Cytokinins and the Germination of *Tagetes minuta* L.

by
John David Gold

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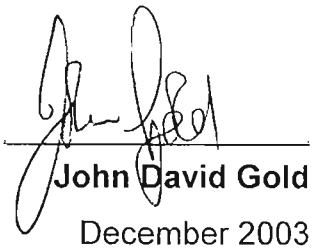
Preface

The work presented in this thesis was carried out from January 2002 to December 2003, in fulfillment of the academic requirements for M.Sc. under the supervision of Professor J. van Staden and Doctor W.A. Stirk in the Research Centre for Plant Growth and Development, School of Botany and Zoology, University of Natal, Pietermaritzburg.

These studies represent my own original work, and have not been submitted in any form for another degree to any other University. The use of the work of others is acknowledged in the text.



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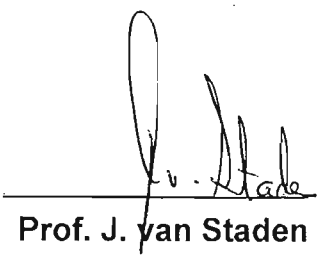
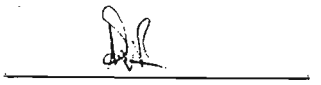
We declare that the above statement is correct.



Prof. J. van Staden
(Supervisor)



Dr W. A. Stirk
(Co-supervisor)

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Papers in Progress

The imposition and maintenance of thermoinhibition in *Tagetes minuta* L. achenes - GOLD, J. D., HILLS, P.N., STIRK, W.A., TAYLOR, N. J. and VAN STADEN, J. (Manuscript ready for submission).

The role of cytokinins in the germination of *Tagetes minuta* L. achenes - GOLD, J. D., STIRK, W. A., STRNAD, M. and VAN STADEN, J. (In preparation).

Abstract

Tagetes minuta L. is a weedy herb that has been a rich source of fragrant oils, used as in the perfume and flavour industry. *T. minuta* achenes germinate erratically under field conditions. However, at the optimal germination temperature of 25 °C, 100 % germination is attained within 48 h of imbibition. The achenes are thermoinhibited at 35 °C. The aims of this project were to assess the role of cytokinins (CKs) in normal germination at 25 °C, and to investigate the factors that regulate thermoinhibition at 35 °C.

CKs were extracted from achenes germinating at 25 °C at 0, 24; 48; 96 and 144 h after imbibition. Two different purification techniques were used, namely Dowex cation exchange resin followed by paper chromatography, or high performance liquid chromatography (HPLC). CK-like activity was tested with the soybean callus bioassay. With both techniques, a peak in CK-like activity appeared 24 h after imbibition, which coincides with the period during which most of the achenes germinated. For quantitative analysis, HPLC\mass spectrometry (MS) techniques were used. The isoprenoid CKs were far more abundant in *T. minuta* achenes than the aromatic CKs. *cis*-Zeatin (*c*Z) and its derivatives were the most abundant CKs. In total, 19 CK compounds were detected, including 4 free bases and a number of corresponding conjugates. Benzyladenine (BA) was the only aromatic CK detected. There was no common time at which active free base maximal concentrations were detected, suggesting that different CKs may have specific roles in the germination process, and thus peak at different times. This in turn suggests that germination is not a single process, but rather a correlative process involving a number of events, with specific CKs having specific roles relating to these correlative events.

There is sufficient evidence obtained from both the soybean callus bioassay and HPLC/MS analysis to suggest that CKs have an active role in *T. minuta* germination. A decline in free BA during germination without corresponding conjugation, suggests that BA is actively used in early germination processes,

possibly in the stimulation of DNA synthesis. Secondly, there was a distinct dihydrozeatin (DHZ) peak obtained at 24 h. Roughly 75 % of the achenes germinate between 16 and 26 h, thus it is likely that DHZ has an active role during the germination of *T. minuta*. Although CKs are probably not involved in the breaking of dormancy *per se*, the distinct peak in CK-like activity obtained in the bioassays, 24 h after imbibition, suggests that CKs have an active role in the germination of *T. minuta*.

With respect to the regulation of thermoinhibition, a number of exogenous treatments were applied, including hormones [gibberellins (GA_{4+7}), abscisic acid (ABA), ethylene and a number of CKs], adenosine triphosphate (ATP) and incubation in 100 % oxygen. ABA was extracted from thermoinhibited and germinating achenes to assess the role of ABA in thermoinhibition and germination. While exogenous 0.1 mg L^{-1} GA_{4+7} application slightly improved normal germination at 25 °C, no treatments were effective in alleviating thermoinhbibition in *T. minuta* achenes. Thermoinhibition in *T. minuta* achenes may be under hormonal regulation, as there is strong evidence for the role of ABA in the maintenance of dormancy and thermoinhbition. High ABA levels were found in dry control samples. Additionally, exogenous ABA application inhibited normal germination, and the commencement of germination was accompanied by a decrease in endogenous ABA levels.

A number of experiments relating to the imposition of thermoinhibition were carried out. Thermoinhibition appears to be very rapidly imposed. Germination is rapidly inhibited following shifting to higher thermoinhibitory temperatures, even after prolonged exposure to optimal germination temperatures. Results suggest active *de novo* biosynthesis of ABA in thermoinhibited achenes. Active biosynthesis of ABA during thermoinhibition suggests that this phytohormone is essential in the maintenance of thermoinhibition of *T. minuta* achenes. It thus appears that ABA is synthesized in the achenes in response to elevated temperatures that are unfavourable for germination to proceed. Unfavourable environmental conditions result in an achene-mediated inhibition of germination, which appears to be initiated and maintained by elevated levels of endogenous ABA.

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Abbreviations

ABA	Abscisic acid
ACC	Aminocyclopropane-1-carboxylic acid
AdoMet	S-adenosyl-L-methionine
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BA	Benzyladenine
BAG_3	Benzyladenine-3-glucoside
BAG_9	Benzyladenine-9-glucoside
BAR	Benzyladenosine
CK	Cytokinin
*c*Z	*cis*-zeatin
*c*ZOG	*cis*-Zeatin-O-glucoside
*c*ZR	*cis*-zeatin riboside
*c*ZROG	*cis*-Zeatin riboside-O-glucoside
*c*ZR5MP	*cis*-Zeatin riboside-5'-monophosphate
DHZ	Dihydrozeatin
DHZOG	Dihydrozeatin-O-glucoside
DHZROG	Dihydrozeatin-O-glucoside riboside
DHZR	Dihydrozeatin riboside
DHZROG	Dihydrozeatin riboside-O-glucoside
DHZR5MP	Dihydrozeatin riboside-5-monophosphate
DHZ9G	Dihydrozeatin-9-glucoside
DMAPP	Dimethylallyl pyrophosphate
dpm	Disintegrations per minute
DNA	Deoxyribonucleic acid
DW	Dry weight
EDTA	Ethylene diamine tetra-acetic acid di-sodium salt
GA	Gibberellins
GA_3	Gibberellic acid
GA_{4+7}	A combination of gibberellins 4 and 7

h	Hour
HPLC	High performance liquid chromatography
IAC	Immunoaffinity chromatography
i.d.	Internal diameter
iP	Isopentenyladenine
iPA	Isopentenyladenosine
iPDP	Isopentenyladenosine-5'-diphosphate
iPMP	Isopentenyladenosine-5'-monophosphate
iPTP	Isopentenyladenosine-5'-triphosphate
iP5MP	Isopentenyladenine-5'-monophosphate
iPA5MP	Isopentenyladenosine-5'-monophosphate
iP9G	Isopentenyladenine-9-glucoside
MBq	Becquerels
min	Minute
mM	Millimolar
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MVA	Mevalonic acid
PDA	Photodiode array detector
PVP	Polyvinylpolypyrrolidone
RNA	Ribonucleic acid
tRNA	Transfer ribonucleic acid
s	Second
SPE	Solid phase extraction
*t*Z	*trans*-zeatin
tZ9G	*trans*-zeatin-9-glucoside
*t*ZOG	*trans*-zeatin-O-glucoside
*t*ZR	*trans*-zeatin riboside
*t*ZROG	*trans*-zeatin riboside-O-glucoside
*t*ZR5MP	*trans*-zeatin riboside-5'-monophosphate
v/v	Volume to volume
w/w	Weight to weight
Z	Zeatin
ZDP	Zeatin diphosphate

ZMP	Zeatin monophosphate
ZOG	Zeatin-O-glucoside
ZOGR	Zeatin-O-glucoside riboside
ZR	Zeatin riboside
ZR5MP	Zeatin riboside-5'-monophosphate
ZTP	Zeatin triphosphate
Z9G	Zeatin-9-glucoside

Chapter 1

Literature Review

1.1 Introduction

1.1.1 Distribution and Morphology of *Tagetes minuta* L.

Tagetes minuta L. is a strongly scented, annual, herbaceous species that grows to a height of between 5 cm and 3 m. All plant parts contain elongated, embedded oil sacs, which result in the strong scent associated with this species. The leaves are opposite or alternate, reaching up to 10 cm in length. Leaf margins are serrated. The flowers are creamy white with a short, broad limb of the solitary ray. The dark brown to black achenes are shaped like a spinning awl, and are 10 to 15 mm in length. Flowering occurs in South Africa in the summer/autumn, between February and June (HILLIARD, 1977).

T. minuta is native to the temperate grasslands and montane regions of southern South America, ranging from Chile, Argentina, Bolivia, Peru and Paraguay. However, the species is now widespread, having been introduced to Europe, Asia, Africa, Australia and Hawaii. *T. minuta* is often found in disturbed sites as part of the early successional stage (SOULE, 1993).

1.1.2 Status in South Africa

In South Africa, *T. minuta* is regarded as a weed. The earliest record of the species in South Africa is at Vlakfontein in 1905. In 1909, it was recorded as a "troublesome weed" in Grahamstown. The first record of *T. minuta* in Natal was at Newcastle in 1910. By 1920, the weed was widespread across South Africa, also occurring in Rhodesia (Zimbabwe) and Mozambique.

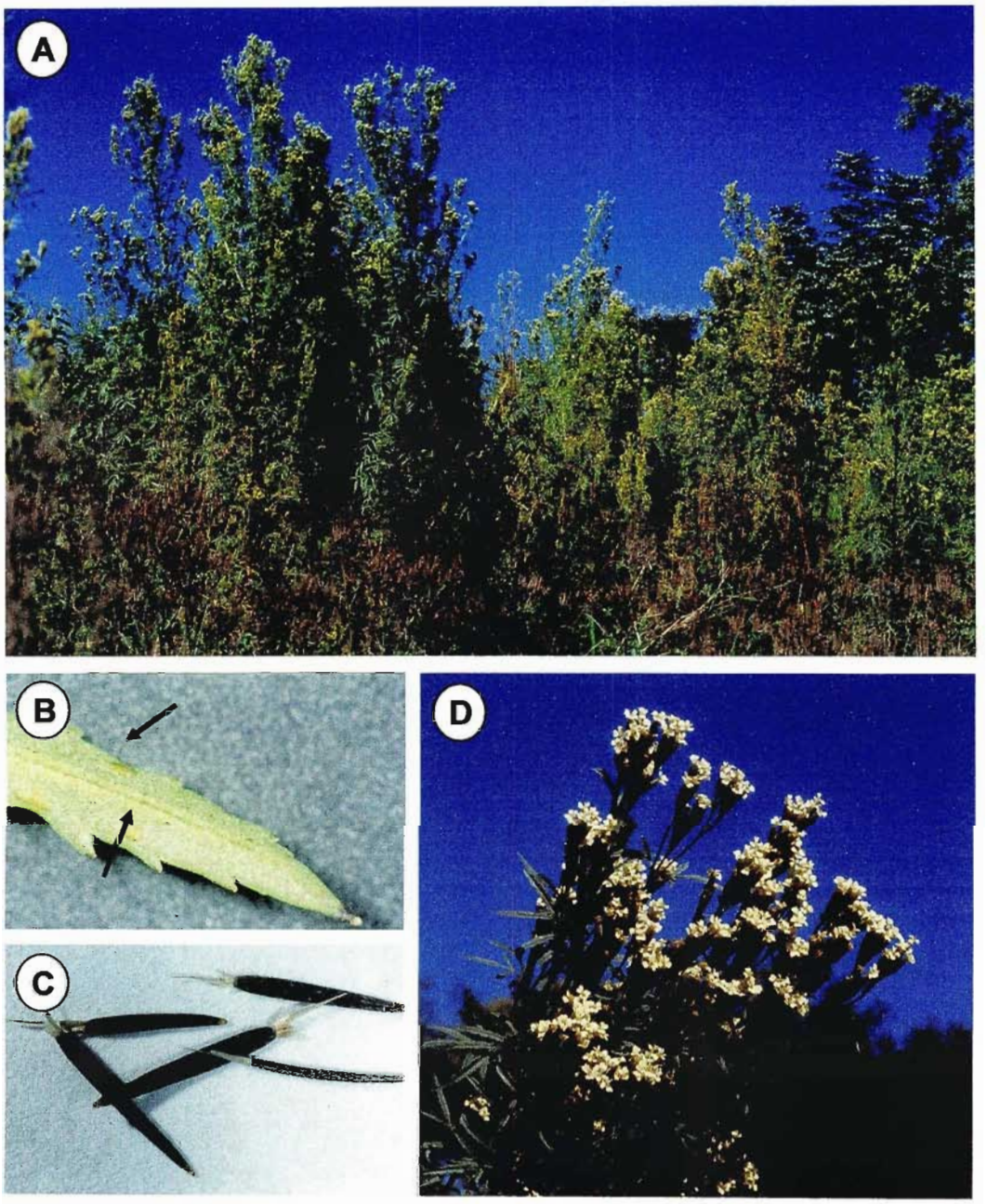

Plate 1: (A) General morphology of *T. minuta*. (B) Oil glands (as indicated by arrows) on abaxial surface of *T. minuta* leaf. (C) *T. minuta* achenes. (D) *T. minuta* inflorescence.

T. minuta is a problem weed in maize crops, with severe infestations occurring in autumn. The weed plants often grow taller than the crop plants and impede harvest operations (MALAN *et al.*, 1981). The roots of *T. minuta* produce a secondary metabolite called terthienyl. This compound may affect seedling growth, depending on its concentration in the rhizosphere. A concentration of 0.4 mg L^{-1} is sufficient to inhibit seedling growth. Follow-on crop growth is often inhibited in fields which have previously been infested with *T. minuta*. Application of aqueous extracts of *T. minuta* to crop seeds may not affect crop germination, but seedling development may be retarded, especially with respect to radicle elongation (MEISSNER *et al.*, 1986).

1.1.3 Uses

Attempts have been made to cultivate *T. minuta* as a crop. The strong scent associated with *T. minuta* is due to a large amount of essential oils and secondary metabolites produced by the plant. These essential oils are widely used in the perfume and flavour industries as "Tagetes oil". The oil is used in many food products, gelatins, condiments and relishes. In 1984, Brazil was the top producer in the world, while worldwide production was estimated at approximately 1.5 million tonnes (SOULE, 1993).

T. minuta also has medicinal properties, and is used to treat inflammation of the respiratory tract, upset stomach, diarrhoea and liver ailments. In its native South America, *T. minuta* is a popular seasoning in rice dishes and stews (SOULE, 1993). Insecticidal properties have also been identified (WEAVER *et al.*, 1994) and in South Africa extracts of the plant are used as a remedy to fleas. However, *T. minuta* is grown primarily for its essential oils.

In South Africa, attempts to cultivate *T. minuta* as a crop have taken place in the eastern Cape. These attempts have generally been unsuccessful due to the erratic germination (FORSYTH and VAN STADEN, 1983) that is regularly associated with weed species as a survival mechanism.

1.2 General Aspects of Germination

BEWLEY (1997) describes germination as "events which commence with the uptake of water by a quiescent dry seed and terminate with the elongation of the embryonic axis". A visible sign that germination has occurred is the emergence of the radicle, an embryonic structure that penetrates the seed structures surrounding it. Germination is associated with the mobilization of nutrient reserves, which are used by the seedling during the early stages of establishment.

1.2.1 Imbibition and the Resumption of Metabolism

Uptake of water by a mature seed is triphasic (Figure 1.1). A rapid initial uptake (Phase I) is followed by a plateau (Phase II). Further water uptake occurs only after germination is complete, as the embryonic axis elongates. The influx of water into a dry seed causes temporary structural perturbations, especially in the membranes. This results in a rapid leakage of solutes with a low molecular weight into the surrounding imbibition solution. Within a short time of rehydration, the membranes return to a stable configuration, which results in solute leakage being curtailed (BEWLEY, 1997).

A rapid resumption of metabolic activity occurs upon imbibition. The structures and enzymes are generally believed to be present within the dry seed tissue, having survived the desiccation associated with maturation. Water reintroduction during imbibition is sufficient to resume metabolic activities. Turnover or replacement of components occurs over several hours as full metabolic status is achieved (BEWLEY, 1997).

The resumption of respiration is one of the first changes that occur after imbibition. After a steep initial increase in oxygen consumption, the rate declines until radicle protrusion. However, another burst of metabolic activity occurs at the same time (BOTHA *et al.*, 1992). The glycolytic and oxidative pentose pathways

both resume activity during Phase I, with the Krebs cycle enzymes becoming active as well (BEWLEY, 1997). Although the dry seed tissues contain mitochondria, they are poorly differentiated as a consequence of maturational drying. However, they contain sufficient enzymes and terminal oxidases to provide adequate amounts of adenosine triphosphate (ATP) to support metabolism for several hours after imbibition (EHRENSHAFT and BRAMBL, 1990; ATTUCI *et al.*, 1991).

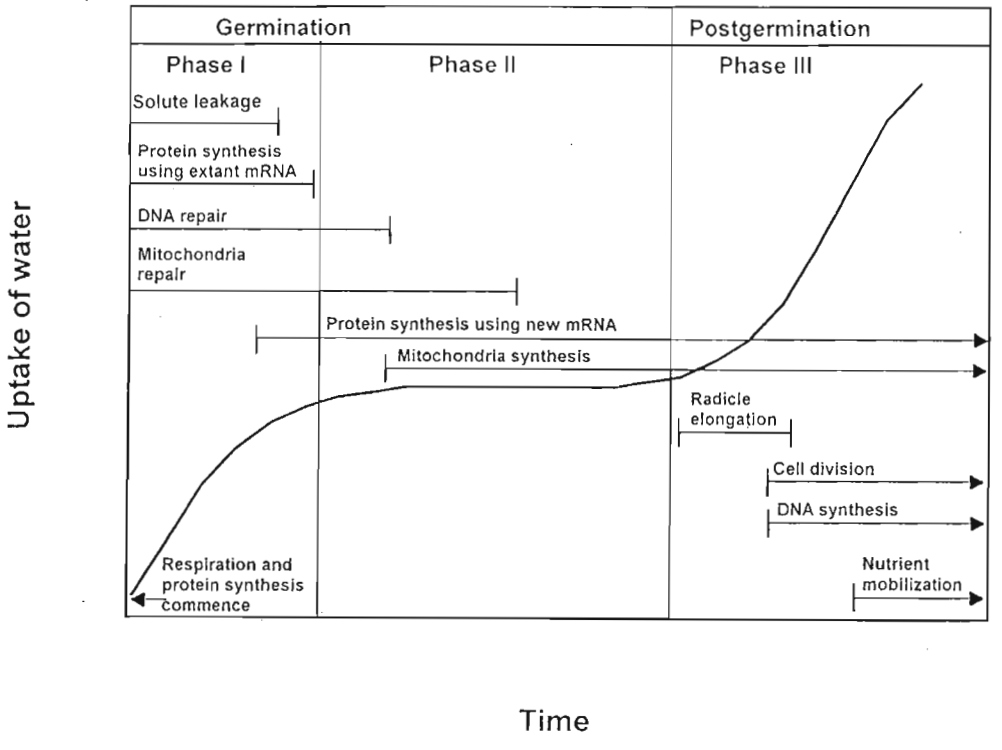

Figure 1.1: Time course of major events associated with germination and postgermination growth. The time scale varies from hours to weeks, depending on plant species and germination conditions (adapted from BEWLEY, 1997).

1.2.2 Protein Synthesis During Germination

Germination is dependent on a number of enzymatic processes relating to the mobilization of nutrient reserves for the growth of the embryo, and the seedling in

the early stages of germination and seedling establishment. Whether the messenger ribonucleic acids (mRNA), needed for the production of enzymes used in these initial processes, are products of *de novo* synthesis or merely pre-existing molecules that become activated is debatable. Carboxypeptidase and isocitrase are synthesized in cotton seed cotyledons, with the required mRNA being transcribed during early embryogenesis (THOMAS, 1980). In contrast, GORDON and PAYNE (1976) found intact, long-lived mRNA strands in the dry seeds of rye, broad bean, pea and oil seed rape.

With respect to enzymes, the proteins produced via *de novo* synthesis upon imbibition may be the proteins involved in germination. There was a need for the *de novo* synthesis of hydrolytic enzymes in the germination of *Cicer arietinum* seeds, namely proteases, amylases, phosphatases and peroxidases (THOMAS, 1980). Thus the general modern concept appears to be that *de novo* synthesis is essential for germination to proceed.

All the components necessary for the resumption of protein synthesis are present in dry seed embryos, despite polysomes being absent. However, within minutes of imbibition, single ribosomes become recruited to form polysomal protein synthesizing complexes. The initial protein synthesis is dependent on these extant ribosomes, but new ribosomes are produced and used within hours of initial polysome assembly (DOMMES and VAN DER WALLE, 1990).

Preformed mRNA is present in the embryo tissues, and may be used transiently during early germination. Message encoding proteins which are important in the maturation of the seed e.g. LEA proteins, are actively degraded upon seed imbibition (JIANG and KERMODE, 1994; HAN *et al.*, 1996). New mRNA transcription occurs as germination proceeds, with the majority of the new transcripts being used to support normal cellular metabolism or growth maintenance reactions, which are not specific to germination (BEWLEY, 1997).

Some proteins are considered to be germination specific, such as the protein germin, which is associated with oxalate oxidase (LANE, 1991). However, a later review of the timing of synthesis and its potential metabolic function suggest that germin is most likely involved with postgermination cell elongation (LANE, 1994). At this stage, few proteins can confidently be considered as germination specific.

1.2.3 Radicle Extension and the Completion of Germination

Radicle emergence marks the beginning of seedling growth and the end of germination. Two discreet phases of nucleic acid synthesis occur in radicle cells. The first phase, which occurs immediately after imbibition, is probably involved in the repair of ribonucleic acids (RNA) that were damaged during maturational desiccation. The second phase, involving deoxyribonucleic acid (DNA) synthesis, occurs during cell division (ZLATNOVA *et al.*, 1987; OSBORNE and BOUBRIAK, 1994).

