUBE *et al.*, 2012b**; **TAIZ and ZEIGER, 2010**). Furthermore, light intensity does not influence the spectral range of light exposure but rather the amount of light energy that callus cultures were exposed to per sec (**TAIZ *et al.*, 2014**). Thus, an incline in light intensity results in the increased exposure of callus cultures to both photosynthetic and harmful wavelengths (**BOURGAUD *et al.*, 2001**). The defensive mechanism employed by plants exposed to UV light

entails the synthesis of secondary metabolites which absorb these deleterious wavelengths (**DIAS *et al.*, 2016**; **NCUBE *et al.*, 2012b**; **SEIBERT *et a*L., 1975**; **TAIZ and ZEIGER, 2010**; **WINK, 1999**). Phenolic compounds provide an optimal protective function, filtering wavelengths through to photosynthetic cells, while simultaneously blocking further infiltration of deleterious UV wavelengths (**NCUBE *et al.*, 2012b**; **TAIZ and ZEIGER, 2010**). This positive correlation between light intensity and the accumulation of phenolic compounds was exhibited by 15 µM 2,4-D (**Figure 3.2 A**), 15 µM and 20 µM PIC (**Figure 3.2 C**), and 10 µM PIC + 2.5 µM KIN (**Figure 3.2 D**) cultures.

This increased exposure to UV light induced the phenyl-propanoid derived (**NCUBE *et al.*, 2012b**) production of phenolic compounds (**DIAS *et al.*, 2016**; **SEIBERT *et al.*, 1975**). However, secondary metabolites produced in response to UV light exposure do not consist solely of phenolic compounds (**DIAS *et al.*, 2016**; **SEIBERT *et al.*, 1975**). Thus, phenolic content of 10 µM PIC (**Figure 3.2 C**), 10 µM and 20 µM 2,4-D (**Figure 3.2 A**), and 2,4-D + 2,5 µM KIN (**Figure 3.2 B**) cultures increased significantly (p = 0.0001) under a 0.75 µmol m^{-2} s^{-1} light intensity, with further increase in light intensity to 1.53 µmol m^{-2} s^{-1} yielding a reduced phenolic content. The production of alternative defence metabolites, such as alkaloids (**MAKKAR *et al.*, 2007**), would result from the allocation of carbon resources to alkaloid production over phenolic production (**WINK, 1999**). However, the absorption of harmful UV wavelengths by these defensive alkaloids does not allow for the selective filtration of wavelengths required for photosynthesis (**NCUBE *et al.*, 2012b**; **TAIZ and ZEIGER, 2010**). Therefore, it is possible that at the lower light intensity of 0.75 µmol m^{-2} s^{-1} fewer UV absorbing metabolites were present, allowing for a limited level of photosynthetic carbon fixation to occur (**CORDELL, 2014**; **DOWNEY *et al.*, 2006**; **NCUBE *et al.*, 2012b**), increasing the availability of carbon resources, providing for carbon allocation to phenolic compound production (**WINK, 1999**).

Finally, the reduction in phenolic content from 0.00 µmol m^{-2} s^{-1} to 0.75 µmol m^{-2} s^{-1}, followed by a substantial increase in phenolic content at 1.53 µmol m^{-2} s^{-1} light intensity observed for 15 µM PIC + 2,5 µM KIN, and 20 µM PIC + 2,5 µM KIN supplemented callus cultures (**Figure 3.2 D**), could be explained by the combined effect of PGR and light intensity treatments. High 2,4-D and PIC concentrations should inhibit the production of secondary metabolites (**DIAS *et al.*, 2016**; **LUCZKIEWICZ *et al.*, 2014**; **PTAK *et al.*, 2013**; **TU *et al.*, 2001**), however both of these synthetically produced PGR's (**BEYL *et al.*, 2015**) have demonstrated a susceptibility to photo-degradation as *in vivo* herbicides, with 2,4-D exhibiting rapid

acceleration of degradation in an *in vitro* environment (**Tu *et al.*, 2001**). This rapid degradation of 2,4-D *in vitro* (**Tu *et al.*, 2001**) could possibly explain why 15 µM 2,4-D + 2,5 µM KIN, and 20 µM 2,4-D + 2,5 µM KIN supplemented callus cultures (**Figure 3.2 B**), did not express a reduction in phenolic content of 0.75 µmol m^{-2} s^{-1} cultures and marked increase in 1.53 µmol m^{-2} s^{-1} cultures, similar to that of PIC (**Figure 3.2 D**). The low phenolic content of 15 µM and 20 µM PIC + 2,5 µM KIN cultures exposed to 0.75 µmol m^{-2} s^{-1} (**Figure 3.2 D**), displayed the minor potential of PIC degradation by photolysis (**Tu *et al.*, 2001**). However, the amplified production of phenolic compounds obtained from these cultures exposed to a light intensity of 1.53 µmol m^{-2} s^{-1} (**Figure 3.2 D**) demonstrates the positive correlation between light intensity and the capacity of PIC to be degraded by photolysis (**Tu *et al.*, 2001**).

3.4. Conclusions

Variation in PGR and light exposure influence the production and accumulation of phenolic compounds under *in vitro* culture conditions (**Taylor and van Staden, 2001a**). This chapter aimed to determine how the variation in PGR concentration and combination influenced the production of phenolic content of callus cultures exposed to three light intensity treatments. Three responses to light intensity were observed, with the concentration and combination of PGR having a significant effect on callus phenolic content. The concentration of 2,4-D had no effect on callus phenolic content, though the additional supplementation of 2,5 µM KIN significantly reduced phenolic production by these cultures. Furthermore, PIC concentration demonstrated an effect on callus phenolic content, though the addition of 2,5 µM KIN diminished this effect. Overall, PIC supplemented callus cultures produced greater quantities of phenolic compounds than 2,4-D cultures, however the intensity of light exposure demonstrated a significant effect on this phenolic production. First, an increase in light intensity was shown to increase phenolic production, presumably in response to the increase in UV radiation exposure. The phenolic compounds produced under these circumstances absorb harmful UV radiation while simultaneously allowing the filtration of wavelengths used by photosynthesis. This results in the fixation of carbon resources which are required for phenolic production. Alternatively, the increased exposure to harmful UV radiation under the 1.53 µmol m^{-2} s^{-1} light intensity, may induce the preferential production of alkaloids. These secondary metabolites however, absorb all wavelengths, preventing the photosynthetic fixation of carbon, further limiting the availability of carbon resources for phenolic production. This resulted in the optimal phenolic content being produced by callus

cultures exposed to the lower light intensity of 0. 75 µmol m^{-2} s^{-1}, with 1.53 µmol m^{-2} s^{-1} cultures producing lower phenolic contents. Finally, due to the inhibition of secondary metabolite production by synthetic 2,4-D and PIC, the phenolic content of all the callus cultures should have been reduced. However, 2,4-D and PIC are susceptible to photo-degradation by photolysis, which enabled the production of phenolic compounds by these callus cultures. Exposure of 15 µM PIC + KIN and 20 µM PIC + KIN cultures to the low light intensity of 0.75 µmol m^{-2} s^{-1} demonstrated this inhibition of secondary metabolite production. However, exposure to the higher light intensity of 1.53 µmol m^{-2} s^{-1} caused the degradation of PIC, resulting in an increased phenolic production.

CHAPTER 4: EFFECT OF THERMAL STRESS ON PHENOLIC PRODUCTION

4.1.Introduction

Exposure to thermal stress has a substantial undesirable effect on native and agricultural plants (**WANG *et al.*, 2003**), with adverse effects on global crop yields (**HALL, 2001**; **WAHID *et al.*, 2007**) impeding the pursuit of sustainable food production (**DUBEY, 1994**). Given that plant development and growth are permanently impaired by extensive thermal stress (**WAHID *et al.*, 2007**), plants have developed numerous response mechanisms (**NCUBE *et al.*, 2012b**; **WAHID *et al.*, 2007**), which enable thermo-tolerance (**WAHID *et al.*, 2007**). Thermo-tolerance is defined as the ability of a plant to survive (**NAGAO *et al.*, 1990**) and produce profitable yields under potentially fatal thermal conditions (**WAHID *et al.*, 2007**). The mechanisms employed by plants exposed to chronic thermal stress are dependent on the plants morpho-anatomical, phenological, and physiological state (**LARKINDALE *et al.*, 2005**; **NCUBE *et al.*, 2012b**; **WAHID *et al.*, 2007**). Furthermore, thermo-tolerance consists of long-term adaptation and short-term avoidance mechanisms (**WAHID *et al.*, 2007**). Long-term adaptations enable the sustainability of a species under continual thermal stress (**WAHID *et al.*, 2007**). These long term adaptations predominantly influence the reproductive functions in plants, having a substantial effect on crop yields (**WAHID *et al.*, 2007**). On the other hand, short-term thermo-tolerance allows for the rapid, yet temporary, assimilation of plant cells to thermal stress events (**LARKINDALE *et al.*, 2005**). These ephemeral thermal stress response mechanisms are predominantly anatomical, or physiological in form, though the provocation of these response mechanisms is not limited to thermal stress (**AGARWAL and ZHU, 2005**; **NCUBE *et al.*, 2012b**; **WAHID *et al.*, 2007**). For example, anatomical response mechanisms, which primarily influence photosynthetic organelles, correspond to drought stress (**NCUBE *et al.*, 2012b**; **WAHID *et al.*, 2007**); and physiological response mechanisms to thermal stress are often mirrored by osmotic / water stress responses (**WAHID *et al.*, 2007**). Disregarding these thermal stress response mechanisms, the quintessential mechanism of abiotic stress response in plants has been attributed to the activity of PAL (**WAHID *et al.*, 2007**).

4.1.1. Influence of thermal stress on metabolic processes

The deleterious impact of thermal stress in plant cells is due to the sensitivity of photosynthetic processes to heat (**LARKINDALE *et al.*, 2005**; **WAHID *et al.*, 2007**). Plant cell

temperature increases with the elevation of atmospheric temperature, or in response to direct thermal radiation exposure (**DOWNEY *et al.*, 2006**). This thermal incline enhances the permeability of the plasma membrane (**WAHID *et al.*, 2007**) as raised temperatures accelerate molecular movement through the plasma membrane (**WALLACE *et al.*, 1996d**) of mesophyll cells. Subsequent deactivation of chloroplast and mitochondrial enzymes (**WAHID *et al.*, 2007**) hamper photosynthetic processes (**LARKINDALE *et al.*, 2005**; **WAHID *et al.*, 2007**), and reduce energy (**WAHID *et al.*, 2007**). This disruption of photosynthetic and mitochondrial processes prevent the regulation of reactive oxygen species (ROS) generation and abstraction (**AGARWAL and ZHU, 2005**), resulting in the accumulation of these deleterious compounds (**DE KLERK, 2007**; **WAHID *et al.*, 2007**). Independent of regulatory mechanisms, ROS accumulation results in the irrevocable fatality of cells, organs, and whole plants (**AGARWAL and ZHU, 2005**), due to the oxidative destruction of lipids, DNA and proteins (**AGARWAL and ZHU, 2005**; **LARKINDALE *et al.*, 2005**; **WAHID *et al.*, 2007**).

4.1.2. Phenolic production induced by ROS

ROS assist the promotion and preservation of short-term thermo-tolerance assimilation (**WAHID *et al.*, 2007**), given that ROS accumulation denotes the detection of thermal stress (**AGARWAL and ZHU, 2005**; **DE KLERK, 2007**; **LARKINDALE *et al.*, 2005**; **WAHID *et al.*, 2007**). Peroxidation of pigments and lipid membranes (**WAHID *et al.*, 2007**) promotes the production of active oxygen species-scavenging enzymes (**NCUBE *et al.*, 2012b**), including phenolic compounds which express antioxidant potential (**NCUBE *et al.*, 2012b**; **WAHID *et al.*, 2007**). Furthermore, superoxide dismutase (SOD) overexpression influences the production of phenolic compounds due to the deterioration of cell wall lignin (**WAHID *et al.*, 2007**). Therefore, the antioxidant capacity of plant cells is amplified by the production of phenolic compounds in response to thermal incline (**WAHID *et al.*, 2007**). This chapter was aimed to determine if thermal stress influences the quantity of phenolic compounds produced by *E. autumnalis* callus cultures, *in vitro* cultured leaf tissue samples, and the leaves of whole *in vivo* plants.

4.2. Materials and Methods

Whole *in vivo* plants, sixteen-week-old *in vitro* cultured plantlets, and eighty-four-day-old callus cultures (described in **section 2.2**) were exposed to three thermal stress treatments. Furthermore, the leaf samples of both *in vitro* cultured plantlets and *in vivo* plants were separated into upper and lower segments. The comparison of phenolic contents between these

leaf segments may provide information regarding the possible transportation of these defensive metabolites from the site of biosynthesis (**CRONIN and HAY, 1996**; **WINK, 1999**) to the location of the cells which require metabolic defence. The upper and lower segments of leaf samples were determined by halving the length of each leaf, from the bulb to the apex of the leaf. Subsequent samples were collected from separate leaves, given that tissue injury may influence the production of phenolic metabolites.