There are a number of theories as to how the radicle emerges. The first theory identifies radicle extension as a turgor driven process, which requires a loosening of the cell walls in the hypocotyl. An accumulation of solutes in the cells is proposed to cause a decrease in the negative osmotic potential of radicle cells, thus facilitating water uptake which results in turgor driven cell expansion. Loosening of the dominant hemicellulose (xyloglucan) cross-links in the cell walls by the protein expansin, is also associated with this theory (COSGROVE, 1997). Another possible mechanism of radicle emergence involves the degradation of tissue adjacent to the radicle. Cell wall hydrolases, produced and secreted by the endosperm, are believed to be involved in weakening the tissues adjacent to the radicle. However, the presence of these hydrolases has not been recorded across all species (BEWLEY, 1997).

1.3 Temperature Effects on Germination

1.3.1 General

Temperature affects a number of physiological and biochemical processes due to its effect on enzyme activity (BEWLEY and BLACK, 1994). Temperature affects the rate of dormancy loss in dry seeds and the pattern of dormancy change in moist seeds. In non-dormant seeds, temperature affects the rate of germination (ROBERTS, 1988) and the capacity for seeds to germinate (BEWLEY and BLACK, 1994). Generally, the germination rate increases with increasing temperature, within the germination range of a particular species (HEYDECKER, 1977).

RILEY (1981) studied high temperature effects on the germination of maize. At 41 °C, seeds did not germinate, while germination occurred at 28 °C. Seeds at 41 °C, experienced similar respiration and mitochondrial efficiency to those of seeds at 28 °C. However, there was a difference in the activities of several enzymes involved in mobilization of embryo reserves and synthesis of new cellular materials, with seeds at 28 °C having higher increases in the activity of such enzymes. This implies that there is a lower rate of protein synthesis at high temperatures. Thus, enzyme synthesis, not merely activity, may be affected by high temperature.

The dependence of the germination-rate on temperature may be one of the most important factors controlling the timing of emergence in field conditions, as germination rate determines whether a seed can use a certain period of time in which environmental conditions favour germination (WASHITANI, 1985). Variation in germination rate results in the spatial distribution of germination in time (BEWLEY and BLACK, 1994). This is important for the survival of wild, weedy species such as *T. minuta*, but highly undesirable if a species is to be cultivated as a crop.

T. minuta is a spring or early summer germinating species. As temperatures rise in spring, the upper soil layers warm, thus fulfilling the temperature requirements for germination. Thus, seeds on the surface will germinate while seeds deeper in the soil remain quiescent, under cooler soil conditions. In autumn and winter, all seeds will remain quiescent in the prevailing unfavourable environmental conditions (BASKIN and BASKIN, 1985).

During dry storage, seeds undergo physiological changes that may result in a decline in the level of innate dormancy, resulting in germination requirements becoming less specific (ROBERTS, 1988). However, secondary dormancy may be induced if seeds are incubated at temperatures outside their germination range for extended periods (BEWLEY and BLACK, 1994). Provided that germination has not proceeded past a certain critical point, drying of a seed after imbibition will not have any adverse effects on seed viability. With respect to *T. minuta*, dry storage of achenes, following thermoinhibition pre-treatment, may improve germination (see Section 1.3.2).

Temperature fluctuations play a role in the breakdown of hardseededness associated with primary dormancy, especially in Mediterranean climates (ROBERTS, 1988). However, thermoinhibition can be used as a positive pretreatment, which may result in accelerated germination and improve breaking of dormancy when seeds are returned to optimal temperatures, as has been observed in *Spergula arvensis* (ROBERTS, 1988). It is believed that following imbibition and storage at supra-optimal conditions, seeds may undergo a number of metabolic processes up to a certain point at which germination is inhibited. The embryos are thus brought up to a similar, advanced physiological/metabolic state, which may account for the more uniform germination that ensues following such a pretreatment. The associated rapidity of germination may be due to a number of the preliminary metabolic processes associated with germination being completed while the metabolically active seeds are incubated at supra-optimal temperatures. Thus, during incubation, the seeds develop a metabolic

state close to that associated with germination, resulting in rapid germination when dormancy or thermoinhibition is removed (HEYDECKER, 1977, MAYER and POLJAKOFF-MAYBER, 1982). Only a final step in the germination process may be blocked by thermoinhibition, resulting in the attainment of these advanced metabolic states during thermoinhibition (HILLS *et al.*, 2001).

GUTTERMAN (1990) stated that the effect of pre-incubation of seeds at 45 °C for 24 h depended on whether the plants originated from areas receiving summer or winter rainfall. Germination was adversely affected in plants that received winter rainfall, while the converse was observed for plants that received summer rain, indicating that a warming of environmental conditions had to occur for the summer-germinating seeds to become metabolically active. Thus, a warming pretreatment may bring varied results between different species, depending on the conditions in which the plants evolved.

The most notable effect that temperature has on seeds is in the determination of the capacity of seeds to germinate. Germination of all seeds will be inhibited by very low or very high temperatures. Different species have their own temperature ranges over which germination can occur, characterized by the minimum, optimal and maximum germination temperatures (BEWLEY and BLACK, 1994). If temperatures fall outside of the range imposed by the minimum and maximum temperatures, germination will be suspended. The optimal temperature is defined as the temperature at which the highest percentage of seed germinates within the shortest time period (MAYER and POLJAKOFF-MAYBER, 1975). It is important to observe that these temperatures vary not only between species, but also between cultivars, as well as different populations of the same species. Factors such as age and genetic differences may also influence these temperatures (MAYER and POLJAKOFF-MAYBER, 1975). In *T. minuta*, germination is inhibited at a maximum temperature of 35 °C and a minimum temperature of 10 °C, while the optimum temperature is 25 °C (FORSYTH and VAN STADEN, 1983).

1.3.2 Thermoinhibition

Thermoinhibition is a phenomenon whereby non-dormant seeds do not germinate at high temperatures, yet germination proceeds when the seeds are returned to optimal temperatures (HOROWITZ and TAYLORSON, 1983). Thermoinhibition is different from thermodormancy in that seeds may develop a secondary dormancy (thermodormancy) due to overexposure to high temperatures, and will thus not germinate when returned to an optimal germination temperature (as occurs in thermoinhibition). Thermodormant seeds thus require a dormancy-breaking stimulus before germination will proceed (VIDAVER and HSIAO, 1975) while thermoinhibited seeds merely require a return to a suitable temperature for germination to proceed. With both thermodormancy and thermoinhibition, thermal death may result if temperatures rise too high or exposure to an elevated temperature is too lengthy.

A number of treatments have been shown to break thermoinhibition, resulting in the proposal of a number of theories relating to the regulation of thermoinhibition. There appears to be a definite interaction between the classical plant hormones in the regulation of thermoinhibition. General trends indicate that the application of cytokinins (CKs), gibberellins (GAs) and ethylene alleviate thermoinhibition, while ABA is involved in the maintenance or initiation of thermoinhibition.

Applications of gibberellic acid (GA_3) (PERSSON, 1993; SMALL *et al.*, 1993; DUTTA *et al.*, 1994 and 1997; CARTER and STEVENS, 1998), GA_{4+7} (DREWES, 1989; MADAKADZE *et al.*, 1993), ethylene (HAUNG and KHAN, 1992; PRUNISKI and KHAN, 1993; GALLARDO *et al.*, 1994; GALLARDO *et al.*, 1995; GALLARDO *et al.*, 1996; CARTER and STEVENS, 1998) and kinetin (REYNOLDS and THOMPSON, 1971; KHAN *et al.*, 1993; PRUNISKI and KHAN, 1993; SMALL *et al.*, 1993; DUTTA *et al.*, 1994) have overcome thermoinhibition in a number of species. The application of ethylene and GA_3 in combination has been shown to have an additive effect in the alleviation of thermoinhibition (CARTER and STEVENS, 1998). A number of references regarding elevated

ABA levels in the initiation and maintenance of thermoinhibition, have been noted (DUTTA *et al.*, 1994 and 1997; LESKOVAR *et al.*, 1999; YOSHIOKA *et al.*, 1998; LESKOVAR *et al.*, 1999).

A major regulatory process in the alleviation of thermoinhibition may be the biosynthesis of ethylene. Increases in the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) or improved utilization of ACC, ultimately resulting in increased ethylene levels, have been attributed to the alleviation of thermoinhibition in a number of cases (HAUNG and KHAN, 1992; KHAN *et al.*, 1993; DUTTA and BRADFORD, 1994; GALLARDO *et al.*, 1994, 1995 and 1996). MATILLA (2000) has proposed that the regulation of the partitioning of S-adenosyl-L-methionine (AdoMet) between polyamine synthesis and ethylene synthesis may be the regulatory mechanism in the alleviation of thermoinhibition. GALLARDO *et al.* (1995) supports this partitioning concept. A similar partitioning of ACC between these two biosynthetic pathways is believed to be the regulatory step in thermoinhibition-alleviating ethylene biosynthesis. Explanations for the alleviating effect attained by CK and GA application may relate toward ethylene synthesis or utilization. KHAN *et al.* (1993) reported that the alleviation of thermoinhibition in tomato seeds by the application of kinetin is due to improved utilization of ACC, as well as an increase in the production of ACC, resulting in an increase in ethylene production. Conversely, the effect of ABA in maintaining thermoinhibition has been attributed to an ABA-induced interference in ethylene production (GALLARDO *et al.*, 1994). YOSHIOKA *et al.* (1998) found that the application of fluridone, an ABA synthesis inhibitor, restored germination in thermoinhibited lettuce seeds. This implies that thermoinhibition may be due to an increase in the rate of ABA biosynthesis.

A number of other factors may be involved in thermoinhibition. The pericarp is believed to regulate thermoinhibition in spinach seeds (LESKOVAR *et al.*, 1999). Removal of the pericarp resulted in 90 % of previously thermoinhibited seeds germinating. It was suggested that the pericarp acted as a physical barrier as

well as a source of inhibitors, such as ABA, during thermoinhibition. DUTTA *et al.* (1997) correlated the alleviation of thermoinhibition following GA_3 application, with a significant enhancement of mannanase activity in lettuce seed cell walls. However, this may merely be a secondary effect of GA-induced germination. SMALL *et al.* (1993) stated that thermoinhibition might be an energy related phenomenon, whereby ATP levels may be insufficient for germination to proceed in thermoinhibited seeds. Lettuce seeds thermoinhibited at 38 °C germinated following incubation in 100 % oxygen and kinetin. Ethylene and oxygen had a lesser effect, while GA_3 and oxygen had no effect. While the oxygen and kinetin treatment allegedly caused the seeds to bypass an ethylene requirement for germination, or increased the seed's sensitivity to ethylene, it was also noted that the treated seeds that germinated at 38 °C had similar ATP and adenylate concentrations to control seeds germinating at 25 °C. These ATP and adenylate levels were considerably higher than those recorded in thermoinhibited seeds in air at 38 °C.

With respect to *T. minuta*, achenes become thermoinhibited at 35 °C and higher. Thermoinhibition was alleviated by the application of GA_{4+7} at 10, 100 and 200 mg L^{-1} after 1 and 24 h pulses, respectively. Applications of GA_3, ethylene, kinetin and benzyladenine (BA) did not alleviate thermoinhibition. A number of hormone combinations were tested, and also proved ineffective in the alleviation of thermoinhibition (DREWES, 1989). The discovery of 10 polypeptides strongly linked to thermoinhibited *T. minuta* achenes suggests that thermoinhibition is a tightly regulated process under positive control in this species (HILLS *et al.*, 2001).

In some species, thermoinhibition may be used as a form of pretreatment to improve germination, resulting in rapid, uniform germination. Under field conditions, the germination of *T. minuta* achenes is erratic and spread over a number of days, with not all achenes germinating. This is typical of a weed species. At optimal temperatures (25 °C) germination is more rapid, with 100 %

germination being attained by 48 h. However, when achenes are thermoinhibited prior to shifting to 25 °C, 100 % germination is attained within 24 h. FORSYTH and VAN STADEN (1983) found that an improvement in germination only occurred if achenes were pretreated for at least 120 h. In contrast, DRENNAN and VAN STADEN (1989) found that no further advantage was obtained with respect to accelerated germination if the achenes were pretreated for more than 24 h. These differences may be indicative of the genetic differences that result in varied germination results from different achene samples.

Dry storage of *T. minuta* achenes, following thermoinhibition and subsequent drying, has produced varied results with respect to the maintenance of accelerated germination following thermoinhibition pretreatment. FORSYTH and VAN STADEN (1983) found that accelerated germination persisted in previously imbibed achenes that had been stored for 25 days. This was confirmed by DRENNAN and VAN STADEN (1989) who noted that accelerated germination persisted after 10, 20 and 30 days. It appeared that the achenes "remembered" their imbibed pretreatment during the subsequent dry storage. This phenomenon has been termed the "memory effect". However, DREWES (1989) found that after 30 days dry storage, achenes germinated in a manner similar to a control, implying that the beneficial effects of imbibed pretreatment had been lost during dry storage. Dry storage for 60 days and longer resulted in the retardation of germination. Figure 1.2 summarises germination in *T. minuta* achenes.

1.3.3 Cytokinin Involvement in Thermoinhibition

Temperature may control the germination of celery seeds by affecting endogenous CK and GA levels. Thermoinhibited seeds experienced improved germination when CK or GA was exogenously applied, while a combination of the two hormones gave a synergistic effect (THOMAS, 1980). SAINI *et al.* (1989) obtained similar results with lettuce seeds. BIDDINGTON and THOMAS (1978) proposed that increased CK levels may enhance the effect of GA in relieving thermoinhibition.

Red or white light relieves the effects of thermoinhibition in celery seeds (BIDDINGTON and THOMAS, 1978). VAN STADEN (1973) has shown that qualitative and quantitative changes occurred in the CK content of lettuce seeds that had undergone red light-induced dormancy breaking. This suggests that phytochrome-mediated processes in germination may in turn be mediated through changes in endogenous CKs.

REYNOLDS and THOMPSON (1971) found that the upper and lower temperature cut-off points, with respect to thermoinhibition in lettuce seeds, could be shifted higher by kinetin application and downwards by ABA application. The degree to which these temperatures were shifted was directly proportional to the amount of exogenous hormone applied. The application of ABA and CK in concert resulted in a balance, with no shift in thermoinhibition temperatures. This supports the inhibitor interaction model put forward by THOMAS (1980; see Section 1.4.2). The levels of ABA appeared to be related to temperature, and high levels of ABA are regularly associated with heat and water stress (MILBORROW, 1987). Thus, at higher temperatures, one would expect higher ABA levels. CK application counters these high ABA levels, resulting in an upward shift in the upper thermoinhibition temperature limit.

1.4 Cytokinins and Germination

1.4.1 General

The possible roles that CKs may have in seed germination were first alluded to when it was discovered that the blocking of enzymatic and GA-induced germination could be overcome by CK application (THOMAS, 1980). The germination of a number of crop seeds can be improved by pretreatment with growth regulator mixtures, of which CKs may be an important constituent (THOMAS, 1980). However, the mode of action is unclear. CKs may be involved in stimulating protein synthesis and membrane permeability phenomena. CKs have also been found as constituents of transfer ribonucleic acid (tRNA) and have been shown to promote protein synthesis in polysome preparations *in vitro* (THOMAS, 1980).

A number of authors have implicated CKs in the development and maturation of cotyledons (LESHAM, 1989; LETHAM, 1978; VAN STADEN, 1983; GAILLI, 1984). In watermelon cotyledons, early synthesis of DNA is promoted by CK application (GAILLI, 1984) while LESHAM (1989) has shown that CK application stimulates greening and growth in cultured watermelon cotyledons.

A number of CKs have been discovered in seeds, including dihydrozeatin (DHZ), *iso*pentenyladenine (iP) (SHINDY and SMITH, 1976), ribonucleotides and ribonucleosides of zeatin (Z), and a number of other Z derivatives. These initial discoveries were made in the caryopses of maize, but CKs have since been isolated from the seeds of numerous other species (THOMAS, 1980).

Generally, CK levels are high in developing fruits and seeds. However, as these tissues mature, these levels decrease (SANDSTEDT, 1971). The decrease in CK levels may be due to a breakdown of CKs or a build up of inhibitors that may affect CK sensitivity. The formation of biologically inactive ribonucleotides from CK bases and ribonucleosides, has been proposed (THOMAS, 1980).

The inactive ribonucleotides, formed during seed development, are believed to be stored until the seed imbibes sufficient water for germination to begin. It is not known whether these CKs are transported from the parent plant into the seed during development, or whether synthesis occurs *in situ* in the embryo. However, VILLALOBOS and MARTIN (1992) identified the embryo as a site of CK synthesis in chickpea seeds. LETHAM (1978) stated that the embryonic axis is the major site of CK synthesis, and demonstrated that the removal of the embryonic axis could be countered by exogenous CK application in *Curcubita* embryos. Thus, it is probable that the embryonic axis is a major site of CK synthesis in a germinating seed.

1.4.2 Cytokinin/Inhibitor Interactions in Seed Germination

In GA-induced germination, CKs and inhibitors have been proposed to be "permissive" and "preventative". Thus, GA-mediated germination cannot occur in the presence of inhibitors unless there are sufficient CK levels to counter the inhibition effect (THOMAS, 1980). Germination inhibitors, particularly ABA, can inhibit the germination of a number of weed seeds. Often, inhibitory effects can be overcome by the application of CK (SANKHLA and SANKHLA, 1968; REYNOLDS and THOMPSON, 1971). GA application is either totally ineffective, or only partially effective in overcoming ABA effects (THOMAS, 1980). If CK and GA are added together, a complete reversal of inhibition may be obtained (POGGI-PELLEGRIN and BULARD, 1975).

ABA treatment inhibits germination through a number of mechanisms, one being through the inhibition of pregermination protein and RNA synthesis. The application of ABA to lettuce seeds results in an increase in the levels of free CK from bound nucleotide forms (MIERNYK, 1979). This illustrates a possible physiological step to counter higher ABA levels with CK, indicating a possible counteractive effect of CK on ABA.

However, BROWN and DIX (1985) suggested that CKs may have more than a permissive role in germination, as was proposed by THOMAS (1980). BROWN and DIX (1985) suggested that CKs have an important role in the early germination of *Leucadendron tinctum* achenes. An increase in CK levels prior to germination followed by a rapid decrease during visible germination, suggests that CK may have a primary role in breaking dormancy. The fact that a low percentage of germination in a sample of achenes correlated to low levels of CK further supports this argument. However, the key aspect in this argument is that no germination inhibitors are involved in the dormancy and germination process of *L. tinctum*, thus CKs are unlikely to have a permissive role in germination phenomena.

GIDROL *et al.* (1994) noted that high viability of soybean seeds correlated to high CK levels, and that germination of stressed seeds could be induced by the application of exogenous CK. In seeds with low viability, high levels of reactive oxygen species were noted. It is believed that these reactive oxygen species are responsible for the oxidation of CK in seeds with low viability. This implies that CK has an active role in the promotion of germination.

1.4.3 Cytokinins and Membrane Permeability

CKs have been proposed to alter many phytochrome-controlled responses, including seed germination (MILLER, 1956). FONDEVILLE *et al.* (1966) indicated that phytochrome is involved in altering the selective permeability of cell membranes through reorientation of the phytochrome molecule within the membrane layers. Chemicals previously shown to affect membrane permeability, such as ethylene diamine tetra-acetic acid (EDTA), simulated the effect of CK in combination with GA. The induced effects included improved germination or production of reducing sugars (PALEVICH and THOMAS, 1975).

1.4.4 Cytokinins and Molecular Control

The *de novo* synthesis of enzymes may be essential for germination to occur, as is the case in lettuce seeds (SCHULTZ and SMALL, 1991). CKs have been observed as having a stimulatory effect on protein synthesis, regulating gene expression at the transcriptional and post-transcriptional levels (SCHMULLING *et al.*, 1997) while a shift in the pattern of protein synthesis from undefined proteins to repair type proteins has been reported (LONGO *et al.*, 1979). VASQUEZ-RAMOS (1999) reported that the addition of BA to germinating maize embryo axes accelerated cell cycle events and DNA metabolism, including DNA polymerase activity and the replication of DNA. CKs were also observed to stimulate mitosis during germination by activating and mobilizing the kinase complex involved in protein kinase phosphorylation (ZHANG *et al.*, 1996). In maize, CK promotes radicle growth (SMITH and VAN STADEN, 1978). CKs have been observed bound to tRNA at the anticodon loop adjacent to the 3' end. Thus, it has been proposed that CK may regulate tRNA functioning and activity through this binding, although this possible mechanism is unclear (ROMANOV, 1990).

In germination, the stimulation of protein synthesis may be involved in the production of enzymes involved in nutrient mobilization in the early stages of germination, as well as embryo-independent nutrient mobilization. The maturation of cotyledons is also dependent on CKs, while this phytohormone has also been implicated in the maturation of chloroplasts. The formation of chloroplasts is essential for the seedling in the early stages of establishment as seed reserves run low, necessitating the need for food production to become independent of the seed (SCHMULLING *et al.*, 1997).

1.4.5 Cytokinins and Nutrient Mobilization

The embryo has been identified as a site of CK synthesis. NANDI and PALNI (1989) observed movement of CK from the embryo to the cotyledons during possible nutrient mobilization in the cotyledons of dicotyledonous plants. Thus,

the presence of CKs may be important in the role of nutrient mobilization in dicotyledonous plants, as is the case with GA in the monocotyledonous plants. In chickpea seeds, CK affects the embryonic axis in mobilizing nutrient reserves to the cotyledons. Zeatin riboside (ZR) was particularly effective in the mobilization of carbohydrates, and less effective in the mobilization of protein, while the opposite was observed with Z. The most efficient CK with respect to lipid mobilization was iP, while it was less so in carbohydrate mobilization (VILLALOBOS and MARTIN, 1992). Thus, there appears to be a mechanism whereby CKs are synthesized and moved to the embryonic axis, where nutrient mobilization is initiated. This may also occur in species other than chickpea.

1.5 Aims

This project focused on germination aspects of *T. minuta* achenes. Initial work included an investigation of the role of CKs in the normal germination of *T. minuta* achenes at 25 °C. A number of techniques were used to analyse CK-like activity and actual endogenous CK levels during *T. minuta* achene germination.

Thermoinhibition of achenes at 35 °C was investigated. Parameters pertaining to the imposition of thermoinhibition, and factors potentially involved in the regulation of the process were studied.

1.5.1 The Role of Cytokinins in the Germination of *T. minuta* Achenes

The initial aims of this project were to assess if endogenous CK had a role in the germination of *T. minuta* achenes. Two basic roles for CK in germination have been proposed, namely an active role (BROWN and DIX, 1985) and a "permissive" role (THOMAS, 1980). Thus, qualitative and quantitative analyses of endogenous CKs in *T. minuta* achenes were carried out throughout the germination process, from dry seeds through to young seedlings. Bioassays were used in conjunction with mass spectrometry, thus allowing for identification

of CK-like activity and accurate quantification of CK, respectively. The results obtained from these different methods were used in conjunction in an attempt to clarify the role of endogenous CKs in the germination of *T. minuta* achenes.