4.2.1. Thermal stress simulation

Sixteen-week-old *E. autumnalis* plantlets and eighty-four-day-old callus cultures were sub-cultured into 100 mℓ Erlenmeyer flasks (**BALDI *et al.*, 2009**) containing 30 mℓ in autoclave sterilized MS suspension media. The suspension media for the callus cultures were additionally supplemented with the respective PGR combinations (as described in **section 2.2.2.1**). These cultures were then placed under a constant (125 rpm) stirring condition to prevent anaerobic fermentation (**LEVA *et al.*, 2012**), in growth rooms of a constant 25 ºC (± 3 ºC) temperature, 0.75 µmol m^{-2} s^{-1} light intensity, and sixteen hour light / eight hour dark cycling for twelve days (**BALDI *et al.*, 2009**). Following this assimilation period these suspension cultures were exposed to three thermal stress temperatures simulated using a Gallenkamp Orbital Incubator, which provided the continuous suspension shake of 125 rpm (**BALDI *et al.*, 2009**), in addition to maintaining a constant thermal stress of the specified temperature. Furthermore, the thermal stress of *in vivo* plants was simulated using Convirons (Controlled environments Ltd, Winnipeg, Manitoba, Canada), which also maintained a constant temperature of the specified thermal stress treatment, yet these samples did not require shaking / stirring. Samples were exposed to temperatures of 30 ºC, 35 ºC, and 40 ºC; for a total duration of twenty hours. Samples were collected in hourly increments for the first five hours, following which, heat shock treatments were imposed by removing samples from the thermal stress environment for 30 min, placing samples in a cool room of 25 ºC (± 3 ºC), following which samples were returned to the thermal stress environment for five hours. This was repeated four times over a total 20 hr period. The phenolic content was determined (as described in **section 3.2.1**) for each sample exposed to the different temperatures for various durations.

4.2.2. Data Analysis

Three samples were randomly selected from each PGR replicate for each temperature treatment following the specified exposure durations. The phenolic content of samples

collected at the specified durations of exposure to 30 ºC, 35 ºC, and 40 ºC thermal treatments, were compiled for callus cultures (**Figure 4.2**), *in vitro* cultured leaf tissue samples, and leaves of whole *in vivo* plants (**Figure 4.4**). Two-way ANOVA's were used to compare how each thermal stress treatment influenced the quantity of phenolic compounds produced by each callus PGR combination (**Figure 4.1**), *in vitro* cultured tissue samples (**Figure 4.3 A**), and leaves of whole *in vivo* plants (**Figure 4.3 B**). Log transformations were used to obtain a normal distribution of residuals, where the data was positively skewed. Duncan's Multiple Range Test's (DMRT) were used to separate the means of homogeneous subset groups which differed at $p = 0.05$, for each sample type (callus PGR combination, *in vitro* cultured tissue sample, and leaves of whole *in vivo* plants). All analyses were performed using IBM SPSS Statistics for Windows, version 24, and all graphs were compiled using GraphPad Prism® version 5.02.

4.3. Results and Discussion

Provided that thermal stress signalling by ROS (**DE KLERK, 2007**; **NCUBE *et al.*, 2012b**; **WAHID *et al.*, 2007**) accelerates PAL activity (**DOWNEY *et al.*, 2006**; **RIVERO *et al.*, 2001**; **WAHID *et al.*, 2007**), the production and accumulation of phenolic compounds should correlate with thermal stress exposure (**DIAS *et al.*, 2016**; **DOWNEY *et al.*, 2006**; **LUCZKIEWICZ *et al.*, 2014**; **NCUBE *et al.*, 2012b**; **PTAK *et al.*, 2013**; **WAHID *et al.*, 2007**). These phenolic compounds assist the thermo-tolerance of plants as deterrents of oxidative stress (**NCUBE *et al.*, 2012b**, **2011**; **WAHID *et al.*, 2007**). However, this thermo-tolerance mechanism is not exclusively dependent on the severity (temperature) of thermal stress (**NCUBE *et al.*, 2012b**; **WAHID *et al.*, 2007**). The production of phenolic compounds in response to thermal stress is dependent on; the severity, and duration of heat exposure (**LARKINDALE *et al.*, 2005**; **NCUBE *et al.*, 2012b**; **WAHID *et al.*, 2007**), and varies relative to the plants phenological phase (**NCUBE *et al.*, 2011**; **WAHID *et al.*, 2007**).

4.3.1. Influence of thermal stress on phenolic content of callus

The control measure of phenolic content produced by callus prior to thermal stress (25 ºC, **Figure 4.1** and 0 hr, **Figure 4.2**) was obtained from callus suspension cultures following a twelve day assimilation period (**BALDI *et al.*, 2009**).

Thermal stress of *E. autumnalis* callus cultures had a significant ($p = 0.0001$) effect on phenolic content, with a thermal incline of 5-10 ºC provoking the production and

accumulation of phenolic compounds as a thermal stress response mechanism (**WAHID *et al.*, 2007**). Overall, the phenolic content of callus cultures exposed to 30 ºC ≥ 35 ºC > 40 ºC > 25 ºC (**Figure 4.1**). Breach of thermal threshold diminishes the production of phenolic compounds (**DOWNEY *et al.*, 2006**) due to the inhibition of rubisco activation during the calvin cycle (**WAHID *et al.*, 2007**) resulting in the reduced fixation of carbon (**CORDELL, 2014**; **DOWNEY *et al.*, 2006**; **NCUBE *et al.*, 2012*b***; **WAHID *et al.*, 2007**). Thus the thermo-tolerance of a sample is reduced at temperatures above the thermal threshold (**WAHID *et al.*, 2007**). The thermal threshold of *E. autumnalis* callus cultures was established at 30 ºC, with the exception of 2,4-D cultures which produced a greater phenolic content at 35 ºC (**Figure 4.1**). This is due to the effect of the culture environment on the samples sensitivity to thermal stress (**HAVAUX, 1992**; **LARKINDALE *et al.*, 2005**; **WAHID *et al.*, 2007**).

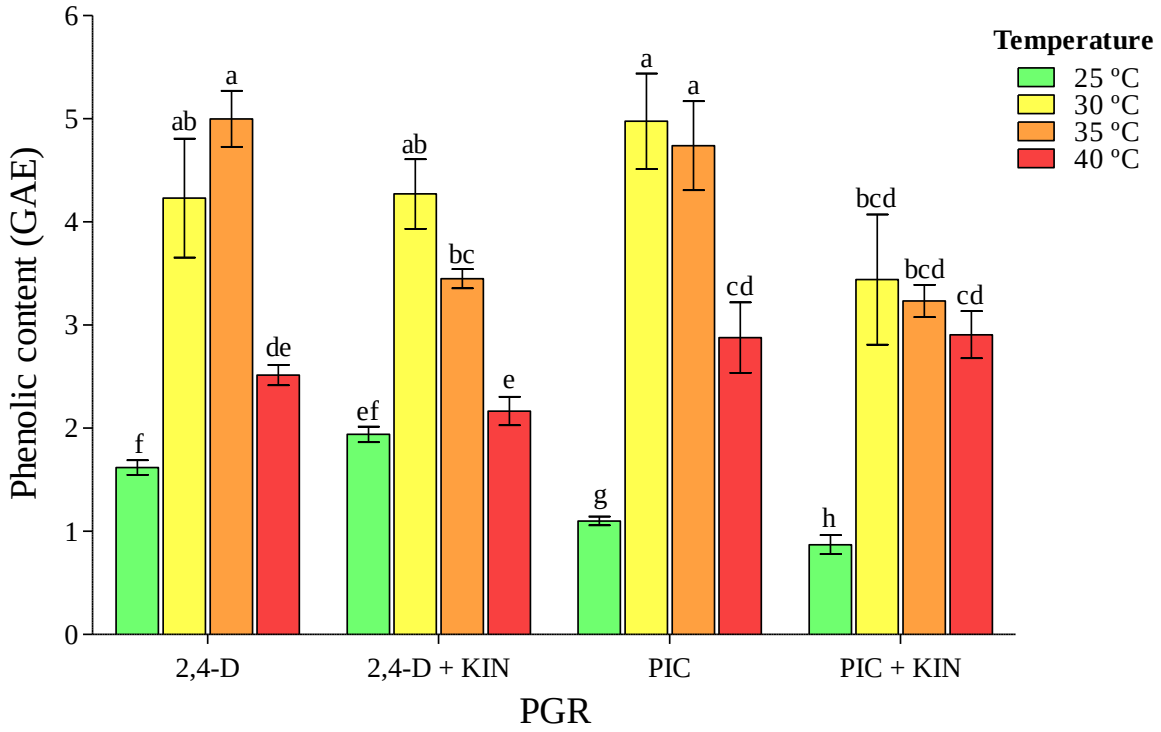

Figure 4.1: Influence of thermal stress temperature on the mean (± SE) phenolic content produced by callus cultures. Phenolic content is measured as gallic acid equivalents (GAE).

Synthetic 2,4-D and PIC inhibit the production of phenyl-propanoid derived phenolic compounds (**DIAS *et al.*, 2016**; **LUCZKIEWICZ *et al.*, 2014**; **PTAK *et al.*, 2013**). The inhibitory activity of PIC > 2,4-D in callus suspension cultures, therefore the phenolic content of 2,4-D > PIC, with the additional supplementation of KIN having opposing effects on phenolic

production (2,4-D + KIN ≥ 2,4-D > PIC > PIC + KIN; at 25 ºC). The exposure of PIC cultures to a thermal incline of only 5 ºC from 25 ºC caused a substantial increase in phenolic compound production and accumulation (**Figure 4.1**). However, this amplified phenolic production in response to thermal incline was substantially greater in PIC cultures than 2,4-D cultures (**Figure 4.1**). This suggests that the inhibition of phenolic production by PIC is significantly reduced by thermal incline, resulting in a significantly greater phenolic production by PIC callus cultures exposed to elevated temperatures. Furthermore, the influence of thermal incline on phenolic production over time (**Figure 4.2**) by callus cultures differs between PGR combinations.

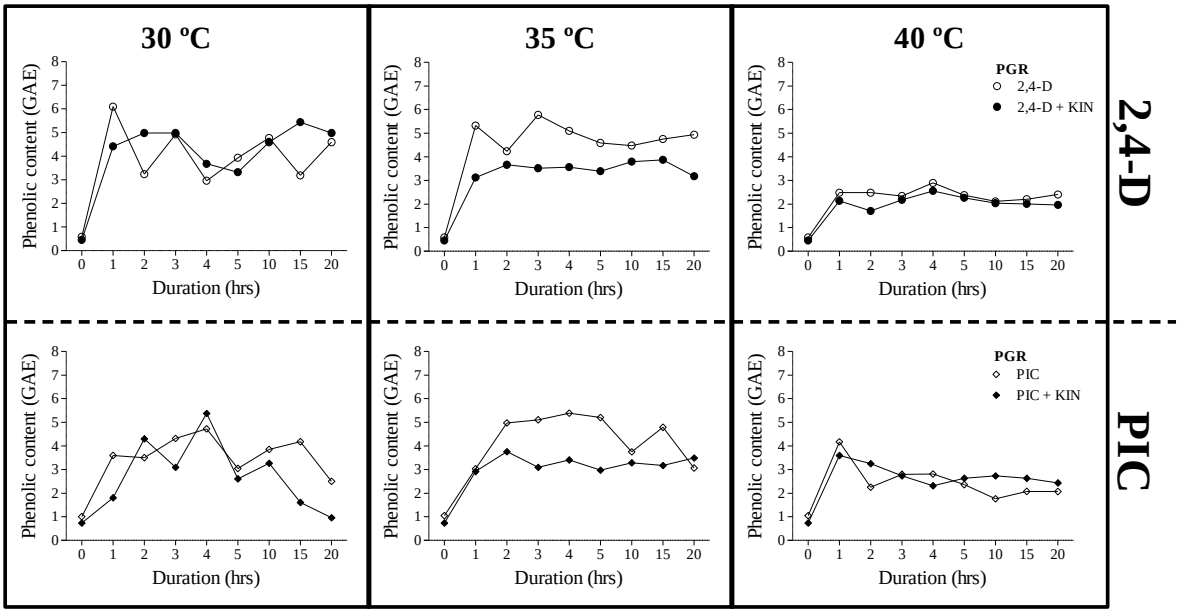

Figure 4.2: Influence of temperature and duration of exposure on the mean phenolic content of callus. Phenolic content measured in gallic acid equivalents (GAE).

The production and accumulation of phenolic compounds is significantly (p = 0.0001) affected by thermal incline within the first hour of exposure (**Figure 4.2**). This is due to the chemical nature of this response mechanism, as metabolic thermo-tolerance provokes an instantaneous response to thermal incline (**POLLOCK *et al.*, 1993**). Subsequent to this initial surge, phenolic accumulation is reduced (**Figure 4.2**), denoting the ephemeral nature of this thermo-tolerance mechanism (**LARKINDALE *et al.*, 2005**). However, this thermo-tolerance is maintained in correlation with the increasing duration of thermal stress exposure (**Figure 4.2**), and does not completely dissipate (**LARKINDALE *et al.*, 2005**), with the exception of PIC + KIN cultures exposed to 30 ºC (**Figure 4.2**). **LARKINDALE *et al.*** (**2005**) suggests that response metabolite accumulation would completely dissipate within twenty-four hours

leaving the cell with no mechanism of thermo-tolerance. However, the retention of phenolic content over time (**Figure 4.2**) suggests that continuous exposure to thermal stress, continued to produce ROS which is regulated by the persistent production of phenolic compounds, maintaining the thermo-tolerance of these cultures. However, this may also be due to the reduced activity of PAL over time, caused by thermal damage of the biosynthetic pathway (**LOAIZA-VELARDE *et al*., 1997**).

The additional supplementation of KIN reduced the variability in phenolic content of 2,4-D and PIC cultures exposed to 35 ºC (**Figure 4.2**). Though phenolic production in *Merwilla plumbea* and *Vitis vinifera* cultures was enhanced by the additional supplementation of KIN (**DIAS *et al*., 2016**), thermal incline resulted in the diminished production of phenolic compounds (**Figure 4.2**), as cytokinin content is reduced by thermal stress (**WAHID *et al*., 2007**). Though the secretion of phenolic compounds into the culture medium might explain the variability in phenolic content observed in cultures exposed to 30 ºC (**GAOSHENG and JINGMING, 2012**), the volatilization potential of 2,4-D and PIC may also influence this variability (**TU *et al*., 2001**). The volatilization of these PGR is simply the conversion of their liquid form into vapour, which is stimulated by the acidic pH and abundance of moisture in these suspension cultures (**TU *et al*., 2001**). Vaporized 2,4-D and PIC is retained within the culture vessel, yet the inhibition of secondary metabolite production by these PGR's is alleviated (**SIDHU, 2010**).