1.5.2 Thermoinhibition and Achene Germination Studies

While it is known that thermoinhibition in *T. minuta* achenes prevails at 35 °C and higher, very little is known about the time taken to impose and break thermoinhibition. With imposition and breaking of dormancy in mind, two experiments were carried out involving the shifting of achenes between thermoinhibited states and the optimal germination temperature of 25 °C. The aims of these experiments were:

(i) To determine the time required at the optimal germination temperature (25 °C) to alleviate thermoinhibition; and

(ii) To determine the time of incubation at the optimal germination temperature for germination to become irreversible, upon shifting to a supraoptimal thermoinhibition temperature.

A non-hormonal factor that may be involved in the onset and maintenance of thermoinhibition is endogenous ATP levels. With the findings of SMALL *et al.* (1993) in mind, a number of ATP-related experiments were carried out. SMALL *et al.* (1993) also mentioned the effect of incubation in oxygen. These factors were investigated by:

(i) Investigating the effect of exogenous ATP application on thermoinhibited achenes; and

(ii) Investigating the effect of incubation in oxygen on thermoinhibited achenes.

Some experiments entailed a combination of factors, for example, applying exogenous ATP to achenes and incubating them in oxygen.

With respect to hormonal factors, the aims were three fold:

(i) To determine the effect of different hormones applied exogenously to thermoinhibited achenes;

(ii) To determine the effect of different hormones applied exogenously to normally germinating achenes; and

(iii) To determine the changes in endogenous ABA levels in response to thermoinhibition and germination.

Ethylene, GAs and CKs have been shown to improve germination as well as alleviate thermoinhibition in some species, while ABA appears to be the primary hormone involved in the initiation and maintenance of dormancy and thermoinhibition in most species. In this study, several experiments were carried out whereby varying concentrations of different hormones were applied exogenously to germinating and thermoinhibited achenes. Thus, the effects of these exogenous hormone applications on germination and thermoinhibition in *T. minuta* achenes could be assessed. Extractions of endogenous ABA from thermoinhibited and normally germinating achenes were also carried out, in an effort to establish possible changes in endogenous ABA levels in response to thermoinhibition and germination in *T. minuta* achenes.

Chapter 2

The Role of Cytokinins in the Germination of *Tagetes minuta*

2.1 Introduction

It is generally accepted that CK have a "permissive" role in germination, with respect to "permissive and "preventative" GA and ABA interactions. While generally not appearing to have a clearly defined, essential role in germination, CK application does overcome or reverse germination inhibitory effects (THOMAS, 1980). However, as with all hormone-related phenomena, there are exceptions to the general trend. For example, BROWN and DIX (1985) showed that CKs do have a major, active role in the germination of *Leucadendron tinctum* seeds. The possible roles of CKs in germination, whether active or 'permissive", were discussed in the review of the literature (Chapter 1).

Thus, in these experiments, it was attempted to identify the CK profile during *T. minuta* achene germination, in an attempt to identify the role, if any, of CKs in the germination of *T. minuta* achenes. A high level of CK-like activity prior to germination may suggest an active role, while an increase in CK-like activity during and/or after germination may suggest a less active, "permissive" role. A variety of different CK extraction and analysis methods were used to analyze CK levels from the start of imbibition and germination, up to 144 h after commencing imbibition, by which time the achenes had developed into small seedlings.

2.2 Materials and Methods

2.2.1 General

The achenes used in the experiments described in this Chapter were obtained in June 1998 from a single location in Pietermaritzburg (29° 36' S; 30° 23' E), KwaZulu-Natal, South Africa, thus reducing the effect of genetic variation.

For each sample, achenes (1 g) were imbibed in 5 mL distilled water in a 55 mm Petri dish, which was lined with two pieces of 60 mm Whatman No. 1 filter paper. Achenes were incubated at 25 °C in continuous light (15 μmol m^{-2} s^{-1}). The achenes were incubated for 24, 48, 96 and 144 h, respectively, before extraction. Dry control achenes were also extracted. Dowex exchange columns and high performance liquid chromatography (HPLC) separation (as described below) were used independently to purify samples for use in the soybean callus bioassay.

A germination curve under these experimental conditions was generated. Five replicates of 25 achenes each were placed in 55 mm Petri dishes lined with two pieces of 60 mm Whatman No. 1 filter paper. Three mL of distilled water were applied to each Petri dish. Achenes were incubated at a light intensity of 15 μmol m^{-2} s^{-1}. Germination was recorded every 2 h, with germination being noted as the time of radicle emergence. Germination was converted to a percentage after counting and the standard errors calculated.

2.2.2 Determination of Cytokinin-Like Activity

Cytokinin Extraction

A modified version of the protocol developed by SMITH and VAN STADEN (1978) was used in the extraction of CKs. The achene sample (1 g) was soaked in 100 mL 80 % ethanol. The ethanol/achene mixture was homogenized using an Ultra-Turrax T25 homogenizer, and subsequently extracted for 24 h at 10 °C. The extracts were then filtered through Whatman No.1 filter paper, and further washed in 100 mL 80 % ethanol. The combined ethanolic extracts were dried *in vacuo* at 35 °C.

Cytokinin Purification

Two different CK purification procedures were used, namely a cation exchange resin column combined with paper chromatography, and HPLC.

a) Cation Exchange Resin Columns and Paper Chromatography

The extracted residue (from Section 2.2.2) was resuspended in 50 mL 80 % ethanol and the pH of the extracts adjusted to 2.5 by adding dilute hydrochloric acid. Following extraction, CKs were purified using Dowex cation exchange resin chromatography. Thirty grams of Dowex 50W-X8 (BDH Chemicals Ltd.) cation exchange resin were soaked overnight in 100 mL 80 % ethanol and then poured into a column with a 3 cm internal diameter, and the air bubbles removed. Extracts were loaded onto the column and passed through the exchange resin at a flow rate of 20 mL h^{-1}, followed by further washing with 100 mL of 80 % ethanol. These ethanolic eluates constituted the aqueous phase, and were discarded, as they had no biological activity in pervious experiments.

Elution of the CKs from the resin was achieved by adding 100 mL of 5 N ammonium hydroxide. The ammonia eluates were dried and concentrated under vacuum and the residues taken up in 3 mL 80 % ethanol. These eluates were strip-loaded onto Whatman No.1 chromatography paper (as a 1 cm strip) and separated in descending chromatography tanks. The solvent system used was *iso*-propanol:25 % ammonium hydroxide:water (10:1:1 v/v). The chromatograms were developed overnight so that the solvent front moved approximately 30 cm.

The chromatograms were dried in an oven for 24 h at 50 °C. Dry chromatograms were divided into 10 R_f zones. The strip of paper, corresponding to each R_f zone, was cut up and placed in a 50 mL Erlenmeyer flask, which were then used in the soybean callus bioassay (see Section "Soybean Callus Bioassay").

b) High Performance Liquid Chromatography (HPLC) Separation

The dried achene extracts (from Section 2.2.2) were resuspended in 500 µL HPLC-grade methanol, and filtered through a 0.22 µm Millipore filter. Each sample was subjected to HPLC fractionation using a Varian 500 instrument (Varian Instrument Group, USA) fitted with a Hypersil 5 ODS 25 cm x 10 mm semi-preparative column (HPLC Technology, UK). A gradient elution series of 95 % 0.2 M acetic acid buffered to pH 3.5 with triethylamine: 5 % methanol to 50 % buffer: 50 % methanol over 90 min at a flow rate of 2.5 mL min^{-1} was used to fractionate the extracts. Compounds were detected at 254 nm (Hewlwtt Packard, Series 1050).

Ninety 2.5 mL fractions were collected and bioassayed for CK-like activity using the soybean callus bioassay (MILLER, 1965). Samples of authentic CK standards were run on the same program to obtain retention times for this system. This technique was used to assess the qualitative CK activity of the achenes in more detail.

Soybean Callus Bioassay

The soybean callus bioassay was used to analyze CK-like activity in samples purified and separated from both Dowex columns (combined with paper chromatography) and HPLC. Four stock solutions (see Appendix) were prepared according to MILLER (1965). Following cation exchange resin chromatography, 30 mL of nutrient medium and 0.3 g agar were added to each Erlenmeyer flask, containing the respective R_f zones obtained from the paper chromatograms. Following HPLC separation and drying off of the separation solvent, 15 mL of nutrient medium were added to each Erlenmeyer flask, with 0.15 g agar. For each bioassay, a set of authentic kinetin standards at 1, 10 and 50 µg L^{-1} were included. The flasks were stoppered with non-absorbent cotton wool bungs, which were covered with aluminum foil. The flasks were autoclaved at a pressure of 1.5 bars for 20 min, before being transferred to a sterile transfer chamber.

Once the agar had solidified, 3 pieces of 3 to 4-week-old soybean callus (*Glycene max* L. Acme) of approximately 20 mg in weight were placed on the medium. The callus is maintained on Millers medium with 50 µg L-1 kinetin and subcultured every 28 days. Flasks were incubated in a growth room at a constant temperature of 26 °C (± 2 °C) in continuous low white light (15 µmol m^{-2} s^{-1}) for 28 days before the callus was weighed. The amount of callus growth in each flask was plotted on a histogram relative to the control value. Figure 2.1 summarizes the method used to extract and test for CK activity in this study.

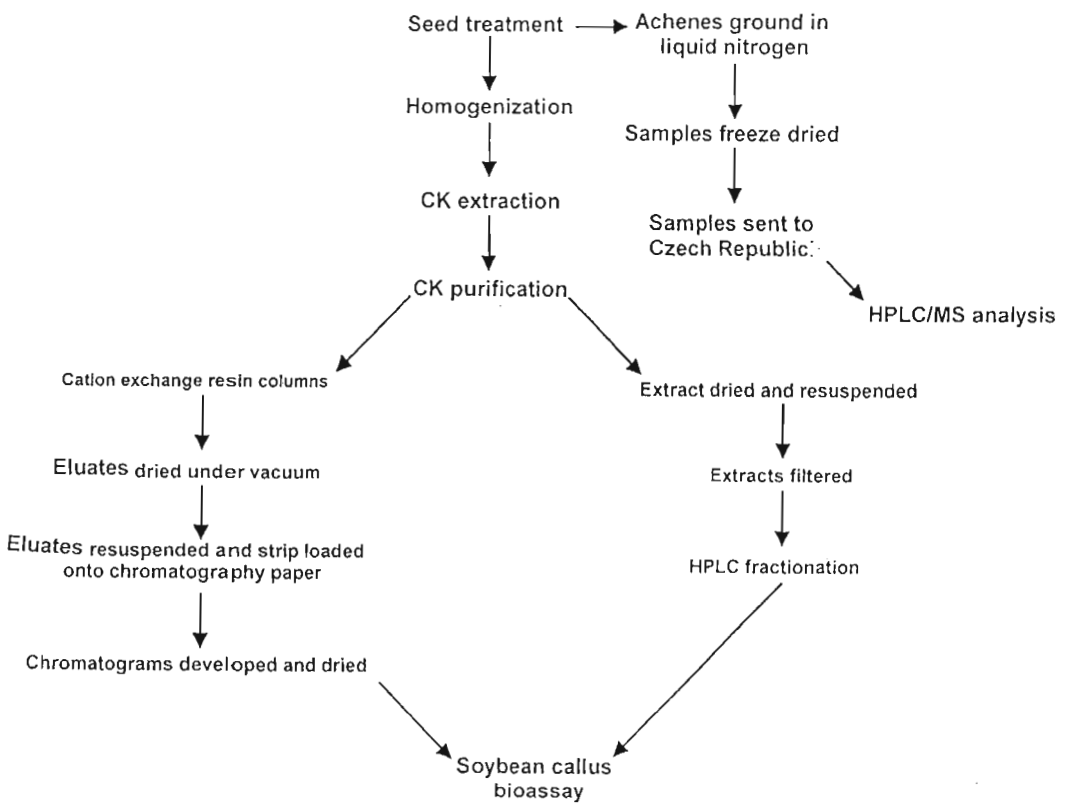

Figure 2.1: Summary of CK extraction, purification and identification.

The soybean callus bioassay is commonly used, as it displays activity over a wide range of concentrations, giving a linear response to the CK concentrations. Thus, callus weights reflect CK-like activity. However, CK concentrations cannot be quantified and there is a degree of variability between bioassays, hence the employment of kinetin standards in each

bioassay. Unless repeated many times, these results do not allow statistical analyses. Where possible, samples were assayed in a single bioassay to allow for comparison of CK-like activity between samples. If this was not possible, assays were performed within the same week using callus from the same subculture.

2.2.3 Identification and Quantification of Cytokinins

HPLC/Mass Spectrometry (MS) Analysis

Achene samples (2 g) were pretreated as previously described in Section 2.2.1, and frozen in liquid nitrogen. After thorough grinding using a pestle and mortar, the samples were freeze dried to a consistent weight. These dry samples were sent to the laboratories of Prof. Miroslav Strnad in the Czech Republic for qualitative and quantitative analysis, using a highly accurate method involving HPLC and MS in combination, as described below.

The frozen plant tissue was ground to a fine powder, under liquid nitrogen. Extraction followed in ice-cold 70 % ethanol (10 mL g^{-1} DW). Deuterium-labeled standards were then added ([$^2H_5$] *trans*-zeatin (*t*Z), [$^2H_5$] *trans*-zeatin riboside (*t*ZR), [$^2H_5$] *trans*-zeatin-9-glucoside (*t*Z9G), [$^2H_3$] DHZ, [$^2H_3$] dihydrozeatin-9-glucoside (DHZ9G), [$^2H_6$] iP, [$^2H_6$] *iso*pentenyladenosine (iPA), [$^2H_6$] *iso*pentenyladenine-9-glucoside (iP9G), [$^2H_5$] *trans*-zeatin-O-glucoside (*t*ZOG), [$^2H_5$] *trans*-zeatin riboside-O-glucoside (*t*ZROG), [$^2H_3$] dihydrozeatin-O-glucoside (DHZOG), [$^2H_3$] dihydrozeatin riboside-O-glucoside (DHZROG), [$^2H_5$] *trans*-zeatin riboside-5'-monophosphate (*t*ZR5MP), [$^2H_3$] dihydrozeatin riboside-5'-monophosphate (DHZR5MP), [$^2H_6$] *iso*pentenyladenine-5'-monophosphate (iP5MP), [$^2H_7$] BA, [$^2H_7$] benzyladenosine (BAR), Olchemim Ltd., Czech Republic), which allowed for the checking of recovery after determination. Centrifugation (15 000 x *g* at 4 °C) took place after 3 h of extraction. The resulting pellets were re-extracted in the same way. Combined extracts were concentrated to approximately 1 mL by rotary evaporation under vacuum at 35 °C.

The samples were diluted to 20 mL with ammonium acetate buffer (40 mM, pH 6.5), and purified using a combined DEAE-cellulose (1.0 x 5.0 cm) octadecysilica (0.5 x 1.5 cm) column and immunoaffinity chromatography (IAC) with polyspecific monoclonal antibodies against CKs. CK bases, ribosides and glucosides retained on a reverse-phase cartridge were eluted in 5 mL 80 % methanol (v/v) and, after drying, the fraction containing the basic CKs was obtained. After washing with 10 mL distilled water, the DEAE-Sephadex column was coupled to another Sep-Pak Plus C_{18} (SPE) cartridge and the CK nucleotides were eluted with 10 mL 6 M HCOOH. The nucleotides initially retained on the C_{18} cartridge were eluted in 5 mL 80 % methanol, dried and dephosphorylated by alkaline phosphatase treatment to obtain the nucleotide fraction. Both fractions were immunopurified on a CK monoclonal antibody column (FAISS *et al.*, 1997). CK O-glucosides occurring in the phosphate buffer solution eluted from the immunoaffinity columns were treated with β-glucosidase and re-purified on the same monoclonal column. The methanolic eluates from IAC columns were evaporated to dryness and resuspended in 75 µL of mobile phase for HPLC analysis. The ammonium acetate eluate from the IAC column was incubated with β-glucosidase and again purified with IAC.

Samples containing CKs were analyzed by HPLC (Waters Alliance 2690) linked to a Micromass single quadrupole mass spectrometer equipped with an electrospray interface [liquid chromatography+electrospray ionization-MS] and a Waters PDA 996 photodiode array (PDA) detector. Samples (35 µL) were injected into a C_{18} reversed-phase column (Waters; Symmetry; 3.5 µm; 150 mm x 2.1 mm) and the elution performed at a flow rate of 250 µL min^{-1} with a methanolic gradient composed of 100 % methanol (A) and 15 mM formic acid (B) adjusted to pH 4.0 with ammonium. The following protocol was used for gradients: 0 min, 10 % A + 90 % B; 25 min, 50 % A + 50 % B; 30 min, 50 % A + 50 % B; then column re-equilibration. A post column split of 1:1 was used to introduce the eluent into the electrospray source (source block temperature 100 °C, desolvation temperature 250 °C, capillary voltage +3.0 V, cone voltage 20 V) and PDA (scanning range 210-300 nm; with 1.2 nm resolution).

Quantitative analyses of the different CK types was thus performed under these conditions in selective ion recording mode. All data was processed by Masslynx software using a standard isotope dilution method. Ratio of endogenous CK to appropriate labeled standard was determined and further used to quantify the level of endogenous compounds in the original extract, according to the knowledge of quantity of added internal standard (NOVAK *et al.*, 2003).

2.2.4 Exogenous Cytokinin Application to Germinating Achenes

Five of the CKs detected in the HPLC/MS analysis were applied to normally germinating achenes at 25 °C. These CKs were selected from positive results of the soybean callus bioassay and the CK HPLC/MS identification and quantification. They were applied at concentrations of 0.1, 1 and 10 mg L^{-1}. The free bases BA, DHZ and Z (a *cZ* [10 %] and *tZ* [90 %] mixture), and the synthetic CK, kinetin. The CK conjugates included zeatin-O-glucoside (ZOG) and zeatin riboside-5'-monophosphate (ZR5MP). Achenes were imbibed with 3 mL of the hormone solution and incubated under continuous light (15 µmol m^{-2} s^{-1}) at a constant 25 °C. Germination was checked every 12 h.

2.3 Results

2.3.1 Germination of *T. minuta* Achenes at 25 °C

These achenes behaved as expected, with nearly 100 % germination being attained by 48 h. Maximal germination was attained by 36 h. Radicle emergence first occurred after 14 h, but the majority of the achenes (approximately 75 %) germinated between 16 and 26 h (Figure 2.2).

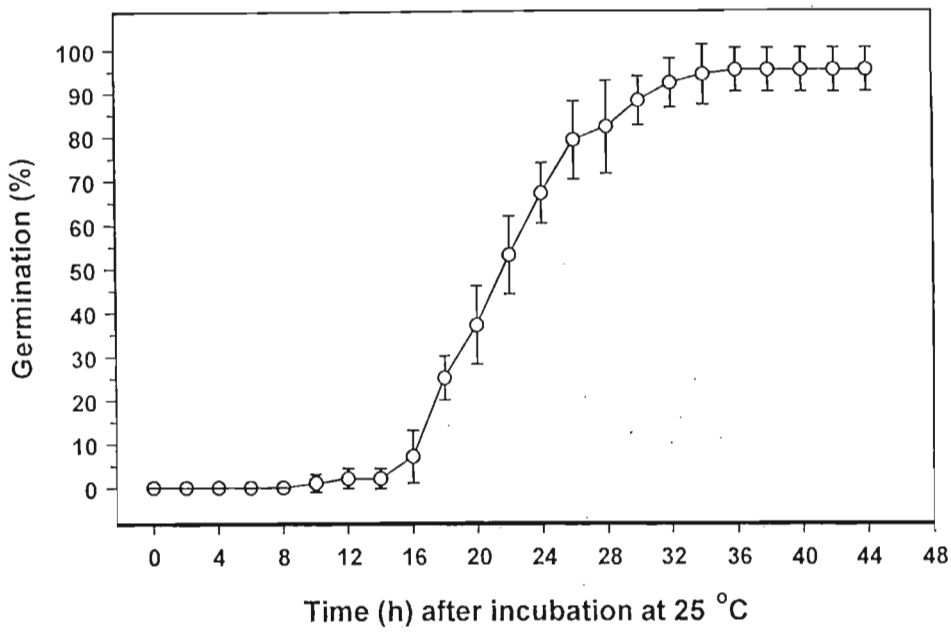

Figure 2.2: Germination curve of *T. minuta* achenes recorded every 2 h.

2.3.2 Bioassay Results (HPLC and Dowex Column Separation)

Table 2.1 shows authentic CK standard HPLC running times, and the R_f zones in which 5 CKs occurred following paper chromatography separation. For both the Dowex cation exchange resin-paper chromatography and HPLC separated samples, there was a large increase in CK-like activity after 24 h (Figures 2.3 and 2.4) in the region of the DHZ (R_f 0.5), Z (R_f 0.8) and iP (R_f 1.0) derivatives. There was a marked increase in CK-like activity in the iP (R_f 1.0) region after 96 h. Separation by paper chromatography allowed all the samples to be assayed in a single bioassay, facilitating comparison between samples. Separation by HPLC allowed for better separation of the compounds that resulted in more activity being detected e.g. there was a larger level of activity that co-chromatographed with iP and iPA after HPLC separation compared to paper chromatography. This may be due to inhibitory compounds no longer being present in these fractions.

Table 2.1: Retention times and R_f zones of various authentic CK standards when separated by paper chromatography or HPLC, respectively. – indiciates that the authentic CK standard was not tested.