4.3.2. Influence of thermal stress on phenolic content of leaves

The susceptibility of plant cells to thermal stress differs between developmental phases (**WAHID *et al*., 2007**). Leaf segments of *in vitro* cultured plantlets produced and accumulated phenolic compounds in response to thermal incline up to a thermal threshold of 35 ºC, with plantlet cultures exposed to 40 ºC producing a significantly (p = 0.003) lower phenolic content (**Figure 4.3 A**). This thermal threshold corresponds with that of callus cultured on 2,4-D supplemented MS media (**Figure 4.1**), the phenolic content of *in vitro* cultured plantlet leaves duplicates that of the callus cultures.

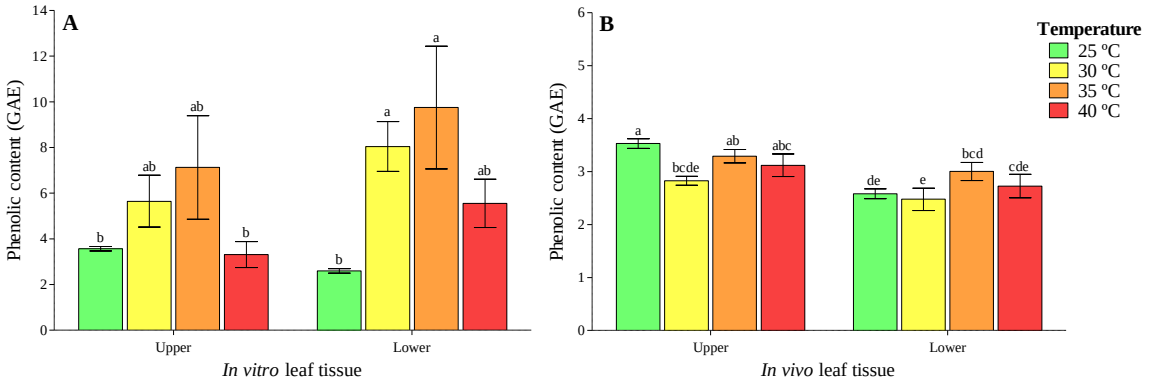

Figure 4.3: Influence of thermal stress temperature on the mean (± SE) phenolic content, produced by (**A**) *in vitro* cultured plantlet leaf segments, and the (**B**) leaf segments of whole *in vivo* plants. *In vitro* plantlets were cultured on PGR free MS media under a constant light intensity of 0.75 µmol m^{-2} s^{-1}, and *in vivo* plants were not subjected to any pre-treatment of PGR or exposure to various light intensities. Phenolic content measured in gallic acid equivalents (GAE).

Though callus formations lack developmental and morphological specialization (**SIDHU, 2010**), and *in vitro* cultured plantlets have developed cellular specialization, the production of phenolic compounds in response to thermal incline does not differ between phenological phases (**WAHID *et al.*, 2007**). However, the quantity of phenolic compounds produced by *in vitro* cultured plantlet leaves (**Figure 4.3**) duplicated that of the callus cultures (**Figure 4.1**). Furthermore, both callus and plantlets have been cultured *in vitro* under extensively regulated environmental conditions (**HUSSAIN *et al.*, 2012**). Due to the lack of exposure to the numerous forms of environmental stress (**NCUBE *et al.*, 2012b**) these samples have not acquired long-term environmental stress response mechanisms (**LARKINDALE *et al.*, 2005**) prior to the simulated thermal stress treatment. Furthermore, though the variation between phenolic content of upper and lower *in vitro* cultured leaf segments (**Figure 4.3**) did not differ significantly (p = 0.119), thermal incline influences the metabolic processes of leaves and bulbs, in a source (leaves) to sink (bulb) mechanism of phenolic compound translocation (**WAHID *et al.*, 2007**).

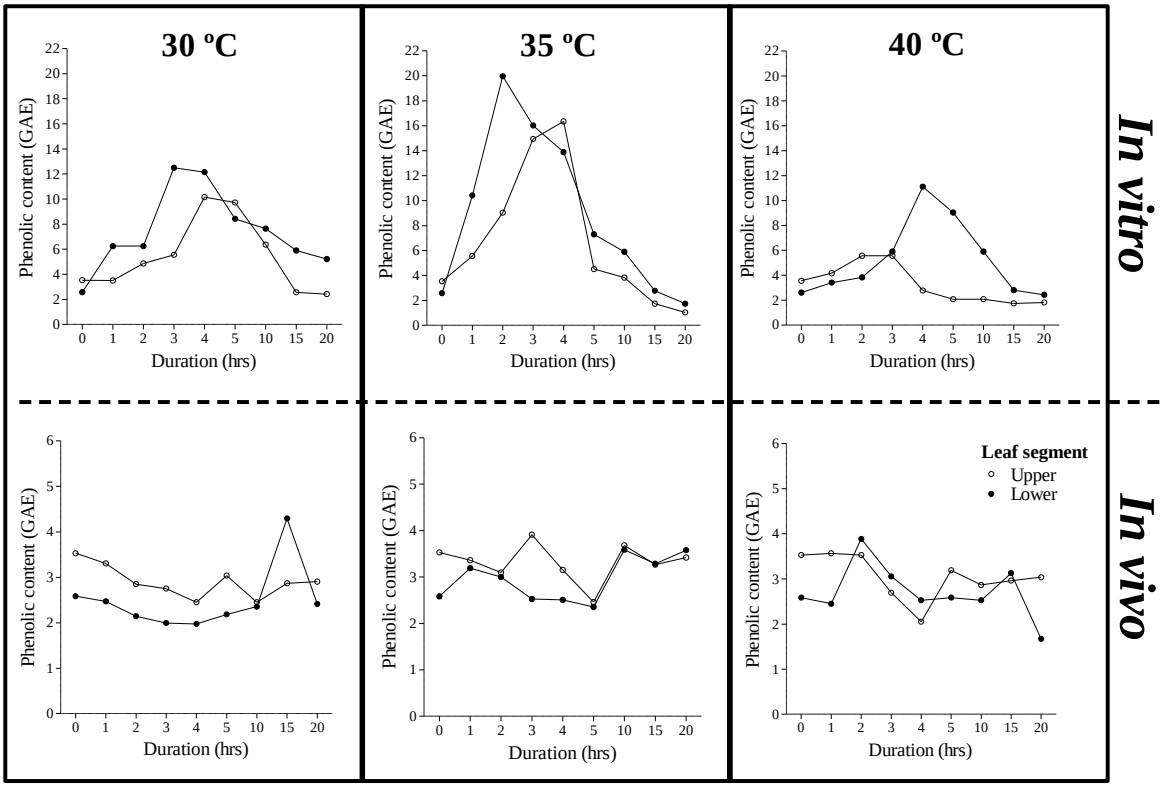

Figure 4.4: Influence of temperature and duration of exposure on the mean phenolic content of *in vitro* cultured plantlet leaves and leaves of whole *in vivo* plants. *In vitro* plantlets were cultured on PGR free MS media under a constant light intensity of 0.75 µmol m^{-2} s^{-1}, and *in vivo* plants were not subjected to any pre-treatment of PGR or exposure to various light intensities. Phenolic content measured in gallic acid equivalents (GAE).

Phenolic content of *in vitro* cultured plantlets was substantially increased by thermal stress initiation (**Figure 4.4**) denoting the metabolic foundation of thermal stress response (**POLLOCK *et al.*, 1993**). Furthermore, subsequent to this accumulation of phenolic compounds, phenolic content was depleted (**Figure 4.4**) due to the extended duration of thermal stress exposure reducing the activity of PAL (**LOAIZA-VELARDE *et al.*, 1997**).

On the other hand, *in vivo* leaf samples exposed to the various temperatures failed to produce a discernible pattern of phenolic production or accumulation in response to thermal incline (**Figure 4.3 B**) or duration of exposure to thermal stress (**Figure 4.4**). Due to the fact that *in vivo* plants are exposed to numerous environmental stresses prior to the thermal stress simulation (**HAVAUX, 1992**), these plants presumably possess inherent long-term stress response mechanisms (**LARKINDALE *et al.*, 2005**), which would deter the production of metabolites that deplete limited resources (**ECONOMOU and READ, 1987**; **NCUBE *et al.*, 2012b**; **STANLY *et al.*, 2011**). Furthermore, the phenolic content of upper and lower leaf

segments of these plants do not significantly differ (**Figure 4.3 B**), though phenolic tannin production is known to increase in response to osmotic stress (**NCUBE *et al.*, 2012b**, **2011**) which is induced by thermal stress in natural environments (**HAVAUX, 1992**).

4.4. Conclusions

Metabolic processes accelerate under thermal stress conditions (**DOWNEY *et al.*, 2006**), which result in the accumulation of ROS among other metabolites (**DE KLERK, 2007**; **DOWNEY *et al.*, 2006**; **NCUBE *et al.*, 2012b**; **WAHID *et al.*, 2007**). ROS accumulation in the cell cytoplasm (**WAHID *et al.*, 2007**) function as heat stress signals (**AGARWAL and ZHU, 2005**) provoking the acceleration of PAL activity (**RIVERO *et al.*, 2001**; **WAHID *et al.*, 2007**). This amplification of PAL activity induces the production of phenyl-propanoid pathway derived phenolic compounds (**DIAS *et al.*, 2016**; **LUCZKIEWICZ *et al.*, 2014**; **NCUBE *et al.*, 2012b**; **PTAK *et al.*, 2013**; **WAHID *et al.*, 2007**), which are quintessential to the acclimation of plant cells exposed to thermal stress (**RIVERO *et al.*, 2001**; **WAHID *et al.*, 2007**).

CHAPTER 5: GENERAL CONCLUSIONS

5.1. Influence of the *in vitro* culture environment

Biotechnological tools such as *in vitro* plant culture have enabled the mass production of endangered species, including the model species *Eucomis autumnalis*. Furthermore, *in vitro* methods have been used to improve the production of desired metabolites, as the manipulation of a culture environment either enhances or inhibits metabolic pressures which influence the induction of secondary metabolite production. The enhanced production of desired phenolic compounds, derived from the phenyl-propanoid pathway, is dependent on the activity of phenylalanine ammonia-lyase (PAL) which is vulnerable to environmental influence. Thus, *in vitro* culture methods provide the ideal condition for environmentally induced phenolic compound production, due to the rigorous regulation of the culture environment. Provided that secondary metabolite production may be induced *in vitro* by the metabolic processes involved in cellular differentiation and specialization, callus cultures of *E. autumnalis* were established under *in vitro* conditions.

Variation in the culture environment had a significant effect on the growth, morphology and phenolic content of *E. autumnalis* callus cultures. The rate of callus growth was greatest for cultures exposed to a light intensity of 1.53 µmol m^{-2} s^{-1}, followed by the lower light intensity of 0.75 µmol m^{-2} s^{-1} with the slowest callus growth rate obtained from cultures exposed to a 0.00 µmol m^{-2} s^{-1} light intensity. However, the final volume of most callus cultures exposed to the lower light intensity of 0.75 µmol m^{-2} s^{-1} succeeded that of the higher light intensity (1.53 µmol m^{-2} s^{-1}) following an exposure duration of seventy to eighty-four days, with the exception of 10 µM PIC + 2,5 µM KIN and 20 µM PIC + 2,5 µM KIN cultures (**Figure 2.2** and **Figure 2.3**). These cultures exposed to the higher light intensity of 1.53 µmol m^{-2} s^{-1} produced a lower final callus volume than the lower light intensity (0.75 µmol m^{-2} s^{-1}) cultures as cultures exposed to 1.53 µmol m^{-2} s^{-1} achieved a maximum growth rate sooner than cultures exposed to the lower light intensity of 0.75 µmol m^{-2} s^{-1}. Due to this rapid progression of callus growth in 1.53 µmol m^{-2} s^{-1} callus cultures, the final callus volumes were substantially reduced due to cellular necrosis.

This was substantially evident in 2,4-D cultures exposed to a 1.53 µmol m^{-2} s^{-1} light intensity. This rate of callus growth was accelerated by the increase in 2,4-D concentration with the additional supplementation of 2,5 µM KIN further accelerating this growth rate across all

three light intensity treatments. This acceleration in growth rate was mirrored by the final callus volume which was measured subsequent to the eighty-four-day growth period (10 µM 2,4-D < 15 µM 2,4-D ≤ 10 µM 2,4-D + 2,5 µM KIN < 20 µM 2,4-D < 15 µM 2,4-D + 2,5 µM KIN < 20 µM 2,4-D + 2,5 µM KIN). 2,4-D cultures exposed to a 1.53 µmol m^{-2} s^{-1} light intensity produced nodular compact callus, with cultures exposed to lower light intensities of 0.75 µmol m^{-2} s^{-1}, and 0.00 µmol m^{-2} s^{-1} producing compact callus which potentially restricted the inner cells from accessing oxygen, subsequently resulting in cell death.

Alternatively, increasing PIC concentrations subsequently reduced the rate of callus growth, with the additional supplementation of 2,5 µM KIN further diminishing the rate of callus growth, which was reflected by the final callus volumes acquired following the eighty-four-day growth period (20 µM PIC + 2,5 µM KIN < 15 µM PIC + 2,5 µM KIN < 20 µM PIC < 10 µM PIC + 2,5 µM KIN ≤ 15 µM PIC < 10 µM PIC). The effect of light intensity and PIC concentration on the callus growth rate and final volume was substantially reduced compared to the 2,4-D cultures. These reduced callus volumes of PIC cultures were obtained due to the friable nature of the callus, resulting in multiple smaller formations as opposed to the singular mass formation of the compact callus.