CK	Retention time (min) following HPLC	R_f zone following paper chromatography
ZR5MP	33.61	-
DHZR5MP	35.18	-
Zeatin-9-Glucoside (Z9G)	35.72	-
DHZ9G	38.11	-
ZOG	38.24	0.4
*t*Z	41.23	0.8
DHZOG	42.39	-
DHZ	44.08	0.5
Zeatin Riboside O-Glucoside (ZROG)	45.53	-
Cis-Zeatin (*c*Z) -	46.89	-
Benzyladenine-3-Glucoside (BAG_3)	48.6	-
DHZROG	52.38	-
Zeatin Riboside (ZR)	52.49	0.7
DHZR	56.08	-
Benzyladenine-9-Glucoside (BAG_9)	71.56	-
iP9G	71.96	-
BA	82.24	-
BAR	83.01	-
iP	86.42	1.0
iPA	86.98	0.9

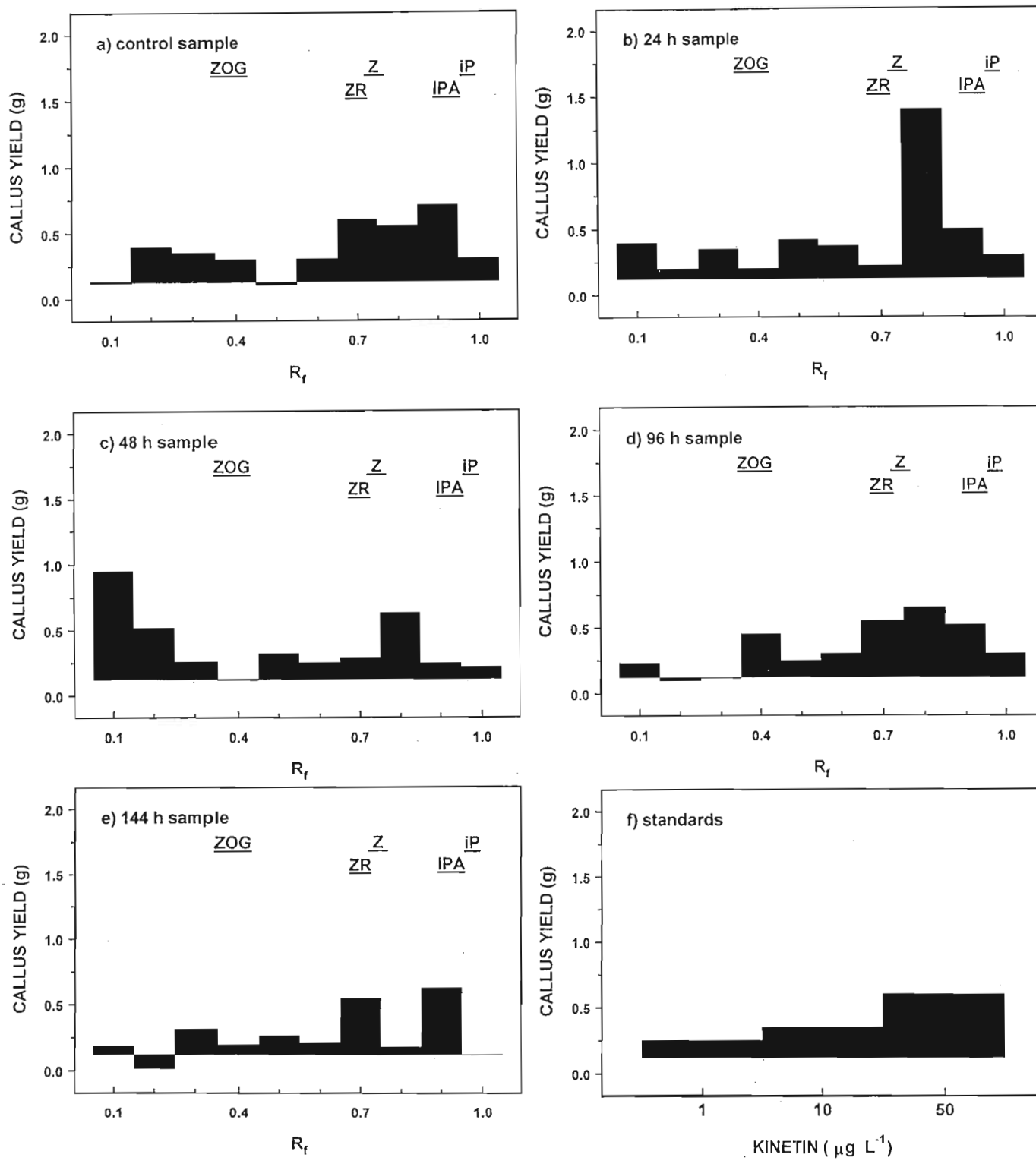

Figure 2.3: CK-like activity detected using the soybean callus bioassay in *T. minuta* achenes during germination and postgermination at 25 °C. Results obtained after purification by Dowex cation exchange resin and paper chromatography separation. Positions of authentic CK standards are (ZOG, ZR, Z, iPA and iP) included.

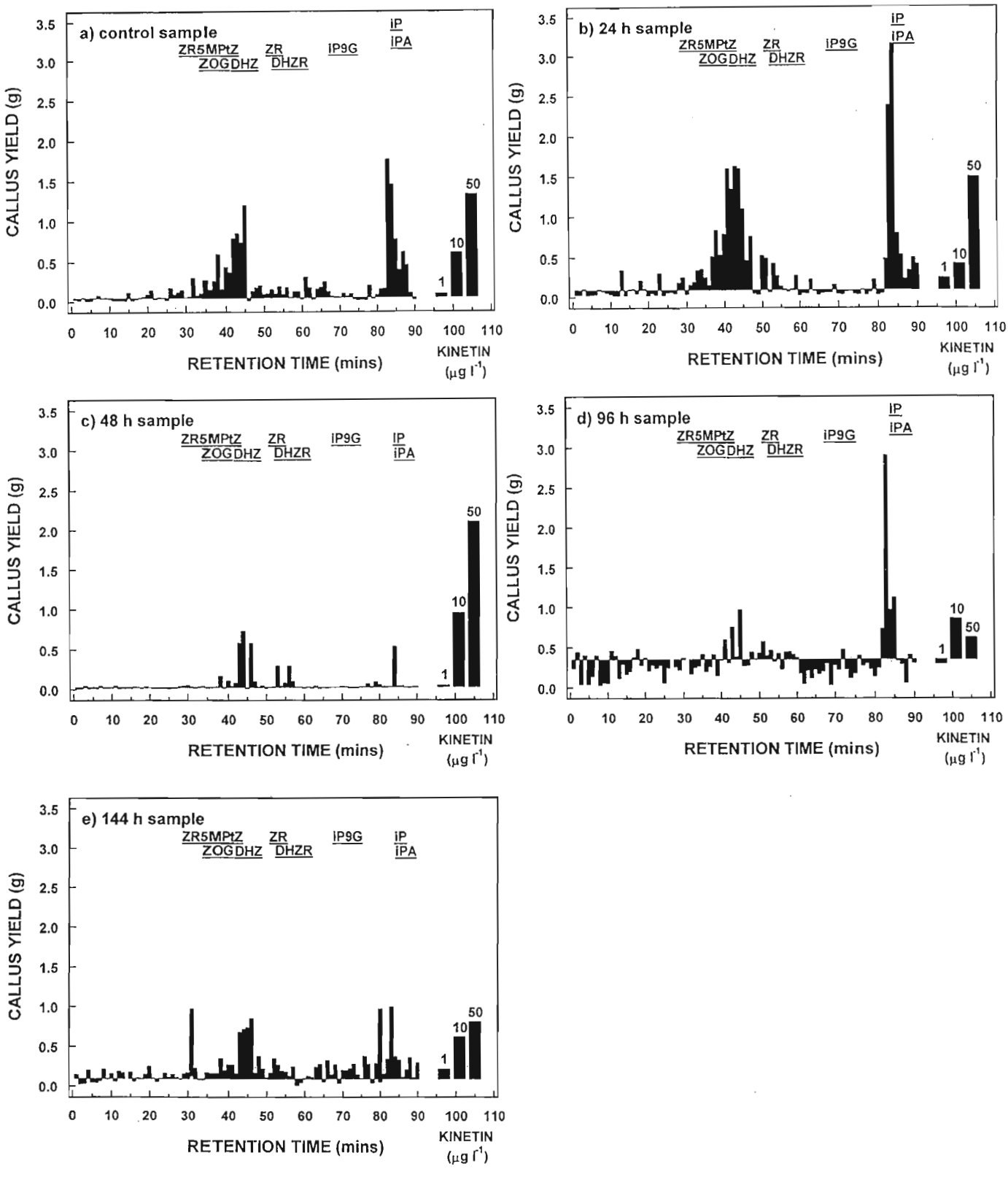

Figure 2.4: CK-like activity detected using the soybean callus bioassay in *T. minuta* achenes during germination and postgermination at 25 °C. Separation was achieved using HPLC. Positions of authentic CK standards are (ZR5MP, ZOG, ZR, DHZ, *t*Z, ZR, DHZR, iP9G, iP and iPA) included.

2.3.3 Cytokinin Identification and Quantification

Nineteen CK types were detectable during the course of germination of *T. minuta* achenes, in samples encompassing dry achenes, germinating achenes and developing seedlings. It is interesting to note that the various CK levels fluctuated independently during germination, with no common period maximal CK levels being obtained throughout. However, 42 % of the observed CKs attained maximal levels at 48 h (Tables 2.2 - 2.7).

Table 2.2: Concentrations of Z and its derivatives (pmol g^{-1} DW) detected in *T. minuta* achenes during germination and subsequent seedling development.

Time after imbibition (h)	*t*Z	*c*Z	*t*ZR	*c*ZR	Z9G
0 (Control)	1.7	8.4	1.7	3.9	2.4
24	2.3	18.6	3.1	23.3	2.2
48	2.3	34.1	6.4	401.2	1.8
96	1.8	5.3	1.0	25.2	5.4
144	3.4	5.4	0.8	1.4	5.0
TOTAL	**11.5**	**71.8**	**13.0**	**455.0**	**16.8**

Table 2.3: Concentrations of DHZ and its derivatives (pmol g^{-1} DW) detected in *T. minuta* achenes during germination and subsequent seedling development (– indicates levels not detectable, < 0.05 pmol 25 μL^{-1}).

Time after imbibition (h)	DHZ	DHZR	DHZ9G
0 (Control)	9.6	27.9	61.2
24	33.5	28.0	68.1
48	9.8	–	67.2
96	2.1	–	74.2
144	2.6	–	79.0
TOTAL	**57.6**	**55.9**	**349.7**

Table 2.4: Concentrations of iP and its derivatives (pmol g^{-1} DW) detected in *T. minuta* achenes during germination and subsequent seedling development (– indicates levels not detectable, < 0.05 pmol 25 μL^{-1}).

Time after imbibition (h)	iP	iPA	iP9G
0 (Control)	15.0	9.6	39.6
24	17.5	13.0	80.2
48	18.4	77.0	165.8
96	18.0	8.4	402.7
144	17.6	–	524.2
TOTAL	**86.5**	**108.0**	**1212.5**

Table 2.5: Aromatic CKs (pmol g^{-1} DW) detected in *T. minuta* achenes during germination and subsequent seedling development. BA derivatives not detected were BAR and BA9G. No topolins were detected.

Time after imbibition (h)	BA
0 (Control)	870.1
24	32.4
48	52.3
96	9.0
144	12.6
TOTAL	**913.4**

Table 2.6: O-glucoside concentrations (pmol g^{-1} DW) detected in *T. minuta* achenes during germination and subsequent seedling development. *t*ZOG, *t*ZROG and DHZOG were not detected (– indicates levels not detectable, < 0.05 pmol 25 μL^{-1}).

Time after imbibition (h)	*c*ZOG	*c*ZROG	DHZROG
0 (Control)	4.0	12.0	30.3
24	3.9	21.6	16.7
48	–	–	–
96	–	–	–
144	–	–	–
TOTAL	**7.9**	**33.6**	**47.0**

Table 2.7: Concentrations of cytokinin monophosphates (pmol g^{-1} DW) detected in *T. minuta* achenes during germination and subsequent seedling development. BAR5MP was not detected.

Time after imbibition	*t*ZR5MP	*c*ZR5MP	DHZR5MP	iPA5MP
0 (Control)	0.4	3.1	8.1	17.3
24	3.9	298.7	23.8	48.3
48	2.6	2192.5	93.3	118.3
96	2.7	492.3	15.0	65.4
144	0.1	23.4	2.9	15.1
TOTAL	**9.7**	**3010.0**	**143.1**	**264.6**

Three CKs (BA, DHZROG and *c*ZOG) were detected at their highest concentrations in the dry controls, the most noteworthy being BA (870.1 pmol g^{-1} DW, Table 2.5). Three CKs attained maximum concentrations 24 h after imbibition, namely DHZ, DHZR (Table 2.3) and *c*ZROG (Table 2.6), with the most notable being DHZ at 33.5 pmol g^{-1} DW. The largest number of CK maximum levels was detected at 48 h (8 out of 19), including *c*Z, *t*ZR, *c*ZR (Table 2.2), iP, iPA (Table 2.4), *c*ZR5MP, DHZR5MP and iPA5MP (Table 2.7). Most notable was the marked increase in *c*ZR5MP (2192.5 pmol g^{-1} DW) and *c*ZR (401.2 pmol g^{-1} DW). *c*ZR5MP concentrations were the highest of any of the CKs detected during germination. While no noteworthy CK concentrations were attained 96 h after imbibition, three CKs attained maximum concentrations 144 h after imbibition, namely *t*Z, DHZ9G and iP9G. The most notable CK concentration at 144 h is that of iP9G, which consistently increased in concentration as germination proceeded, resulting in 524 pmol g^{-1} DW of iP9G at 144 h (Table 2.4).

A number of different CK forms were detected, including the free bases *c*Z, *t*Z, DHZ, iP, and BA (Figure 2.5 A). Riboside conjugates of these free bases were prominent, including *c*ZR, *t*ZR, DHZR and iPA (Figure 2.5 E), as were the 9-glucosides (Z9G, DHZ9G and iP9G, Figure 2.5 D). Three O-glucosides were detected, although at low concentrations and not throughout the entire

germination period (*c*ZOG, *c*ZROG and DHZROG, Figure 2.5 C). The highest concentration of any isoprenoid CK conjugate recorded was the monophosphate conjugate, *c*ZR5MP. Others included *t*ZR5MP, DHZR5MP and iPA5MP (Figure 2.5 B). With respect to levels of activity, most of the free bases attained maximal concentrations early in germination (48 h and earlier, Figure 2.5), while the CK conjugates (besides the O-glucosides) were prominent in later germination (48 h and later; Figure 2.5).

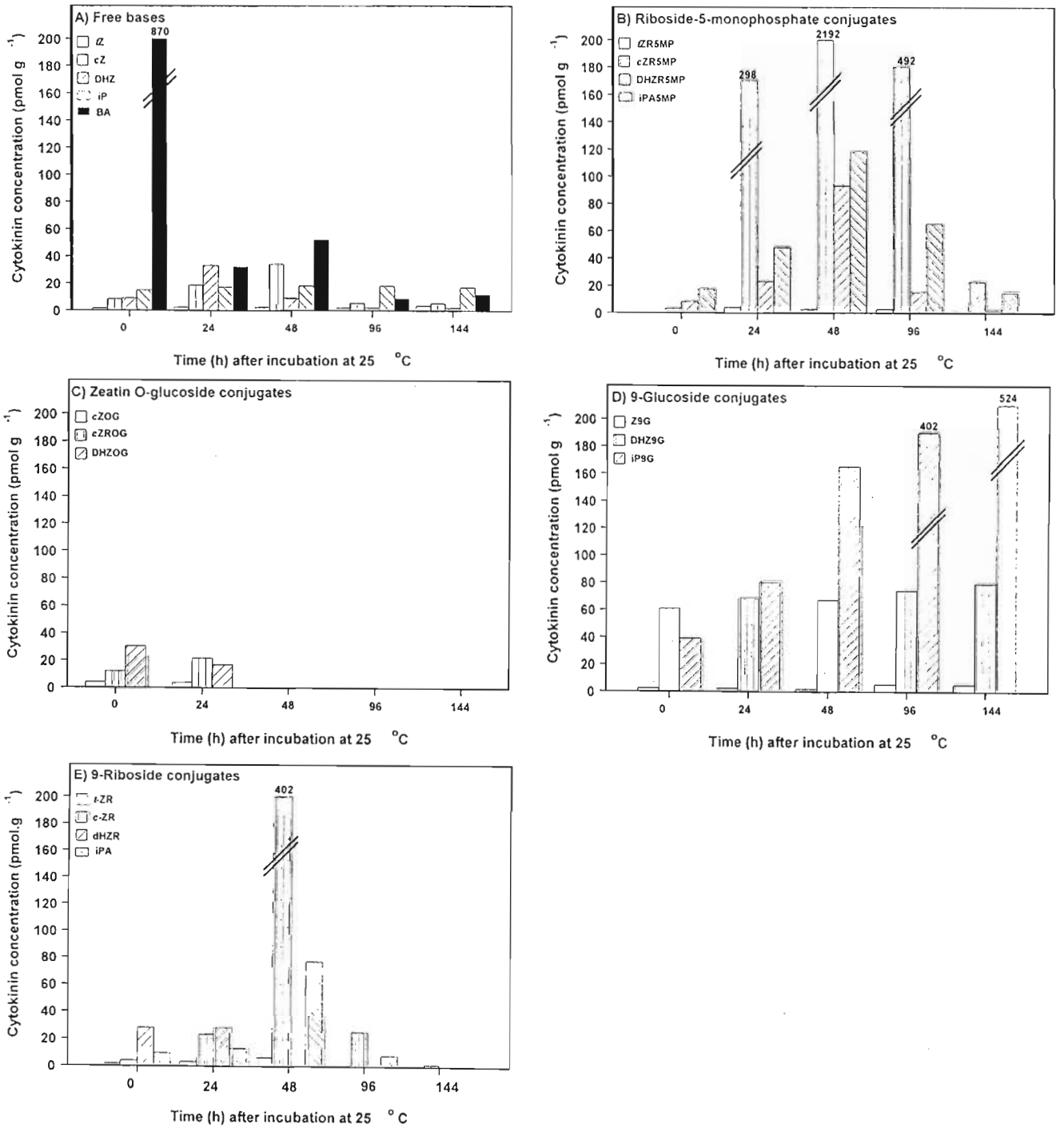

Figure 2.5: CK concentrations (pmol g^{-1} DW) in *T. minuta* achenes during germination and postgermination events.

With respect to qualitative trends, isoprenoid CKs and their derivatives, dominated the CK profile, with 18 isoprenoid CKs detected as opposed to one aromatic CK. No topolins were recorded. Table 2.8 shows that of the total CK content detected (6867.6 pmol g^{-1} DW), the isoprenoid CKs dominated, comprising 86.7 % of the total CK content. The highest isoprenoid CK derivative recorded was *c*ZR5MP (2192.5 pmol g^{-1} DW at 48 h; 31.9 % of the total CK content, Table 2.7) while the highest aromatic CK recorded was BA (870.1 pmol g^{-1} DW in the dry control, 12.6 % of the total CK content, Table 2.5).

Table 2.8: Total isoprenoid and aromatic CK concentrations detected in *T. minuta* achenes during germination and subsequent seedling development.

	Isoprenoid	Aromatic
Total (pmol g^{-1} DW)	5954.2	913.4
Percentage of total (%)	**86.7**	**13.3**

2.3.4 Exogenous Cytokinin Application

There was no improvement in achene germination attained by the application of any exogenous CK. All CK application at a concentration of 0.1 mg L^{-1} had no effect on germination, while the application of all CKs at concentrations of 1 and 10 mg L^{-1} impaired germination (Figures 2.6 and 2.7).

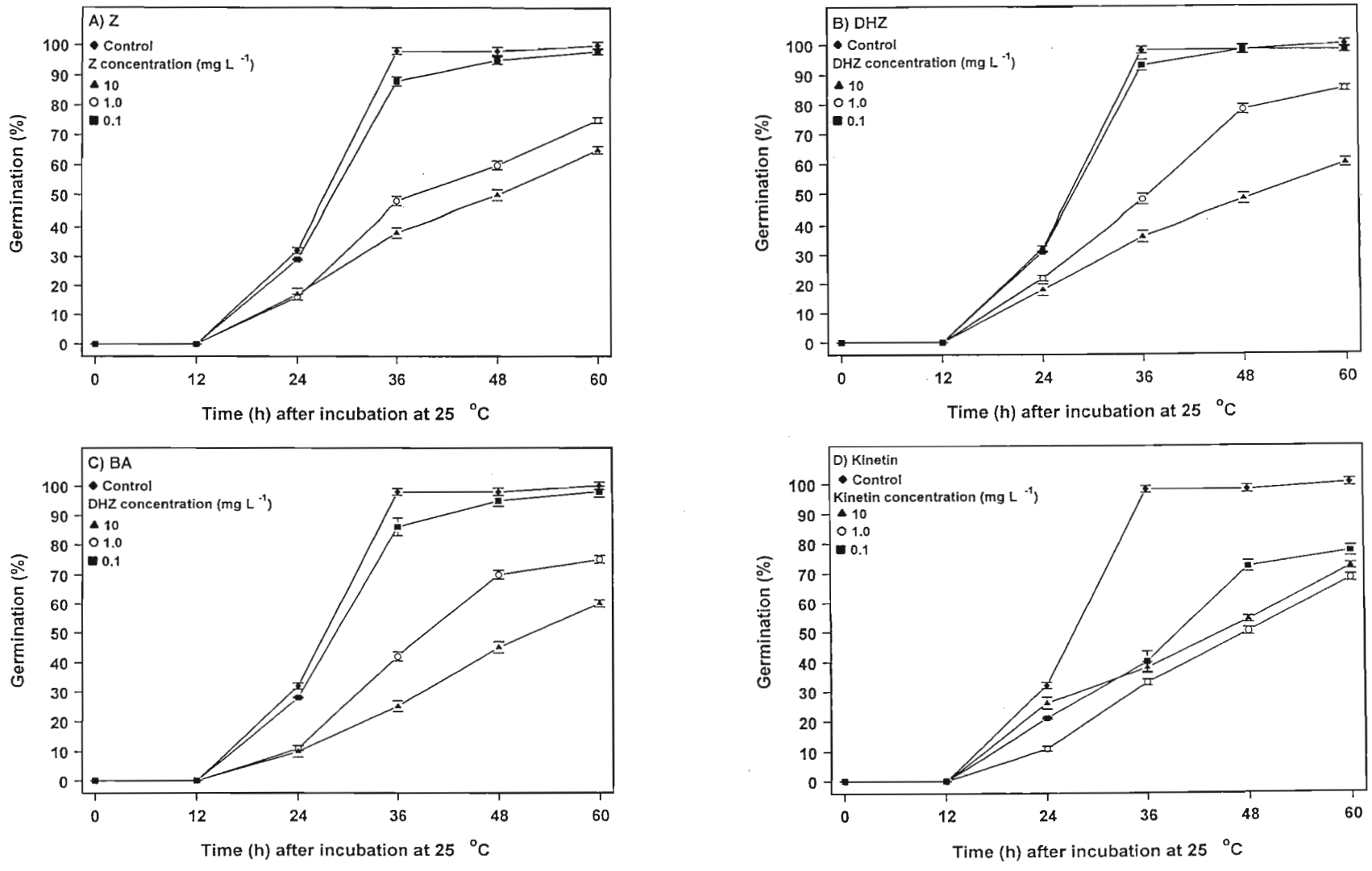

Figure 2.6: Germination of *T. minuta* achenes following exogenous CK free base application of (A) Z, (B) DHZ, (C) BA and (D) kinetin at various concentrations.

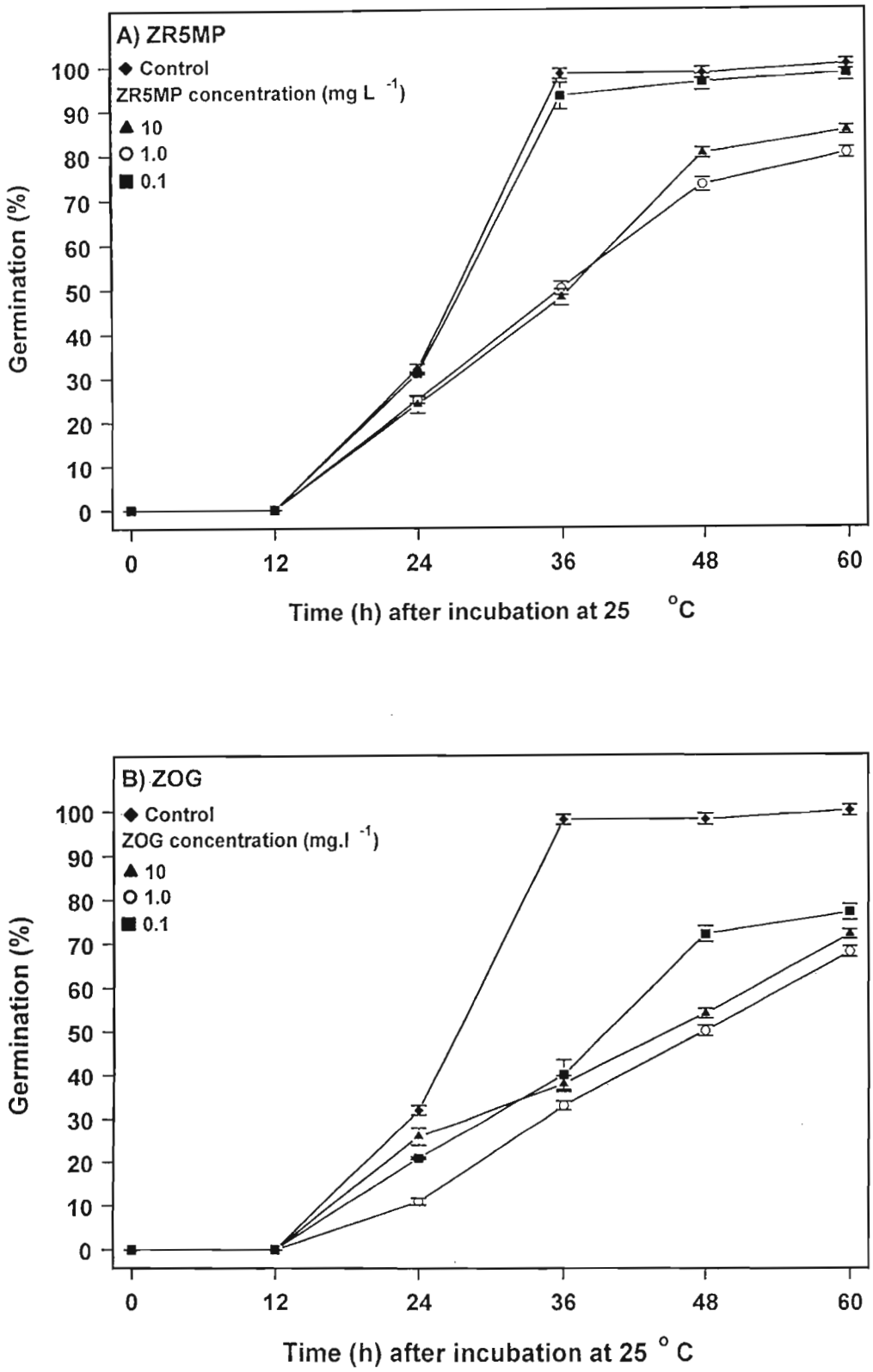

Figure 2.7: Germination of *T. minuta* achenes following exogenous CK conjugate application of A) ZR5MP and B) ZOG at various concentrations.

2.4 Discussion

2.4.1 Endogenous Cytokinins in Germinating *T. minuta* Achenes

Tables 2.2 - 2.7 and Figure 2.5 revealed that there was no definite trend with respect to specific CK maximal concentrations in germinating *T. minuta* achenes, although 8 out of the 19 detected CKs attained maximal levels at 48 h. This lack of a definite trend in CK maximal levels suggests that one cannot group this diverse hormone group collectively to a common role(s). Instead, particular CKs may have specific roles that differ from the roles of other CKs found in the plant. This notion of different roles is supported by KAMINEK (1992), who stated "different CKs show different biological activities, rates of conversion, solubility and transport across membranes, indicating that these compounds differ in their biological functions". Furthermore, a general trend exists between the two basic CK groups, namely the aromatic and isoprenoid CKs, with respect to activity and functioning. Generally, the isoprenoid CKs (particularly Z) have a greater effect on cell cycling and display potent activity in the soybean and tobacco callus bioassays, which are based on cell division. The aromatic CKs display higher activity with respect to growth and developmental processes, and show higher activity in tissue culture and senescence based bioassays (HOLUB *et al.*, 1998). However, there is opposition to the notion that specific CKs have specific biological activities and roles. MOK and MOK (2001) state that plants responses to CKs are broad and unspecific, and that CK action is often masked by other hormones. CHEN (1997) states that it is unknown if CK interconversions are specifically regulated, rather possibly merging with the general metabolism of purines.

In order to interpret these varying CK concentrations found in *T. minuta* samples, one needs to understand the functioning, with respect to activity, of these various CK conjugates. LETHAM and PALNI (1983) summarized the basic functional significance of the various CK conjugates into the following groups 1) active forms that evoke a growth response, 2) translocation forms, 3) storage forms, and 4) detoxification products that are formed when endogenous CK concentrations rise so high as to become toxic. Thus, a high

concentration of an endogenous CK in *T. minuta* may not necessarily confer high CK activity, and needs to be compared to the results of the soybean callus bioassay that measures biological activity. One needs to evaluate the conjugate in question, and assess its function in relation to its activity.

It is widely considered that the free bases are the most active forms of any CK (KAMINEK *et al.*, 1987; STRNAD, 1997; HOLUB *et al.*, 1998). Conjugation invariably leads to reduced activity, with some conjugates reducing activity more than others. Thus, the rate of conversion, and the type of conjugates formed, are important in homeostatic control of CK activity (KAMINEK *et al.*, 1987; STRNAD, 1997; HOLUB *et al.*, 1998). As mentioned, different conjugates have different roles, and specific conjugation may be directed towards these roles in relation to the plant's needs at any given time.

Glycosylation of a free base, at the 3-,7- or 9-N position, results in a metabolically stable conjugate, but with greatly reduced biological activity. These conjugates cannot be hydrolyzed back to the active base form. Reports on the reduction of activity following glycosylation at the 9-position, vary from a complete reduction in activity to a halving of activity (HOLUB *et al.*, 1998). Generally though, it is agreed that biological activity is significantly reduced, and thus, glycosylation is thought to be an irreversible inactivation reaction used to regulate free base levels (KAMINEK, 1992; STRNAD, 1997; HOLUB *et al.*, 1998). In a number of experiments in which intracellular concentrations of glucosides were measured in relation to BA-induced growth, LETHAM *et al.* (1982) found that although BA-glucoside concentrations remained constant, growth rates declined rapidly in correspondence to declining free BA concentrations. This form of conjugation may occur readily in detoxification reactions at supraoptimal CK concentrations.

O-glucosides display high biological activity (KAMINEK, 1992) in tissue culture and senescence bioassays (TAYA *et al.*, 1978; VAN STADEN and DREWES, 1991). O-glucosides are readily hydrolyzed to provide a free source of the active base, with ZOG being hydrolyzed to provide free Z (LETHAM and PALNI, 1983). Despite displaying biological activity, the primary role of the O-

glucosides is thought to be that of a storage role, as they can be rapidly hydrolyzed back to the highly active free bases (LETHAM and PALNI, 1983). Evidence to support this theory is strong. When plant tissues accumulate CK or display higher CK-like activity, it is often associated with an increase in O-glucoside metabolites (PALMER *et al.*, 1981). Additionally, when O-glucosides are applied exogenously to plants, a rapid conversion to free bases has been observed (SUMMONS *et al.*, 1980). Finally, and perhaps most appropriately, following a developmental process requiring CK, the level of O-glucosides has been observed to rapidly drop e.g. during the germination of maize seed (SMITH and VAN STADEN, 1978) and bud development in bean seedlings (VAN STADEN and DIMALLA, 1978). A similar phenomenon was observed in this study, where O-glucoside concentrations were highest in the dry control achene samples, and decreased upon imbibition and the initiation of germination, suggesting that germination is a correlative process.

It has long been regarded that ribosylation also decreases free base activity (SKOOG *et al.*, 1967) although HOLUB *et al.* (1998) found that ribosylation of BA and Z at the 9-position did not affect the activity of the BA and Z free base significantly. Thus, ribosylation is possibly a reversible form of conjugation. Although riboside conjugates may display activity, the primary role of this conjugate is believed to be for inactive transportation in the xylem (SAENZ *et al.*, 2003). Although conjugation may occur at a number of different positions, and subsequently result in different activity levels, the most common pattern of glycosylation and ribosylation is that of 9-conjugation (HOLUB *et al.*, 1998; SAENZ *et al.*, 2003).

The role of CK-ribosibe-5'-monophosphates (also referred to as ribotides, but simply referred to as monophosphate conjugates in this thesis) has been widely discussed. Monophosphate conjugates are thought to play a central role in the regulation of levels of the various CK metabolic forms, as they are readily converted to both the less active riboside and the highly active free base forms (LALOUE and PETHE, 1982; PALMER *et al.*, 1984). Inter-conversion pathways in an active CK pool are thought to favour monophosphate conjugate formation in the overall equilibrium, resulting in

high concentrations of these monophosphate conjugates (LALOUE *et al.*, 1981). More attention should be given to monophosphate conjugates as naturally occurring, active CKs with central roles in CK activity, following the discovery of a correlation between the division of tobacco callus cells with high concentrations of CK-monophosphates and low concentrations of ribosides and free bases, irrespective of whether the free bases or ribosides were supplied (LALOUE *et al.*, 1981). SCOTT and HORGAN (1984) used mass spectrometric techniques to show that monophosphate conjugates are more abundant than previously thought, proving to be more abundant than most riboside conjugates. This same trend was observed in the endogenous CKs found in germinating *T. minuta* achenes as presented in this study, following HPLC/MS analysis. The monophosphate fraction was discarded with the ethanol fraction following Dowex cation exchange resin purification, as the ethanol fraction did not display any biological activity in the soybean callus bioassay when previously tested (results not shown).

However, a number of roles not related to central activity have been proposed for the CK-monophosphates. They appear to be the storage form of CKs in lettuce (PIETRAFACE and BLAYDES, 1981) while CK-monophosphates may also be associated with uptake and transport across the cell membrane (LETHAM and PALNI, 1983). A translocatory role has been proposed by VAN STADEN and CROUCH (1996) following free base application to bean roots followed by high recovery of CK-monophosphates in the stem. VONK and DAVELAAR (1981) proposed that monophosphates may be involved in phloem transport.

Three CKs (BA, DHZROG and *c*ZOG) had their highest concentrations in the dry control achenes of *T. minuta*, the most noteworthy, both qualitatively and quantitatively, being the free base, BA, with a maximum level of 870.1 pmol g^{-1} DW. The marked decrease in BA in germinating *T. minuta* achenes from 870.1 pmol g^{-1} DW in the dry control to 32.4 pmol g^{-1} DW at 24 h, suggests that BA plays an important role in the initiation of germination, and is rapidly used in the first 24 h of germination. As no BA conjugates were formed during the rapid decline in BA, it is suggested that BA was actively used, and not

merely conjugated into an inactive form. However, when 0.1 mg L^{-1} BA was fed exogenously to *T. minuta* achenes, no improvement in germination was recorded and germination was in fact hampered by BA concentrations of 1 and 10 mg L^{-1} (Figures 2.6 and 2.7). However, an excessively high concentration of a particular hormone may prove to be toxic, as may have been the case at 1 and 10 mg L^{-1}.

The possibility that BA is important in the initiation of germination is supported by PANDEY *et al.* (2000), who found that exogenous BA application at a relatively low concentration (250 μM) improved germination in both *Aconitum heterophyllum* and *A. balfourii*. The O-glucoside concentrations, although much lower in comparison to the BA concentration, were also no longer detectable after 24 h, suggesting that they may be involved in the initiation of germination. As aromatic CKs are more active than the isoprenoid CKs in the promotion of growth processes (HOLUB *et al.*, 1998), this further supports the theory that BA (an aromatic CK) is involved in the initiation of germination. However, VILLALOBOS and MARTIN (1992) found eight isoprenoid CKs in germinating chickpea seeds, namely Z, ZR, iP, iPA, Z9G, ZROG, DHZ and DHZR. These authors believe that these CKs are involved in the breaking of seed dormancy and the stimulation of germination in chickpea seeds, by having a strong effect on reserve metabolism and mobilization in the cotyledons.

There is further evidence for the involvement of BA in early germination. BA is believed to promote DNA synthesis during early germination in maize axes (VASQUEZ-RAMOS and JIMENEZ, 1990) while cotyledons accumulated DNA from more than 1 cycle of DNA replication (GAILLI, 1984). With respect to embryonic axes, protein and RNA synthesis inhibitors were used in γ-radiated and non-irradiated axes. These inhibitors reduced DNA synthesis equally in both tissue treatments, suggesting that the mechanism of stimulation of DNA synthesis and polymerase activity is the same in both treatments. This also implies that the production of DNA is dependent on the production of new proteins as a result of stimulation at the

transcription/translation level (VASQUEZ-RAMOS and JIMENEZ, 1990). It is thus thought that the stimulation of DNA synthesis by BA during early germination is due to enhanced DNA polymerase activity. This increased DNA polymerase activity is believed to be the result of a conformational change on the polymerase molecule that is induced by BA. An increase in DNA polymerase activity is not due to an increase in levels of DNA polymerase. The fact that BA application resulted in increased polymerase activity without an increase in the protein itself, suggests that activity is increased and not the polymerase level (VASQUEZ-RAMOS and JIMENEZ, 1990). Secondly, DNA polymerase is not synthesized during early germination (VASQUEZ-RAMOS *et al.*, 1988).

Figure 2.8 shows that imbibition in *T. minuta* achenes is rapid, with a 50 % increase in mass being attained 6 h after imbibition. Metabolism and cell functioning would therefore be expected to be underway shortly after imbibition. This suggests that BA could be active in the promotion of DNA synthesis and repair.

There are three key points which strongly suggest that BA is involved in the promotion of DNA synthesis during the early stages of germination in *T. minuta* achenes: 1) Following imbibition, DNA synthesis and repair is one of the first events associated with germination (Figure 1.1); 2) A high BA concentration was detected in dry control achenes followed by a marked drop in BA without subsequent conjugation at 24 h; and 3) BA has been linked to the promotion of DNA synthesis (GAILLI, 1984; VASQUEZ-RAMOS and JIMENEZ, 1990). These 3 points suggest that BA is rapidly utilized in the initiation of germination, or early germination, via the promotion of DNA synthesis.

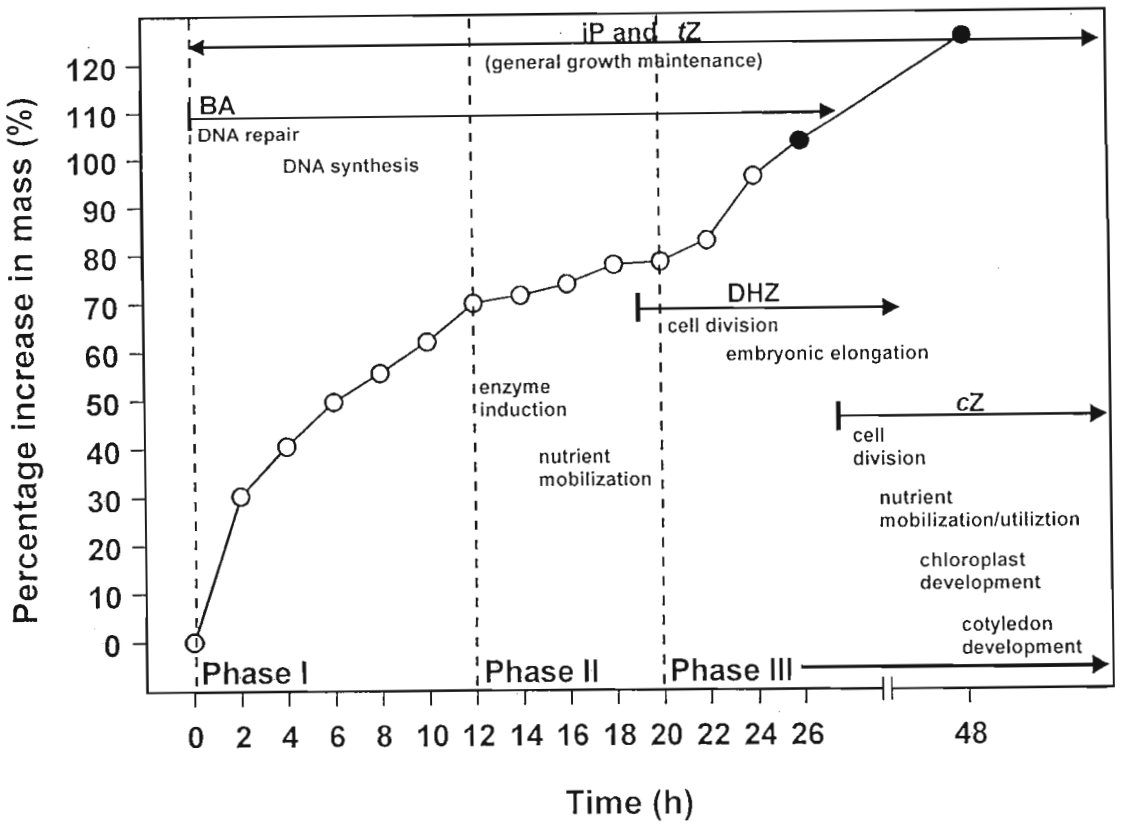

Figure 2.8: Imbibition curve of *T. minuta* achenes. CK free base maximum concentrations are shown sequentially with possible function(s) in germination. Points 0-24 h (O) are actual data courtesy of HILLS (2003), points 26 h and 48 h (●) are estimated by the author, based on a generalized imbibition curve provided by BEWLEY (1997).

Three maximum CK concentrations were attained 24 h after imbibition of *T. minuta* achenes in the HPLC/MS analysis, namely DHZ, DHZR and *c*ZROG (Tables 2.3 and 2.6). All three of these compounds have biological activity in the soybean callus bioassay (VAN STADEN and DREWES, 1991). Bearing in mind that roughly 95 % of the achenes had germinated by 36 h, with approximately 75 % of the achenes germinating between 16 and 26 h (Figure 2.2), it is possible that these compounds are involved in the actual germination events, such as embryonic elongation. As CKs, particularly the isoprenoid CKs (HOLUB *et al.*, 1998), are known primarily for their role in promoting mitotic events in cell division (ZHANG *et al.*, 1996), it is likely that these compounds are involved in the initiation and/or maintenance of cell division during embryonic elongation. However, the fact that the concentrations of these compounds decrease markedly by 48 h (Figure 2.5),

once the achenes have germinated (Figure 2.2), suggests that these CKs are used in the maintenance of actual germination events. Additionally, during such a period of high physiological activity and growth, CKs would be expected to be involved in the high levels of enzyme induction and nutrient mobilization that would undoubtedly be under way at this point.

The CK concentrations detected in the dry controls and achenes after 24 h imbibition support the theory of BROWN and DIX (1985) that CKs have a major, active role in germination and not merely a strictly permissive role, as suggested by THOMAS (1980). However, both theories are plausible and may hold true for different species.

In the bioassay analyses, increases in CK-like activity occurred 24 h after imbibition in *T. minuta* achenes (Figures 2.3 and 2.4). Although there is a degree of variation between each bioassay, these bioassays do have an important role in showing actual biological activity, as the numerous conversions and interactions between bases and their corresponding conjugates are difficult to interpret. Secondly, numerical values obtained from HPLC/MS analysis do not necessarily relate to biological activity, as different compounds display different potency with respect to biological activity.

These results do show high CK-like activity in the DHZ and Z region, at 24 h. This corresponds to the accurate HPLC/MS results that showed a significant DHZ concentration at 24 h. DHZ displays high biological activity in bioassay tests, in some cases higher than Z (VAN STADEN and DREWES, 1991). Activity in the corresponding DHZ and Z conjugate regions may be explained by the hydrolysis of these conjugates to their corresponding free base forms, as described earlier (VAN STADEN and DREWES, 1991).

VAN STADEN and DREWES (1991) found that "iP displayed little activity and may be very difficult to detect using the soybean callus bioassay." Thus, the high biological activity observed in the iP region was difficult to interpret, as this did not correlate with the quantification analysis, which did not show significant increases in iP or iP-conjugates at similar times.

Having stated that bioassays produce variable results, it is encouraging to note that these results were similar for two different extraction and separation methods (Figures 2.3 and 2.4). Both Dowex Column separation (followed by paper chromatography) and HPLC separation produced distinct increases in activity in the soybean callus bioassay at 24 h.

The associated maximal CK-like activity at 24 h supports the theory of BROWN and DIX (1985) that CKs have an active role in the germination of some species. This may possibly have been the time when CK activity was the highest. It is possible at this point that a large amount of readily available CK was required and used rapidly in actual germination events, such as embryo axis elongation. In the postgermination events described earlier, it is possible that a "slow release" of CKs from their associated conjugates (VAN STADEN and DREWES, 1991) was sufficient to drive postgermination events. This "slow release" of CK may have not been detectable in the bioassay. The biological potency of DHZ has been mentioned, and the maximum concentration of this free base obtained at 24 h with HPLC/MS analysis may be reflected by the bioassay results with a DHZ-associated increase in CK-like activity at 24 h.

The most CK types were detected 48 h after imbibition of the *T. minuta* achenes. Besides *c*Z (34.1 pmol g^{-1} DW), the majority of these CKs were conjugated CKs, which have lower activity, corresponding to the decrease in CK-like activity detected using the soybean callus bioassays. Quantitatively, the most significant increases were attained with *c*ZR5MP (2192.5 pmol g^{-1} DW, Table 2.7) and *c*ZR (401.2 pmol g^{-1} DW, Table 2.2) at 48 h, with the high monophosphate conjugate concentrations dominating.