The phenolic content of callus cultures exposed to these culture environment variations was determined subsequent to the eighty-four-day growth period. The phenolic content of callus cultures was significantly influenced by certain variations in the PGR combination and concentration, and by exposure to the three light intensities. Callus phenolic content was not dependent on 2,4-D concentration, yet the additional supplementation of 2,5 µM KIN reduced the phenolic content of 2,4-D cultures significantly. On the other hand, the phenolic content of PIC cultures was influenced by concentration, though this effect was diminished by the addition of 2,5 µM KIN. In general, the greater phenolic content was obtained from PIC cultures, however, phenolic production was substantially influenced by the intensity of light exposure. An increase in exposure to UV radiation was assumed when cultures were exposed to amplified light intensities, potentially resulting in the enhanced production of phenolic compounds. These compounds permit the filtration of photosynthetic wavelengths while simultaneously absorbing UV radiation which is harmful. Through photosynthesis carbon resources become fixed, providing for the production of phenolic compounds further. However, this production of phenolic compounds was reduced when cultures were exposed

to the 1.53 µmol $m^{-2}s^{-1}$ light intensity, as the elevated exposure to harmful UV radiation potentially induced the preferential production of alternative secondary metabolites such as alkaloids. Such metabolites prevent the filtration of all wavelengths to photosynthetic processors, halting the fixation of carbon resources, thus limiting the production of phenolic compounds. Therefore, a light intensity of 0.75 µmol m^{-2} s^{-1} was determined to result in the optimal production of phenolic compounds in callus cultures. Given that synthetic auxin substitutes such as 2,4-D and PIC inhibit the production of secondary metabolites, a reduced phenolic production was anticipated, however these auxin substitutes are broken down by photolysis, which enabled phenolic production. This inhibition of phenolic production was observed in 15 µM PIC + KIN and 20 µM PIC + KIN cultures exposed to the light intensity of 0.75 µmol m^{-2} s^{-1}, though this inhibition effect was reduced at 1.53 µmol m^{-2} s^{-1} due to the photo-degradation of the PIC, resulting in a greater phenolic content.

The production of high callus volumes and low phenolic contents by 2,4-D callus cultures demonstrates the prioritization of primary metabolic growth over the production of protective secondary metabolites which is typically induced by auxin-like PGR's. However, PIC cultures produced low callus volumes which contained high concentrations of phenolic compounds. This could possibly be due to the morphological character of each callus culture. The compact callus formations of 2,4-D cultures restricts the inner cells access to oxygen among other resources which are required for the production of phenolic compounds. However, the friable formation of PIC callus cultures provided for the effortless acquisition of required resources. Furthermore, in the event of phenolic production in response to elevated UV radiation exposure, the compact formation of 2,4-D callus cultures demonstrate a restricted photosynthetic capability of the inner cells, thus fixed carbon resources would be limited, resulting in the preferential synthesis of alternative protective metabolites.

5.2. Stimulating phenolic compound production by heat stress

Despite the susceptibility of *in vitro* cultures to their environment, the production of phenolic compounds derived from the phenyl-propanoid pathway may be enhanced by thermal stress. The chloroplasts and mitochondrial enzymes responsible for the production and removal of reactive oxygen species (ROS) are adversely influenced by thermal incline. ROS among other metabolites are accumulated under thermal stress due to the acceleration of metabolic processes. PAL activity accelerates when ROS accumulates in the cell cytoplasm, stimulating the production of scavenging enzymes. Furthermore, the accelerated activity of PAL induces

the production of phenolic compounds via the phenyl-propanoid pathway. These phenolic compounds elevate the plant cells antioxidant capacity, and are thus quintessential to plant cell acclimation under thermal stress conditions. Over a 20 hour period the accumulation of phenolic compounds was determined for callus, and leaves of intact *in vivo* plants and *in vitro* cultured plantlets. The phenolic content of callus cultures and the leaves of *in vitro* cultured plantlets was increased by thermal incline, though breach of the thermal threshold resulted in reduced phenolic production. Furthermore, abiotic heat stress is dependent on thermal incline and exposure duration, resulting in a rapid production of phenolic compounds, providing a response mechanism of thermo-tolerance. However, this thermo-tolerance mechanism is short-lived, resulting in the subsequent reduction in phenolic compound production. The ephemeral nature of this thermal stress response mechanism is evident in the complete reduction of phenolic content produced by *in vitro* cultured plantlet leaves subsequent to an initial surge in phenolic production. On the other hand, subsequent to an initial surge, the phenolic content of callus cultures declined, yet was retained throughout the 20 hour period of thermal stress exposure, sustaining the cultures thermo-tolerance. However, callus culture PGR combinations influenced the rate at which thermo-tolerance was achieved, and the level of thermo-tolerant phenolic production. A rapidly achieved level of thermo-tolerance was reduced in callus cultures by both the additional supplementation of KIN, and the breach of thermal threshold. Though thermal stress resulted in a relatively constant phenolic compound production in callus cultures, this phenolic content was duplicated by that of the *in vitro* cultured plantlet leaves, where the leaves of intact *in vivo* plants failed to display any significant relationship between thermal stress and the production of phenolic compounds.

5.3. Enhancing the production of phenolic compounds

The manipulation of *in vitro* culture environments has influenced the production of these desired medicinal metabolites. Though the greatest quantity of phenolic compounds produced was obtained from *in vitro* cultured plantlet leaves, the *in vitro* production of callus cultures remains valuable. Callus cultures could be used as a source for the proliferation of more callus and/or the growth medium could be manipulated to initiate organogenesis and whole plantlet growth *in vitro*. Furthermore, the exposure of callus cultures to environmental variation demonstrated a significant effect on phenolic compound production. Given that the manipulation of culture environments has influenced the production of phenolic compounds in callus cultures, further research is required to determine if this physiological response is

transferred from pre-treated totipotent callus to the plantlets produced by these callus cultures. Though 2,4-D has been preferentially used for the induction of medicinal plant callogenesis, the morphological characteristics of these callus cultures has hampered their sustainability, proliferation ability, and enhancement of phenolic production. Alternatively, callus cultured on PIC supplemented MS media were friable, which is ideal for callus proliferation, and produced significantly greater quantities of phenolic compounds, which were substantially increased in response to thermal manipulation. The friable nature of these PIC cultures became problematic in the collection of samples as the callus often broke apart during extraction from the suspension medium. The difficulty in separating the callus from suspension medium meant that phenolic assays may have been assessing the quantity of phenolic compounds of the callus and any phenolic compounds which may have been released into the suspension media. On the other hand, if 2,4-D cultures released any phenolic compounds into the suspension media, the ease of separating the callus from the medium meant that the phenolic content was assessed for the callus alone. This could easily be corrected in future studies by assessing the phenolic content of the callus and the medium. Furthermore, the sustainability of callus cultures was greatest in the absence of light exposure. Therefore, despite the rapid production of small phenolic quantities by 2,4-D callus cultures, the gradual accumulation of phenolic compounds produced by PIC callus cultures is favoured. The results of this dissertation determined that biotechnological methods of *in vitro* manipulation may be used to enhance the production of secondary metabolites in medicinal plants. However, extensive knowledge and understanding of the desired plant species micro-propagation protocol, and the secondary metabolite pathways of biosynthesis are required.