In *T. minuta* achenes, maximal germination had been attained 48 h after imbibition (Figure 2.2), and the majority of the achenes had developed into young seedlings. The potential role of CK-monophosphates, with respect to uptake and transport across the cell membrane is well documented (LETHAM and PALNI, 1983) while a translocatory role has been proposed (VAN STADEN and CROUCH, 1996). Thus, it is probable that the conjugated

monophosphate forms were performing a transport role, so that CK could be rapidly transported to parts of the seedling where they are required, and then again move easily across cell membranes for use intracellularly. These conjugates may also have a storage role, suggesting that the majority of endogenous CKs in *T. minuta* are not actively biosynthesized, but rather metabolized and stored until required. These monophosphate conjugates display great versatility as they may be rapidly converted into active free bases, or conversely into inactive riboside-conjugates (LALOUE and PETHE, 1982; PALMER *et al.*, 1984), supporting the notion that this may be the primary storage form used in the homeostatic control of active CK. However, LALOUE *et al.* (1981) believe that a potential role related to central activity may exist. VAN STADEN and DREWES (1991) reported biological activity for a number of monophosphate conjugates, including ZR5MP. However, they believed that these conjugates may not actually have had biological activity, but rather act as a "slow release" of CK. It is possible that the monophosphates may be either directly or indirectly involved in a number of physiological processes. This is a period of high physiological activity in *T. minuta* achenes as numerous growth and developmental processes are occurring as the seedling rapidly develops into a mature plant. Such processes include rapid cell division and DNA replication, expansion and development of the cotyledons, chloroplast development and nutrient mobilization. Even if the monophosphate conjugates display mild biological activity, their concentrations are so high in *T. minuta* achenes that they may exert a physiological effect. There was a sharp decline in *c*ZR5MP concentration, after 48 h. This suggests that these monophosphate conjugates (or possibly a free base derived from conversion of the monophosphate conjugates) were actively used and not merely converted to another form. In addition to the very high monophosphate concentrations, *c*Z, *c*ZR and iP also attained a maximal concentration at 48 h. This supports the theory of the monophosphates being slowly converted into more biologically active forms to be used in the maintenance of physiological processes during seedling development.

It is possible during this period of high physiological activity that CK biosynthesis occurs. There are two possible pathways for CK biosynthesis where CK monophosphates are important product forms (Figure 2.9). KAKIMOTO (2001) describes in a potential CK biosynthetic pathway, whereby the first reaction in the formation of isporenoid CKs involves the addition of dimethylallyl diphosphate to ADP and ATP, to form isopentenyladenosine-5-diphosphate (iPDP) and isopentenyladenosine-5-triphosphate, respectively, which is in turn dephosphorylated to form isopentenyladenosine-5-monophosphate (iPMP). Additionally, ASTOT *et al.* (2000) proposes an iPMP independent pathway whereby *t*Z5MP is the first product formed. These monophosphate are then also converted to a number of other forms (Figure 2.9). Thus, active CK biosynthesis may account for the high level of CK-5-monophospahte conjugates observed at 48 h.

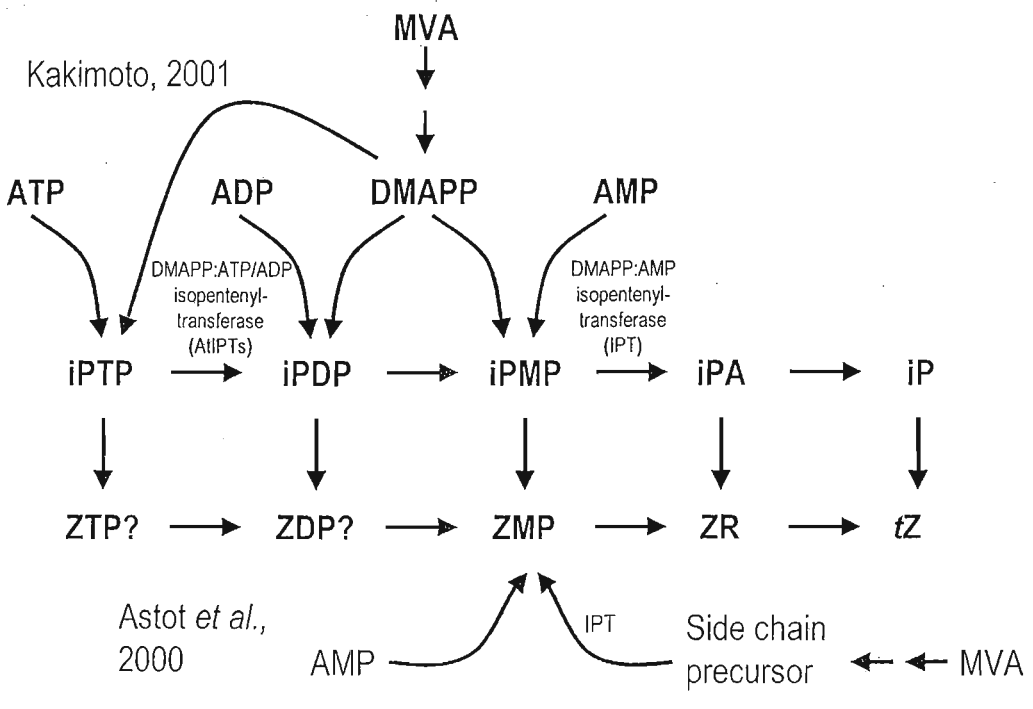

Figure 2.9: Proposed CK biosynthetic pathways involving monophosphates as primary intermediates (adapted from TAYLOR *et al.*, 2003).

The majority of the CKs detected in *T. minuta* were isoprenoid CKs. While isoprenoid CKs have a stronger effect on cell division than aromatic CKs, it does not mean that they will not have any effect on a number of growth and developmental processes. Isoprenoid CKs have been implicated in the expansion and development of cotyledons (RIJVEN and PARKASH, 1970; FANTELLI *et al.*, 1984), the stimulation of reserve metabolism and mobilization (VILLALOBOS and MARTIN, 1992; DUTTA *et al.*, 1994; DUTTA *et al.*, 1997). LONGO *et al.* (1979) confirmed that aromatic CKs are important in the development of chloroplasts. Thus, besides being important in cell division in the growing seedling, these endogenous CKs may have roles in other growth and developmental processes.

Normally the *trans*-isomer of Z (*tZ*) displays greater biological activity than the *cis*-isomer (*cZ*), as shown by bioassay results. In these *T. minuta* results, *cZ* and its derivatives are far more dominant than *tZ* and its derivatives. A few exceptions have been recorded where *cZ* is more common and displays higher activity than *tZ*. EMERY *et al.* (1998) found concentrations of *cZ* and its isomers to be higher than *tZ* and its isomers in chickpea seeds. EMERY *et al.* (1998) found high concentrations of *cZ* during the early stages of development which involved periods of rapid cell division. These concentrations dropped rapidly once the cotyledons expanded. This again displays the potency of isoprenoid CKs in cell division, and suggests that *cZ* displays activity in cell division during development in chickpea seeds. No isomerization of their (*trans*) [^{2}H] Z internal standard occurred, suggesting that the high concentrations of *cZ* were not due to isomerization during extraction from the *tZ* to the *cZ* form. EMERY *et al.* (1998) suggest that Z isomer activity may be species specific, with *cZ* displaying greater activity than *tZ* in certain species, such as chickpea. Tables 2.2; 2.6 and 2.7 show that a similar situation may occur in *T. minuta*. An overall 3578.3 pmol g^{-1} DW *cZ* and *cZ* conjugates were detected in comparison to 34.2 pmol g^{-1} DW *tZ* and *tZ* conjugates. In addition, 5 *cZ* CKs were detected as opposed to 3 *tZ* CKs.

Two 9-glucoside conjugates attained maximum concentrations 144 h after imbibition in *T. minuta* achenes. These conjugates have been reported to

display greatly reduced activity, and cannot be hydrolysed back into an active form (HOLUB *et al.*, 1998). Thus, the 9-glucoside conjugates accumulate through the time sequence as they cannot be reconverted and used. A prime example of this is iP9G, where concentrations steadily increased as time progressed.

The fairly constant concentrations of iP and *tZ* throughout the time sequence of the *T. minuta* sample may reflect a general, maintenance role during germination and seedling development. Distinct maximal levels of DHZ and *cZ* indicate a period of increased activity for a specific function, while iP and *tZ* concentrations remained fairly constant throughout (Figure 2.5 A). DHZ and *tZ* must be performing a role throughout germination and postgermination, even though their concentrations do not fluctuate significantly. The potency of *tZ* (EMERY *et al.*, 1998; HOLUB *et al.*, 1998) and DHZ (VAN STADEN and DREWES, 1991) has frequently been noted in bioassays.

The concentrations of conjugated CK forms in *T. minuta* achenes were much higher in relation to the free bases at any one time (Figure 2.5). This may highlight the fact that most phytohormones are required in minute quantities to be effective, and that the activity of free CK bases is under tight regulation. However, it is possible that some of the conjugated forms may display higher activity than anticipated, particularly the O-glucosides (Figure 2.5 C) and the monophosphate (Figure 2.5 B) conjugates. The versatility and potential activity of the monophosphate conjugates may account for their high concentrations. The steady but gradual increases in the 9-glucoside conjugates (Figure 2.5 D) highlights the detoxification role of these irreversibly inactive conjugates. In a number of bioassay tests, VAN STADEN and DREWES (1991) reported that a number of CK conjugates displayed higher biological activity than their respective free bases. They attributed this to the fact that these conjugates may be far more resistant to CK oxidase, as it is well known that applied CK may be rapidly enzymatically oxidized (VAN STADEN and DREWES, 1991). Thus, while not displaying activity in the conjugated form, subsequent hydrolysis to their corresponding free base states would ensure a continued supply of free CK over a prolonged period.

Thus, these CK conjugates, besides the glucosides, may act as "slow release" CKs. This again, would explain the high concentrations of monophosphate and riboside conjugates. However, there is no increase in corresponding free base or conjugate concentration associated with the high concentration of *c*ZR5MP, suggesting "slow release" of free bases or conversion to other conjugate forms. This is also true following the decline in *c*ZR5MP after 48 h, thus suggesting rapid use of *c*ZR5MP.

With respect to qualitative analysis, the dominance of isoprenoid CKs over aromatic CKs is not uncommon. SAENZ *et al.* (2003) stated that the isoprenoid CKs are generally more common across the plant kingdom, and while the occurrence of both isoprenoid and aromatic CKs in a species is rare, in such cases the isoprenoid CKs are most abundant. Examples include *Elaeis guineensis* and *Medicago sativa* (SAENZ *et al.*, 2003). Table 2.8 shows that such a trend prevails in *T. minuta* achenes. However, as technology improves, more aromatic CKs are being discovered in natural plant systems.

2.4.2 Exogenous Cytokinin Application

The CKs applied in these experiments were chosen in relation to their biological activity (Figures 2.3 and 2.4) and their abundance in the *T. minuta* achenes (confirmed from quantitative analysis, Figure 2.5). Thus, a number of active free bases were applied. Bearing in mind that exogenously applied free bases may be metabolized before uptake, and that conjugates may act as a CK "slow release", ZOG and ZR5MP were also applied.

The fact that exogenous CK application did not improve germination does not mean that CKs do not have a function in the germination of *T. minuta* achenes. It has already been stated that exogenously supplied CKs are readily hydrolyzed and metabolized. Exogenously supplied free bases are readily N-and O-glycosylated, respectively, if concentrations become excessively high (STRNAD, 1997). At a concentration of 1 and 10 mg L^{-1}, germination was impaired markedly (Figures 2.5 and 2.6), probably as the CK concentration was too high, and thus toxic. Future research should identify

and quantify potential detoxification conjugates present after exogenous application to determine if higher CK-conjugates, especially N-glucosides, occur after exogenous CK application.

However, at 0.1 mg L^{-1}, germination was not affected at all, suggesting that this concentration was not toxic, yet no effect on germination was observed. As mentioned, the excess CK supplied exogenously may have been metabolized, or else there was sufficient endogenous CK to fulfill the requirement for germination, and excess CK may have been superfluous. At the higher concentrations, conjugation may not have been enough to prevent toxicity prevailing, and thus germination was inhibited.

2.5 Conclusions

HPLC/MS analysis is the most accurate method of analysis. A full spectrum of CKs were detected with this method, including both aromatic and isoprenoid CKs and a number of biologically less active CK conjugates. Additionally, HPLC/MS analysis gives a neatly quantified numerical value of the actual CK level. However, bioassays do have an important role to play in displaying biological activity, as the interactions and conversions between free bases and their corresponding conjugates are at times difficult to interpret, and numerical values do not always relate to the actual biological activity of the compound.

The isoprenoid CKs are far more abundant in *T. minuta*, than the aromatic CKs. *c*Z and its derivatives are the most abundant CK, with *c*ZR5MP being the most abundant CK conjugate. The detailed HPLC/MS analysis detected 19 CK compounds, including 4 free bases and a number of their corresponding conjugates. BA was the only aromatic CK observed, and interestingly, none of its derivatives were detected. This suggests active use of BA.

There was no distinct, common time at which high concentrations of the active free bases were detected, suggesting that different CKs may have different,

specific roles in the germination process, and thus attain maximum concentrations at different times. This in turn suggests that germination is not a single process, but rather a correlative process involving a number of events, with specific CKs having specific roles relating to these correlative events. Figure 2.8 attempts to summarize this correlative process and the roles that particular CKs may have during the entire germination and postgermination process.

A number of potential postgermination roles have been mentioned in conjunction with high concentrations in the free bases, *c*Z and iP, at 48 h once the achenes had completed germination. These postgermination roles were discussed in relation to the role of the very abundant monophosphate conjugates, particularly *c*ZR5MP, which also attained a maximum concentration at 48 h. It is also possible that CK biosynthesis may occur at 48 h, resulting in high monophosphate levels at this point, which may be intermediate products in a variety of biosynthetic reactions.

While *t*Z has consistently been reported to be more abundant and to display higher biological activity than *c*Z in most plant species, species-specific exceptions have been reported (EMERY *et al.*, 1998). The present data for *T. minuta* is a new example of another *c*Z dominant species

There is sufficient evidence obtained from the HPLC/MS analysis to support the theory of BROWN and DIX (1985) that CKs do have an important, active role to play in germination. The dramatic decline in free BA observed in germination without corresponding conjugation, following the high concentration observed in dry achenes, suggests that BA is actively used in the initiation of germination or in early germination processes, possibly in the stimulation of DNA synthesis (GAILLI, 1984; VASQUEZ-RAMOS and JIMENEZ, 1990). However, while authors have reported exogenous BA application improving germination (PANDEY *et al.*, 2000) this was not the case with *T. minuta*. Secondly, there was a distinct, high DHZ concentration obtained at 24 h. The potency of DHZ with respect to biological activity has been mentioned (VAN STADEN and DREWES, 1991). Bearing in mind that

roughly 75 % of the achenes germinate between 16 and 26 h (Figure 2.2), it is likely that DHZ has an active role during the germination of *T. minuta*. Although CKs are probably not involved in the breaking of dormancy *per se* (BEWLEY, 1997), the distinct increase in CK-like activity obtained in the bioassays, at 24 h during germination, suggests that CKs have an active role in the germination of *T. minuta*.

In an effort to more clearly define the roles of the various CKs in the actual germination process, smaller extraction intervals are recommended between 0 and 48 h. This may more clearly define CK changes and optimal concentrations, and how rapidly CK compounds are used/metabolized. Unfortunately due to time restrictions, this was not possible in this study. Further research is needed to better understand the role of CKs in germinating *T. minuta* achenes.

Chapter 3
Germination and Thermoinhibition Studies

3.1 Introduction

Factors that are associated with thermoinhibition may be divided into hormonal and non-hormonal factors. Under normal field conditions, non-hormonal factors may be associated with the induction of thermoinhibition e.g. elevated temperature, while hormonal factors may relate to the maintenance of thermoinhibition in response to non-hormonal factors.

While it is known that thermoinhibition in *T. minuta* prevails at 35 °C and higher, very little is known about the time taken to impose and break thermoinhibition. Thus, a number of experiments were carried out involving the shifting of achenes between the optimal germination temperature of 25 °C and thermoinhibition at 35 °C, in an attempt to clarify the time parameters involving the initiation and breaking of dormancy.

A non-hormonal factor that may be involved in thermoinhibition is endogenous ATP. SMALL *et al.* (1993) reported an alleviation of thermoinhibition in lettuce seeds following incubation in oxygen and kinetin, which was believed to be due to increased endogenous ATP and reduced aldehyde levels. Thus, a number of experiments involving the effect of exogenous ATP application and incubation in oxygen were carried out.

ABA is the hormone most commonly associated with the imposition and maintenance of thermoinhibition (DUTTA *et al.*, 1994 and 1997; YOSHIOKA *et al.*, 1998; LESKOVAR *et al.*, 1999). GA_3 (PERSSON, 1993; SMALL *et al.*, 1993; DUTTA *et al.*, 1994 and 1997; CARTER and STEVENS, 1998), GA_{4+7}

(DREWES, 1989; MADAKADZE *et al.*, 1993) and ethylene (HAUNG and KHAN, 1992; PRUNISKI and KHAN, 1993; GALLARDO *et al.*, 1994; GALLARDO *et al.*, 1995; GALLARDO *et al.*, 1996; CARTER and STEVENS, 1998) are the phytohormones most commonly associated with the alleviation of thermoinhibition. ABA/ethylene interaction models have been proposed where ABA interferes in the production of ethylene (GALLARDO *et al.*, 1994). There are also reports of CKs, particularly kinetin, alleviating thermoinhibition (REYNOLDS and THOMPSON, 1971; KHAN *et al.*, 1993; PRUNISKI and KHAN, 1993; SMALL *et al.*, 1993; DUTTA *et al.*, 1994). However, ethylene and GA are more commonly associated with the alleviation of thermoinhibition.

3.2 Materials and Methods

3.2.1 Materials

For experiments investigating non-hormonal factors, two seed lots were used. They were both collected from the same site (29° 36' S; 30° 23' E), but from different generations, with seed lot (A) harvested in June 1998, and seed lot (B) harvested in June 2002. For the experiments investigating hormonal factors, only seed lot (A) was used as it generally gave slightly better germination results and had previously been used in other CK-related experiments.

For all germination experiments, germination was recorded every 12 h, with germination being noted as the time of radicle emergence. For each experiment, five replicates of 25 achenes were placed in a 55 mm Petri dish lined with two pieces of 60 mm Whatman No. 1 filter paper. Three mL of treatment solution were applied to each Petri dish. Achenes were incubated at a light intensity of 15 μmol m^{-2} s^{-1}. Germination was converted to a percentage and the standard errors calculated.

3.2.2 Non-Hormonal and Hormonal Factors Affecting Thermoinhibition

a) Investigation of Germination at 25 °C

Germination curves were plotted for the two seed lots at 25 °C, using 3 mL distilled water. Materials and methods were as described above, except that germination was recorded every 2 h.

b) Determination of the Rapidity of Thermoinhibition Imposition

Achenes were imbibed with distilled water and incubated at the optimal temperature of 25 °C for 1; 2; 4; 8 and 16 h respectively, and then shifted to 35 °C. Shifting to 35 °C was recorded as time 0 h, and germination was recorded every 12 h thereafter for 72 h. Controls were maintained continuously at 35 °C, thus ensuring that any germination occurring after shifting to 35 °C was purely as a result of previous exposure to 25 °C.

c) Determination of the Time Required at the Optimal Germination Temperature to Alleviate Thermoinhibition

Thermoinhibition was induced by imbibing the achenes in distilled water and incubating them at 35 °C for 72 h. The thermoinhibited achenes were then shifted to 25 °C for 1; 2; 5; 10; 30; 60 min and 3; 4; 5; 6 and 7 h, respectively. Following exposure to 25 °C, achenes were shifted back to 35 °C. Shifting thermoinhibited achenes to 25 °C was considered as time 0 h, and germination was recorded every 24 h for 72 h. A control was included when thermoinhibited achenes were shifted to 25 °C, which was allowed to germinate at 25 °C. Additionally, a control was maintained constantly at 35 °C.

d) Effect of Exogenous ATP Application on Thermoinhibition

Five concentrations of ATP (0.01; 0.1; 1; 10 and 100 mg L^{-1}) were applied to the achenes, which were incubated at 35° C. Germination was recorded every 12 h for 72 h. A distilled water control was also observed.

e) Effect of Incubation in Oxygen with Exogenous Hormone Applications

Treatments included a distilled water control, 0.1 mg L^{-1} GA_{4+7}, BA, ZR5MP, kinetin and ATP solutions, respectively. A further treatment entailed combining the ATP and GA solutions, with 1.5 mL of each solution being applied to the achenes. Achenes were imbibed and incubated at 35° C in a 5 L desiccator in 100 % oxygen. A distilled water control was also maintained outside the desiccator. Achenes were checked for germination every 24 h through the glass desiccator, without removing the lid, thus ensuring that the achenes remained in oxygen. After 72 h, the lid was removed and a final germination count carried out.

f) Application of Exogenous Hormones to Germinating Achenes at 25 and 35 °C

ABA, GA_{4+7} (Pro Vida 2 % ww) and ethylene (in the form of Ethephon®, 400 g L^{-1} ethephone, Rhone-Paulena) were applied to the achenes at three concentrations (0.1; 1 and 10 mg L^{-1}). In addition, a number of CKs, namely BA, kinetin, DHZ, *t*Z, ZOG and ZR5MP were applied. The achenes were incubated at 25 or 35 °C, respectively. Germination was recorded every 12 h for 48 h and 72 h, respectively. Distilled water controls were included in each experiment.

g) Application of GA_{4+7} to Thermoinhibited Achenes

Following thermoinhibition for 72 h at 35 °C, achenes were treated with GA_{4+7} at three concentrations (0.1; 1 and 10 mg L^{-1}), thus ensuring that the achenes were already thermoinhibited upon GA_{4+7} application. Incubation at 35 °C was maintained. Germination was recorded every 24 h for 72 h. A distilled water control was included.

3.2.3 Endogenous Abscisic Acid Analysis in Germinating and Thermoinhibited Achenes

a) Achene Treatment in the Analysis of Endogenous Abscisic Acid Levels

To determine ABA levels during the course of germination, achenes were imbibed with distilled water and incubated at 25 °C. Extraction was performed at 12, 24 36 and 48 h after imbibition and incubation at 25 °C. A dry control was also extracted (0 h). For each treatment, two 1 g replicates were used.

To determine the role of ABA in thermoinhibition, achenes were imbibed in distilled water and incubated at 35 °C for 36 h and then shifted to the optimum germination temperature of 25 °C for a further 12 h, until germination was complete. This resulted in a combined incubation time of 48 h during which time endogenous ABA samples were extracted. Extraction occurred 6, 12, 24, and 36 h after imbibition and incubation at 35 °C. Following shifting to 25 °C, extraction was performed 39, 42, 45 and 48 h after initial imbibition and incubation at 35 °C, (3, 6, 9 and 12 h after shifting to 25 °C). A dry control sample was also analyzed (0 h). For each treatment, two 1 g replicates were used.

b) Purification of Abscisic Acid Extracts Using C_{18} Sep-Pak and MCX Cartridges

Achenes were homogenized (Ultra-Turrax T25) in 10 mL 80 % methanol with 10 % (w/w) polyvinylpolypyrrolidone (PVP, Polyclar AT) for two min. A labeled internal standard (1.85 MBq, 50 µCi DL-*cis/trans* [H^3] ABA; Amersham International, Buckinghamshire, UK) was added to the extraction solvent (50 000 dpm $sample^{-1}$) to correct for losses. The sample was extracted overnight at -20 °C, followed by centrifugation (20 000 *g* at 4 °C for 20 min; Beckman, Avanti J-251). The supernatant was collected with a Pasteur pipette, and the pellet re-extracted in 5 mL 80 % methanol at -20 °C for 30 min, followed by centrifugation as before. The resulting supernatant was collected, and pooled with the original supernatant, and purified using the method described below (DOBREV and KAMINEK, 2002).

Sep-Pak C_{18} solid phase extraction (SPE) cartridges (Waters Corporation, USA) were activated with 5 mL methanol followed by 5 mL of distilled water, and then equilibrated with 20 mL 80 % methanol. The pooled supernatants were passed through the cartridge, under pressure, at a flow rate of 5 mL min^{-1}. The resulting eluates were dried down to the water phase *in vacuo* at 35 °C, and then made up to 5 mL with 1 M formic acid. This 5 mL sample was then purified using a Waters Oasis® MCX 6 cc (150 mg) extraction cartridge (Waters Corporation, USA).

The MCX cartridges were pre-conditioned with 5 mL methanol and equilibrated with 5 mL 1 M formic acid. The sample was then passed through the cartridge under pressure at 5 mL min^{-1}, and the cartridge rinsed with a further 5 mL 1 M formic acid. The resulting eluate was discarded. The cartridge was subsequently eluted with 5 mL 100 % methanol to recover the ABA fraction. This eluant was dried under nitrogen, and resuspended in 100 µL methanol. All procedures were carried out in low light conditions as ABA is considered to be photosensitive, and thus degraded by light.

c) Ethereal Diazomethane Preparation and Sample Methylation

Ethereal diazomethane was generated without co-distillation, on ice, by hydrolysis of N-nitroso-N-methylurea with 5 N NaOH in a Wheaton Diazomethane Generator (Pierce, Illinois, USA) using the method described by FALES *et al.* (1973).

Dry diethyl ether was prepared by passing diethyl ether through a charcoal column. The dry diethyl ether was subsequently stored in a bottle containing iron filings to remove peroxides. Peroxides and water may cause an explosion during ethereal diazomethane generation.

N-nitroso-N-methylurea (350 mg) was placed in the inner tube together with 0.5 mL water (for the dissipation of generated heat). Dry diethyl ether (3 mL) was placed in the outer tube. The unit was cooled in ice for 15 min after which 1 mL 5 N NaOH was injected through the teflon rubber septum of the

Wheaton Diazomethane Generator. The reaction was allowed to proceed for approximately 45 min until the ether took on a deep yellow colour.

The resuspended ABA sample was methylated by adding 2 mL ethereal diazomethane. Methylation is important as methyl esters are stable and can be purified easily prior to analysis, thus enhancing HPLC separation and detection limits (CROZIER and MORITZ, 1999). After 30 min, the ether phase was removed under nitrogen. The dried samples were resuspended in 2 mL ethyl acetate, and partitioned against 2 mL distilled water. Partitioning was repeated until the ethyl acetate phase became colourless. The combined ethyl acetate phase was passed through a Nucleosil strata (NH_2) cartridge (Phenomenex®, Torrance, CA, USA) which had been preconditioned with 5 mL methanol and equilibrated with 8 mL ethyl acetate. The resulting eluate was dried under nitrogen, resuspended in 1 mL 20 % methanol and filtered through a 0.22 μm Millipore filter. These samples were subsequently used in HPLC analysis.

d) HPLC for Abscisic Acid Separation

The ABA sample was further separated by HPLC (Varian 5000, Varian Instrument Group, USA) and a 5 μm C_{18} column 250 mm x 10 i.d., ODS 1 (HPLC Technology, United Kingdom). A linear gradient starting with 20 % methanol in water (System B) run for 55 min into 100 % methanol, at a flow rate of 2 mL min^{-1}. Compounds of interest were detected at 260 nm using a Spectra System UV/VIS 1000 detector (Thermo Separation Products, USA) and quantified after calibration with the authentic standards of ABA-methylester (Sigma, USA). Losses incurred during extraction were accounted for by mixing 1 mL of the HPLC separated sample with 3 mL scintillation fluid (Ready Value, Beckman Coulter, USA). This mixture was allowed to stand in the dark for 1 h before counting (Beckman LS 6000 LL, Beckman, USA).

3.3 Results

3.3.1 Non-Hormonal and Hormonal Factors Affecting Thermoinhibition

a) Investigation of Germination at 25 °C

The first signs of radicle emergence occurred at 14 h (seed lot A) and 20 h (seed lot B). In both seed lots, most of the achenes germinated between 20 and 36 h, with approximately 75 % of the sample germinating during this period. In both seed lots, maximal germination was attained by 44 h (Figure 3.1).

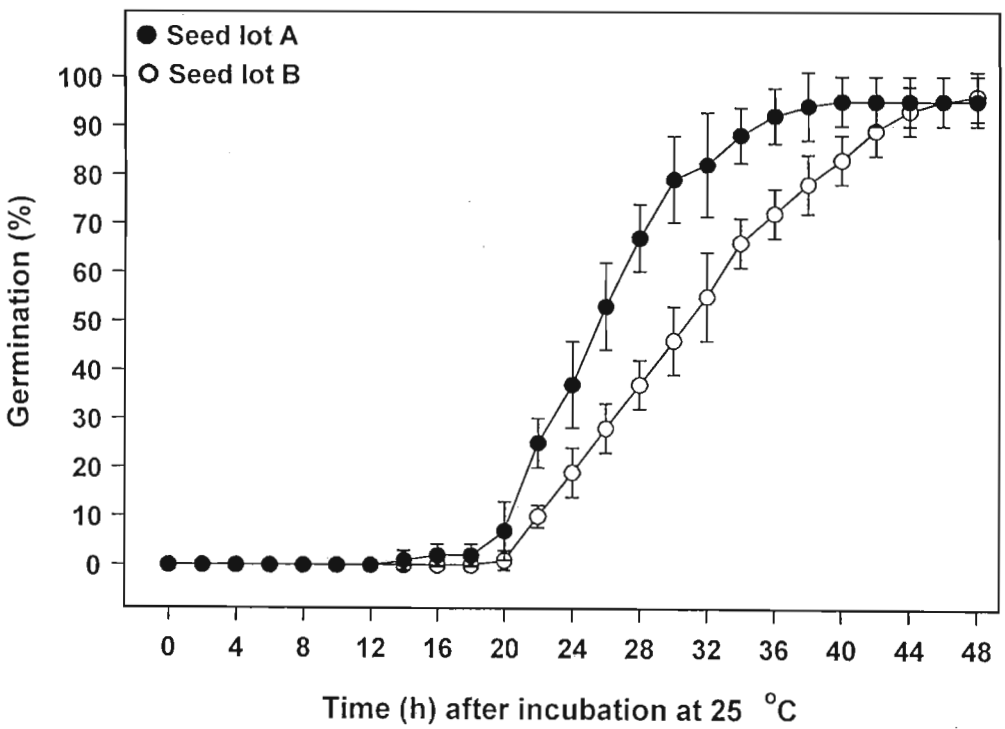

Figure 3.1: Germination curves of two *T. minuta* seed lots at 25 °C.

b) Determination of the Rapidity of Thermoinhibition Imposition

No germination was observed in either seed lot upon shifting the achenes to 35 °C. Even 16 h incubation at 25 °C resulted in no germination upon shifting to 35 °C.

c) Determination of the Time Required at the Optimal Germination Temperature to Alleviate Thermoinhibition

Figure 3.2 shows that thermoinhibited achenes from both seed lots required at least 3 h (180 min) at 25 °C to alleviate thermoinhibition. No germination was observed if exposure to 25 °C was less than 3 h. The germination observed occurred during the exposure to 25 °C.

Seed lot (A) displayed better germination than seed lot (B). Germination corresponded to the length of exposure at 25 °C. In seed lot (A) (Figure 3.2), germination increased following longer exposure to the optimal germination temperature of 25 °C, with the highest germination percentage (78 %) being recorded following the longest exposure to 25 °C (7 h). Conversely, only 4 % germination was observed following a 3 h exposure to 25 °C. Seed lot (B) displayed a similar trend, despite lower levels of germination following 7 h exposure to 25 °C (Figure 3.2).

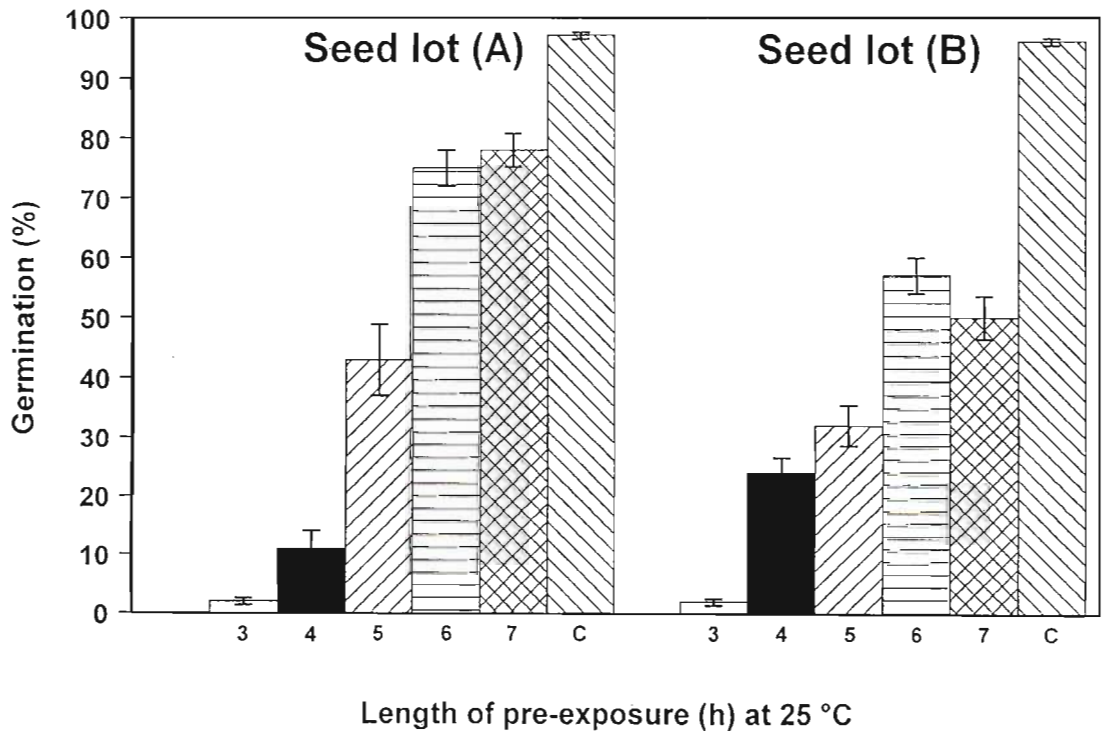

Figure 3.2: Germination of two *T. minuta* seed lots following thermoinhibition at 35 °C for 72 h, shifting to 25 °C for various lengths of time and subsequent shifting back to 35 °C (C= control at 25 °C).

d) Effect of Exogenous ATP Application on Thermoinhibition

The application of exogenous ATP did not alleviate thermoinhibition, with no germination being recorded at any concentration.

e) Effect of Incubation in Oxygen

No treatments had a significant effect on alleviating thermoinhibition. Imbibition with 0.1 mg L^{-1} GA_{4+7} resulted in the highest germination, with 33 % germination after 72 h (Table 3.1). However, all observed germination following such treatments was abnormal, with the cotyledons emerging instead of the radicle (Plate 2). No further seedling development occurred following cotyledon emergence.

Table 3.1: Germination of *T. minuta* achenes at 35 °C following various hormonal and ATP treatments, and subsequent incubation in 100 % oxygen.

Treatment (0.1 mg L^{-1})	Germination (%)
Control outside desiccator	0
Control inside desiccator	8
ATP	12
GA_{4+7}	33
ATP and GA_{4+7}	24
BA	8
ZR5MP	12
Kinetin	12

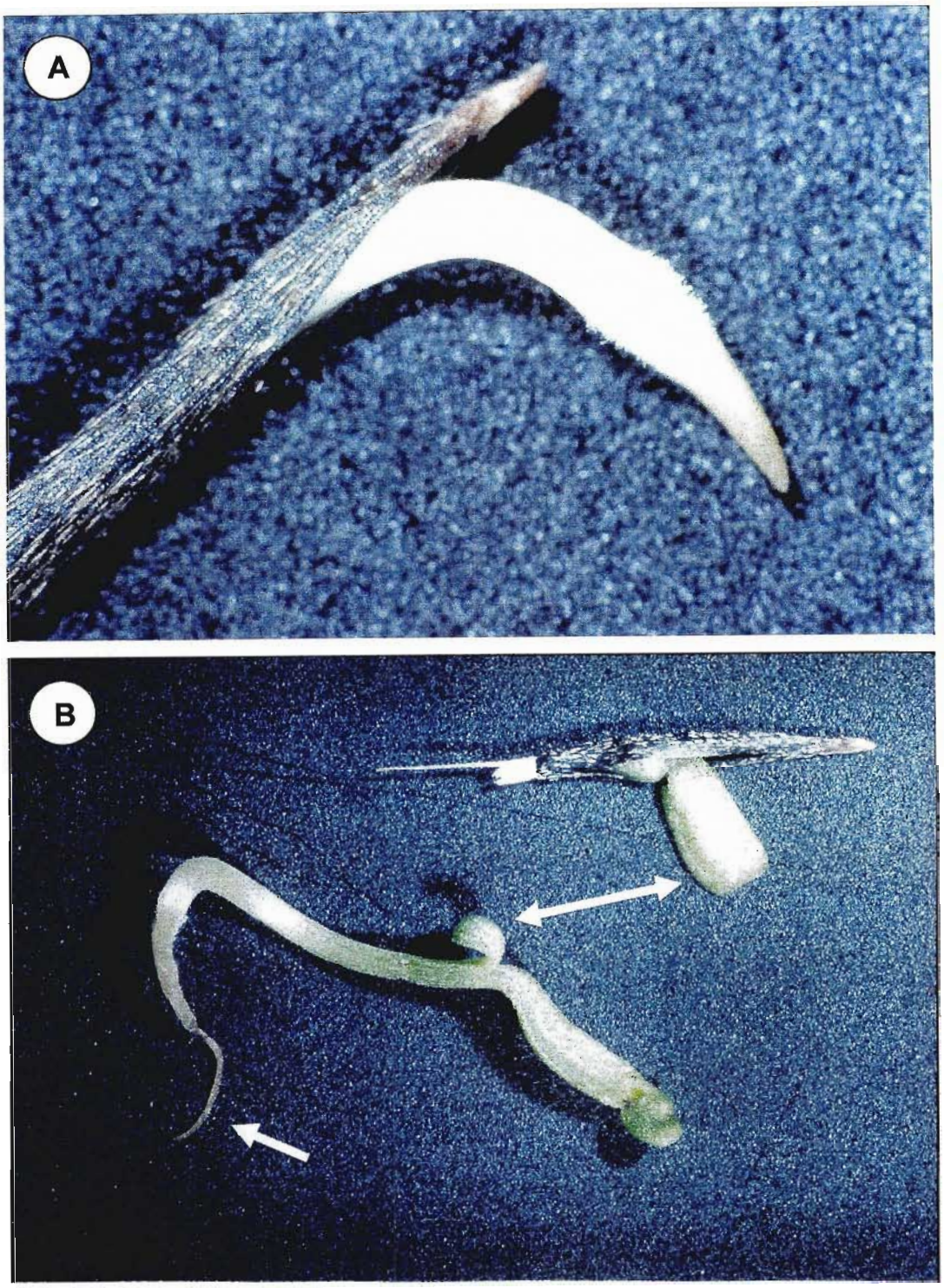

Plate 2: (A) Normal germination of *T. minuta* achene with radicle emergence (80x). (B) Abnormal germination of *T. minuta* achene with cotyledon emergence (6.6x). Note the reduced radicle and amputated cotyledon (as indicated by arrows). Both photos were taken with a Photomakroskop M 400 (Wild, Switzerland).

f) Application of Exogenous Hormones to Germinating Achenes at 25 and 35 °C

Exogenous ABA application inhibited germination at 25 °C (Figure 3.3A). The initiation of germination was delayed and maximal germination was reduced. Germination did not proceed at 10 mg L^{-1}, while germination was significantly reduced at 1 and 0.1 mg L^{-1}, with only 52 % (1 mg L^{-1}) and 63 % (0.1 mg L^{-1}) germination being attained. Application of GA_{4+7} at 0.1 mg L^{-1} to germinating achenes at 25 °C had a promotory effect on the initiation of germination, with germination proceeding more rapidly between 12 and 24 h. However, after 24 h, germination was similar to the control. Germination was reduced at 1 and 10 mg L^{-1}, with 75 % of the achenes germinating at 1 mg L^{-1} and no germination proceeding at 10 mg L^{-1} (Figure 3.3B). Exogenous ethylene application, at any of the three concentrations, did not have an effect on germination, with all germination being similar to the control (Figure 3.3C). No exogenous CK application improved germination. The higher CK concentrations of 1 and 10 mg L^{-1} impaired germination, while the lowest CK concentration of 0.1 mg L^{-1} had no affect on germination (See Chapter 2, Figures 2.6 and 2.7).

g) Application of Exogenous Hormones to Achenes at 35 °C

None of the exogenously applied hormones had any alleviatory effect on thermoinhibition, with no germination being observed during the 72 h incubation period.

h) Application of GA_{4+7} to Thermoinhibited Achenes

Application of GA_{4+7} to thermoinhibited achenes had no alleviatory effect, and no germination was recorded at any concentration during the 72 h incubation period.

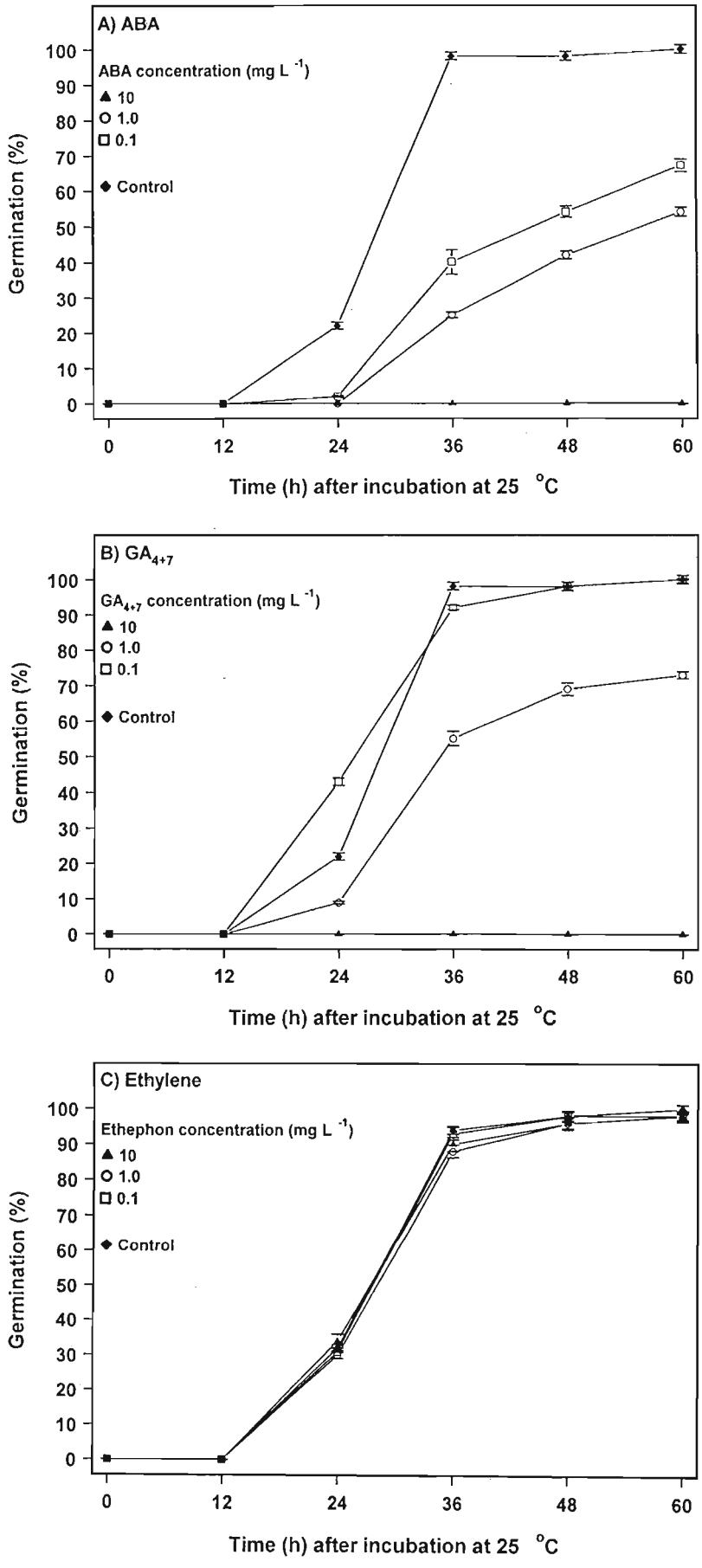

Figure 3.3: Germination of *T. minuta* achenes at 25 °C following exogenous applications of (A) ABA, (B) GA_{4+7} and (C) ethylene at various concentrations

3.3.2 Endogenous Abscisic Acid Analysis in Germinating and Thermoinhibited Achenes

During germination at 25 °C, a marked decrease in ABA levels was observed. Figure 3.4 shows highest levels of ABA (99 nmol g^{-1} FW) were found in the dry control achenes (0 h). Upon imbibition and the initiation of germination (12 h), ABA levels dropped sharply (15 nmol g^{-1} FW) and remained at this level (< 12 nmol g^{-1} FW) throughout the germination and postgermination periods (24 - 48 h) at 25 °C.