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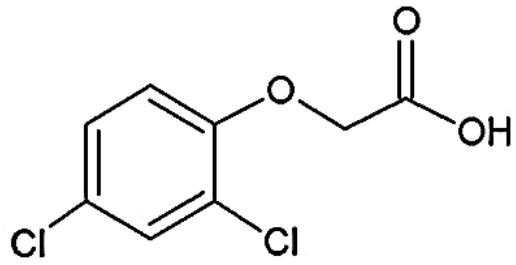
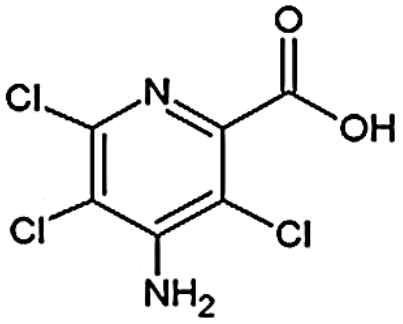
APPENDIX 1 – MURASHIGE AND SKOOG (1962) BASAL GROWTH MEDIUM COMPOSITION

Nutrient component	Mass (g) / 1 ℓ stock	Vol. stock (mℓ/ℓ) final medium
MACRO-NUTRIENTS		
Ammonium nitrate (NH_3NO_3)	165.0	10
Potassium nitrate (KNO_3)	95.0	20
Calcium chloride ($CaCl_2.2H_2O$)	44.0	10
Magnesium sulphate ($MgSO_4.7H_2O$)	37.0	10
Potassium phosphate (KH_2PO_4)	17.0	10
MACRO-NUTRIENTS		
EDTA disodium salt dehydrate (NaFeEDTA)	4.0	10
Boric acid (H_3BO_4)	0.62	10
Zinc sulphate heptahydrate ($ZnSO_4.7H_2O$)	0.860	10
Potassium iodide (Kl)	0.083	10
Manganese sulphate monohydrate ($MnSO_4.4H_2O$)	2.230	10
Sodium molybdate ($NaMoO_4.2H_2O$)	0.025	10
Copper sulphate pentahydrate ($CuSO_4.5H_2O$)	0.0025	10
Cobalt chloride ($CoCl_2.6H_2O$)	0.0025	10
VITAMINES		
Thiamin HCl (B1/Aneurine)	0.01	10
Niacine (Nicotinic acid)	0.05	10
Pyridoxine HCl (B_6)	0.05	10
Glycine	0.2	10
Myo-inositole	0.1	-
CARBOHYDRATE		
Sucrose	30	-

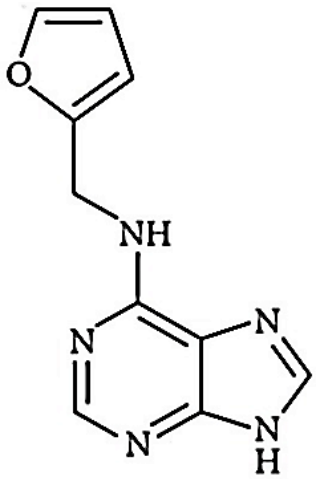
APPENDIX 2 –CHEMICAL STRUCTURE OF PLANT GROWTH REGULATORS USED IN THIS STUDY



2,4-Dichlorophenoxy acetic acid

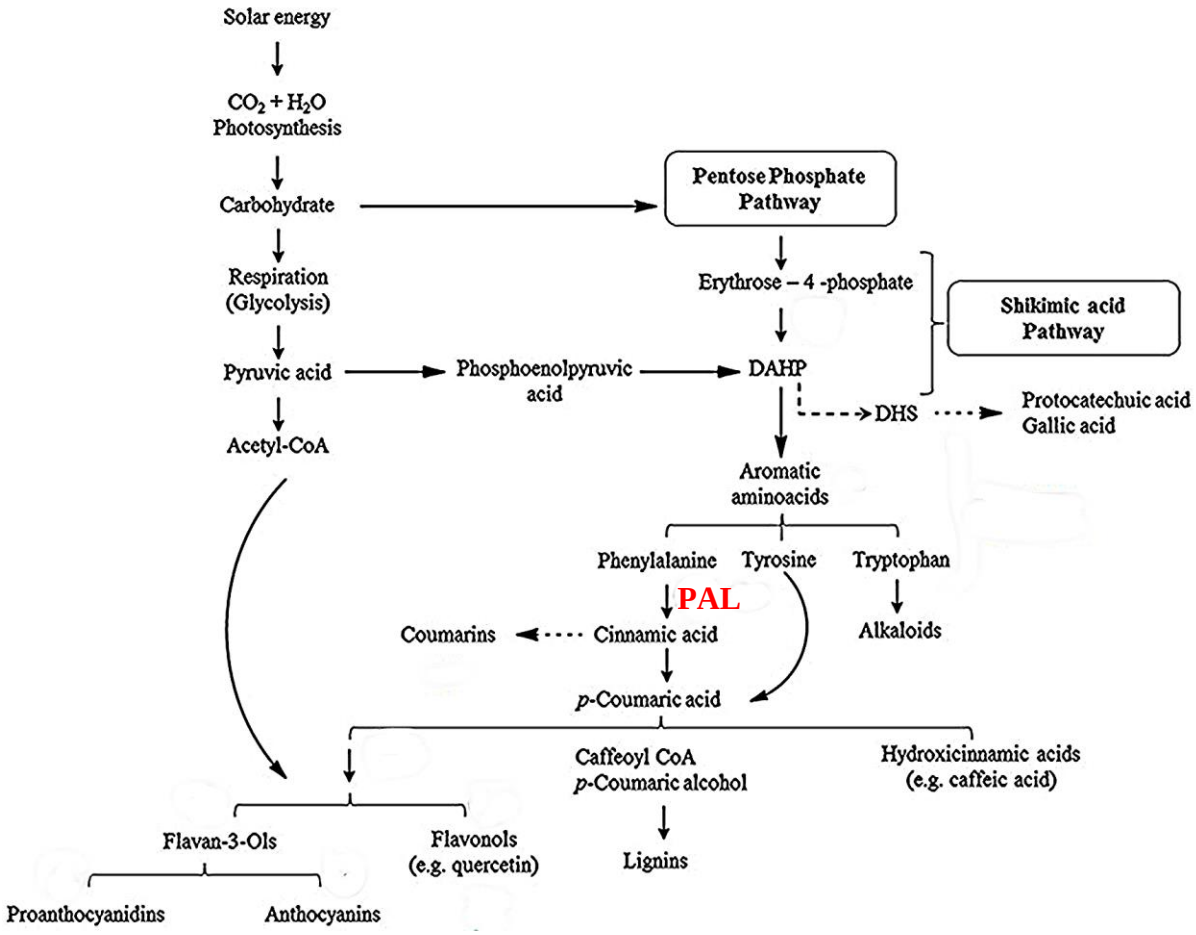


Picloram



Kinetin

APPENDIX 3 – PHENOLIC COMPOUND PRODUCTION IN PLANTS



(adapted and modified from: **DIAS *et al.*, 2016**)