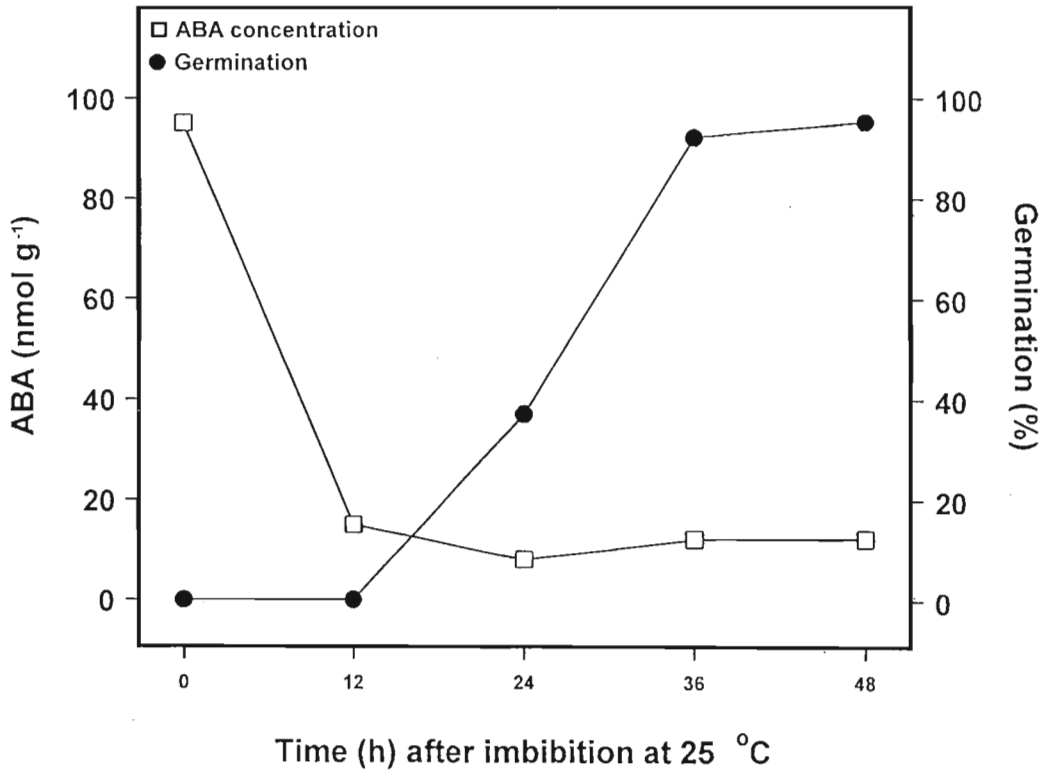

Figure 3.4: Endogenous ABA levels during germination of *T. minuta* achenes at 25 ° C.

Similarly, Figure 3.5 shows the highest ABA levels (95 nmol g^{-1} FW) in dry achenes (0 h). Following imbibition at 35 °C, ABA levels dropped markedly to the lowest level recorded (3 nmol g^{-1} FW at 6 h). This rapid decrease in ABA was followed by a steady increase in ABA between 6 h (3 nmol g^{-1} FW) and 36 h (70 nmol g^{-1} FW) while the achenes remained thermoinhibited at 35 °C. ABA levels dropped sharply again once the achenes were shifted to 25 °C and germination commenced (from 70 nmol g^{-1} FW at 36 h to 28 nmol g^{-1} FW

at 39 h, 3 h after shifting to 25 °C). The decrease in ABA levels after 36 h upon the commencement of germination corresponds with the trend observed in germinating achenes in Figure 3.4. In both endogenous ABA analysis experiments, the highest levels of ABA were observed in the dry controls (Figures 3.4 and 3.5).

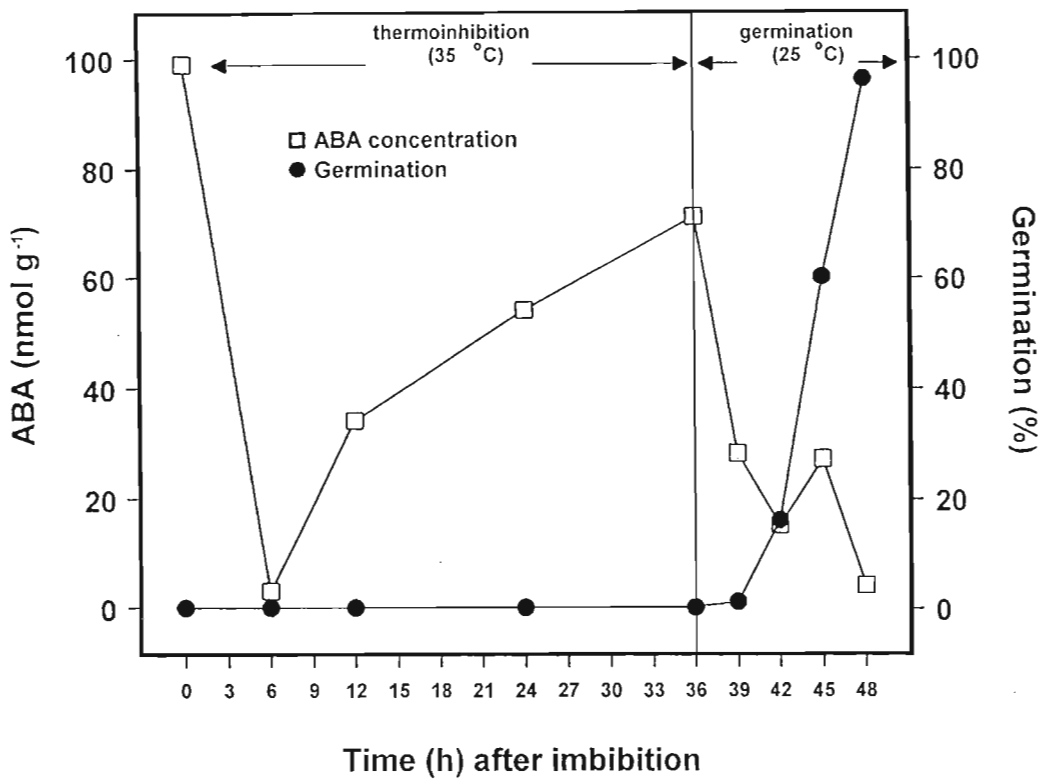

Figure 3.5: Endogenous ABA levels in *T. minuta* achenes during thermoinhibition (35 °C) and subsequent germination (25 °C).

3.4 Discussion

The results obtained from the experiments outlined in this Chapter suggest that thermoinhibition is tightly regulated and very rapidly imposed. No germination occurred following shifting to 35 °C in Section 3.3.1 (b), even after 16 h exposure to the optimal germination temperature of 25 °C. In the germination curves of *T. minuta* at 25 °C, the first signs of radicle emergence occurred 14 h (seed lot A) and 20 h (seed lot B) after commencing imbibition (Figure 3.1). Thus, after 16 h at 25 °C, physiological germination processes are well under way in all the achenes. The fact that no germination occurred upon subsequent shifting to 35 °C suggests that thermoinhibition is rapidly imposed.

Germination proceeds very rapidly in *T. minuta* achenes when shifted to optimal germination conditions following thermoinhibition, with near 100 % germination being attained within 12 h of shifting to 25 °C (Figure 3.5 and HILLS *et al.*, 2001), as opposed to 36 h in a control (which did not receive any thermoinhibition pretreatment prior to imbibition at 25 °C, Figure 3.1). This is believed to be due to the achenes being "primed" for germination during thermoinhibition, due to a number of metabolic advances being made towards germination. Thus, all the achenes are in a similar, advanced, near-germinating physiological state following such "priming", and germination proceeds rapidly upon shifting to 25 °C. The germination curve is the same as for normally germinating achenes at 25 °C; it is just brought forward by roughly 24 h (HILLS *et al.*, 2001). This accounts for the higher (than in a normally germinating control at 25 °C) levels of germination following short exposure (<7 h) to 25 °C. Germination only occurs between 14 h and 20 h in the control (imbibed at 25 °C without any thermoinhibitory pretreatment, Figure 3.1). Longer exposure to 25 °C following priming resulted in higher germination (Figures 3.2 and 3.5). Despite the advanced physiological state of the achenes, germinating primed achenes are very rapidly thermoinhibited upon shifting back to 35 °C. Neither the stage of germination that the achene had reached, nor the length of the exposure at 25 °C, affected the imposition of thermoinhibition (Figure 3.2). The fact that no germination occurred following shifting back to 35 °C, suggests that thermoinhibition is readily and rapidly imposed. These results also show that at least 3 h is required at 25 °C to alleviate thermoinhibition. The difference in the germination of the two seed lots shows the variation in germination that may occur between different generations of seed from the same population.

SMALL *et al.* (1993) found that incubation of lettuce seeds in 100 % oxygen, following imbibition in kinetin, resulted in the alleviation of thermoinhibition. The alleviation of thermoinhibition was believed to be partly due to kinetin/oxygen treatment having an effect on the endogenous ATP concentration, as thermoinhibited seeds incubated in oxygen had ATP levels similar to or higher than control seeds, while thermoinhibited seeds incubated in air had ATP levels lower than control seeds. However, exogenous ATP

application had no alleviatory effect on thermoinhibition in *T. minuta* achenes. It is possible that exogenous ATP application proved ineffective due to poor ATP uptake or ATP metabolism prior to uptake. Alternatively, endogenous ATP levels may not regulate thermoinhibition in *T. minuta* achenes.

Additionally, SMALL *et al.* (1993) concluded that the alleviation of thermoinhibition following kinetin/oxygen treatment may be due to the seeds bypassing an ethylene requirement for germination, or an increase in seed sensitivity to ethylene. Figure 3.3C shows that *T. minuta* achene germination is not affected by ethylene, and exogenous ethylene application had no alleviatory effect on thermoinhibition in *T. minuta* achenes. Thus, if increasing sensitivity to ethylene or bypassing an ethylene requirement is the mechanism through which thermoinhibition is alleviated in lettuce seeds following oxygen and kinetin treatments, then the failure of these treatments to alleviate thermoinhibition in *T. minuta* achenes is possibly explained, as *T. minuta* achenes do not appear to have an ethylene requirement for germination.

HEYDECKER and ORPHANOS (1968) stated that a shortage of oxygen was the basic cause of failure of germination in wet, intact spinach "seeds" (actually fruits). In wet, intact seeds, a mucilage layer is secreted which is believed to inhibit diffusion of oxygen to the embryo, and thus inhibit germination. An increase in oxygen pressure or a removal of a part of the fruit coat covering the radicle, induced germination. Additionally, SMALL *et al.* (1993) found that thermoinhibited lettuce seeds, which were incubated in air, displayed high levels of ethanolic fermentation, while thermoinhibited seeds incubated in oxygen (which germinate following kinetin/oxygen treatment) respired aerobically. This implies that higher oxygen levels improve achene respiration. Incubation in 100 % oxygen did not have a significant effect on the alleviation of thermoinhibition in *T. minuta* achenes, despite 33 % germination being attained following imbibition with 0.1 mg L^{-1} GA_{4+7} solution and incubation in 100 % oxygen (Table 3.1). However, all germination observed was abnormal and was recorded over 72 h. A normally germinating control sample at 25 °C attains 100 % germination within 48 h (Figure 3.1), with the radicles emerging ahead of the cotyledons (Plate 2).

While no exogenous hormone applications alleviated thermoinhibition in *T. minuta* achenes, there appears to be strong evidence for an active role of ABA in the hormonal maintenance of thermoinhibition. Only exogenous GA application, at the low concentration of 0.1 mg L^{-1}, improved germination, while exogenous ABA application, at all concentrations, inhibited germination. Much has been written on the involvement of ABA with respect to seed development and maturation (HILHORST and KARSSEN, 1992), the maintenance of seed dormancy (REYNOLDS and THOMPSON, 1971; KOORNEEF *et al.*, 1984; GROOT and KARSSEN, 1992 and LEON-KLOOSTERZIEL *et al.*, 1996), as well as the initiation and maintenance of thermoinhibition (DUTTA *et al.*, 1994 and 1997; YOSHIOKA *et al.*, 1998; LESKOVAR *et al.*, 1999).

Figure 3.3A shows that exogenously applied ABA does inhibit germination at 25 °C. No germination was recorded following the application of ABA at 10 mg L^{-1}. Despite ABA being a known germination inhibitor, the prevention of germination following the application of ABA at 10 mg L^{-1} may be due to the high levels of an exogenously applied hormone proving to be toxic (discussed below with GA results). While germination did proceed at the lower ABA concentrations of 1 and 0.1 mg L^{-1}, it was significantly less than that of the control. Inhibition of germination due to ABA may be due the prevention of radicle extension. BEWLEY (1997) found that seeds that were in the late stages of germination would not germinate if incubated in ABA solutions. This is believed to be due to the prevention of cell wall loosening, which thus inhibits radicle growth. This is supported by SCHOPFER and PLACY (1985) who found that ABA application prevented cell wall loosening in *Brassica napus* embryos. Analysis of water relations indicated that neither the osmotic potential, nor the embryo's potential to take up water, was affected. Thus, the prevention of radicle extension was attributed to the prevention of cell wall loosening. Cell wall loosening is the primary step in all growth processes. Cell expansion is not possible without a loosening of the cell wall fibers, as cell wall loosening results in a relaxation of the cell wall pressure, thus allowing cell expansion. Cell wall loosening is mediated by a group of proteins called

expansins, which are activated by auxins (COSGROVE, 1997). Thus, cell wall loosening is auxin-induced. ABA is well known to counter auxin effects (THOMAS, 1980; YOSHIOKA *et al.*, 1998), thus potentially explaining the inhibitory effect of ABA on cell wall loosening, and thus radicle extension and germination.

The higher GA_{4+7} (1 and 10 mg L^{-1}) concentrations had a negative effect on the germination of *T. minuta* achenes. Supraoptimal hormone levels are toxic, even if the hormone generally promotes germination. This is likely to be the case at 1 and 10 mg L^{-1} GA_{4+7} application. There was a slight improvement in germination at 25 °C after treatment with 0.1 mg L^{-1} GA_{4+7} solution (Figure 3.3B). This is to be expected, as the promotory effect of GA on germination is well documented (THOMAS, 1980; BEWLEY and BLACK, 1994). The primary role of GA during germination has been linked to nutrient mobilization and the control of *de novo* endo-β-mannanase synthesis. This hydrolytic enzyme is thought to be the limiting enzyme required for the complete hydrolysis of the endosperm galactomannan backbone (HILHORST and KARSSEN, 1992; DUTTA *et al.*, 1997; LESKOVAR *et al.*, 1999). GA application has been shown to counter ABA inhibitory effects, thermoinhibition and counter ABA-induced dormancy (DUTTA *et al.*, 1997). However, no exogenous hormone application, including GA_{4+7}, alleviated thermoinhibition in *T. minuta* achenes. This suggests that GA is not involved in the control of dormancy and thermoinhibition *per se* (despite countering ABA effects), but rather appears to be important in the promotion and maintenance of germination once ABA mediated dormancy or thermoinhibition has been overcome (BEWLEY and BLACK, 1994). This timing of action upon the commencement of germination may also hold for the other exogenously applied hormones, which failed to alleviate thermoinhibition in *T. minuta* achenes.

The marked decreases in ABA observed at the onset of germination (Figures 3.4 and 3.5) were expected and confirms ABA as a germination inhibitor, supporting the results obtained from exogenous ABA application (Figure 3.3). The inhibitory effects of ABA on germination are well documented (SCHOPFER and PLACY, 1985; HILHORST and KARSSEN, 1992, and

BEWLEY, 1997), thus a decrease upon the commencement of germination would be expected. High ABA levels are to be expected in dry, mature, achenes, due to the role of ABA in the seed maturation and desiccation. However, for germination to proceed, ABA levels need to decrease upon imbibition. Endogenous ABA levels decrease more rapidly under germination conditions than under conditions that are inhibitory to germination (BRAUN and KHAN, 1975). However, they went on to state that a decrease in endogenous ABA levels does not always correlate with germination. YOSHIOKA *et al.* (1998) showed a decrease in ABA content shortly after imbibition in germinating lettuce seeds, highlighting the requirement for a decrease in endogenous ABA levels prior to germination. This reduction in ABA activity and/or levels may occur via a number of processes, which are discussed later. The commencement of germination in *T. minuta* achenes coincides with the marked decrease in ABA levels at 39 h (Figure 3.5) and 12 h (Figure 3.4), and as germination proceeds, ABA levels remain low.

The high ABA levels observed in the dry *T. minuta* controls (Figures 3.4 and 3.5) are not unexpected, as there are a number of processes in seed development that are ABA-related. The presence of ABA during seed development is essential in the initiation and maintenance of primary dormancy (KOORNEEF *et al.*, 1984, GROOT and KARSSEN, 1992, KOORNEEF and KARSSEN, 1994, LEON-KLOOSTERZIEL *et al.*, 1996; BEWLEY, 1997), the synthesis and accumulation of storage peptides, and increasing desiccation tolerance during the rigors of seed maturation and drying (HILHORST and KARSSEN, 1992). Thus, the high levels of ABA detected in the dry controls of *T. minuta* achenes are probably due to residual ABA from ABA-related developmental processes such as seed maturation and desiccation, as *T. minuta* achenes are quiescent and do not display primary dormancy.

There are two possible explanations for the marked initial decrease in ABA, namely leaching and metabolism (including conjugation and catabolism). Oxidative catabolism of ABA is a common form of ABA degradation (LOVEYS and MILBORROW, 1984). This oxidative catabolism is a spontaneous,

enzymatic reaction, which ultimately results in the formation of dihydrophaseic acid, following the initial formation of phaseic acid from ABA (ZEEVAART, 1999). These breakdown products are biologically less active than ABA, thus reducing the biological effectiveness of ABA (WALTON and LI, 1995). This catabolic reaction is catalyzed by ABA-8'-hydroxylase. The presence of this enzyme is dependent on the presence of ABA, and the level of enzyme induction is dependent on ABA concentration (CUTLER *et al.*, 1997). Additionally, the level of phaseic acid formation increases with ABA treatment (BABIANO, 1995), suggesting that this reaction is autocatalytic. ABA-8'-hydroxlase has been reported to be expressed at high levels in developing seeds and seedlings (BABIANO, 1995; GARELLO and LE PAGE-DEGIVRY, 1995).

An additional form of ABA metabolism is the formation of a sugar conjugate. ABA glucose ester is a widespread conjugate, and once formed, appears to be irreversible (ZEEVAART and BOYER, 1984). Upon formation, the conjugate is sequestered in the vacuole (LEHMANN and GLUND, 1986).

ABA is readily leached from achene pericarps (YAMBE *et al.*, 1992). The presence of ABA in the pericarp is known to inhibit germination, and thus, propagators have taken measures to actively facilitate leaching of ABA from the pericarp to improve germination, despite YOSHIOKA *et al.* (1998) stating that leaching of ABA from lettuce seeds did not contribute significantly to a decline in endogenous ABA levels. YAMBE *et al.* (1992) has provided the protocol for the leaching of ABA from rose achene pericarps, which markedly increase final germination percentages. Although not marked, leaching of ABA does occur in water (YAMBE *et al.*, 1992; LESKOVAR *et al.*, 1999). The presence of activated charcoal improved the leaching of ABA from rose pericarps (YAMBE *et al.*, 1992).

If leaching was not the cause of the reduction in endogenous ABA in *T. minuta* achenes, it is possible that the decrease in ABA at 6 h was due to rapid metabolism of ABA in response to imbibition. Even though the temperature was not suitable for germination, ABA may nonetheless have

been metabolized in a predetermined germination response to imbibition. The fact that endogenous ABA levels decrease rapidly upon the commencement of germination (at 39 h, 3 h after shifting to 25 °C; Figure 3.5) indicates that metabolism may have occurred very rapidly, which is supported by KROCHKO *et al.* (1998), who confirmed that ABA metabolism is under tight regulation and that degradation can be very rapid. In these results, it is debatable whether this potential metabolism is as a result of catabolism or conjugation, although catabolism is common and spontaneous (LOVEYS and MILBORROW, 1984).

It is likely that the increase in ABA levels during thermoinhibition is due to *de novo* biosynthesis. Oxidative catabolism is autocatalytic, thus, an increase in ABA levels would result in increased ABA-8'-hydroxylase activity, and ultimately higher rates of hydrolysis of ABA to phaseic acid. However, ABA-8'-hydroxylase activity can be down regulated in times of stress (CUTLER *et al.*, 1997). Secondly, conjugation to an ABA-sugar conjugate is irreversible, thus conjugates could not be converted back to active forms. Finally, ABA that has been leached from the seed could only be replaced by *de novo* biosynthesis.

The possibility of ABA biosynthesis during dormancy is supported by LE PAGE-DEGIVRY and GARELLO (1992). These authors reported that while dormant sunflower seeds contained high levels of ABA, the continued synthesis of ABA is required to maintain dormancy in sunflower embryos. Although these seeds were not thermoinhibited, this is a case where ABA biosynthesis was essential in the maintenance of a dormant state. However, YOSHIOKA *et al.* (1998) have found evidence that suggests that ABA biosynthesis is essential for the maintenance of thermoinhibition in lettuce seeds (*cv.* Grand Rapids). At 33 °C, Grand Rapid lettuce seeds became thermoinhibited, and maintained high ABA levels after imbibition. In contrast, seeds imbibed at 23 °C showed decreased ABA levels shortly after imbibition, and readily germinated. The application of fluridone, an ABA biosynthesis inhibitor, restored germination in thermoinhibited lettuce seeds, following a decrease in ABA content. This suggests that the maintenance of high ABA levels is responsible for the maintenance of thermoinhibition. The high ABA

levels are due to an increase in the rate of ABA biosynthesis at the higher temperature.

Additionally, there is sufficient evidence relating to the rapidity and specificity of ABA biosynthesis (XIONG and ZHU, 2003) and metabolism (KROCHKO *et al.*, 1998) to suggest that ABA is involved in the regulation of rapid thermoinhibition responses. ABA biosynthesis occurs very rapidly and is under tight regulation in the embryo (XIONG and ZHU, 2003), suggesting that rapid *de novo* ABA biosynthesis may be sufficient to rapidly impose thermoinhibition, which may account for the rapid imposition of thermoinhibition observed in *T. minuta*. Conversely, ABA may be very rapidly metabolized (KROCHKO *et al.*, 1998), which may thus account for the rapid germination responses observed in *T. minuta* following priming and the decrease in endogenous ABA at 6 h (Figure 3.5).

Thus, ABA does appear to be involved in the initiation and maintenance of thermoinhibition of *T. minuta* achenes. There is sufficient evidence correlating elevated ABA levels to dormancy and thermoinhibition in other species, while ABA biosynthesis and metabolism is tightly and rapidly regulated. The steady increase in endogenous ABA levels during thermoinhibition, following a marked decrease shortly after imbibition, suggests the necessity for active *de novo* biosynthesis of ABA in the maintenance of thermoinhibition in *T. minuta* achenes.

3.5 Conclusions

Thermoinhibition Regulation: Hormonal or Non-Hormonal?

If thermoinhibition was broken by exogenous ATP application and/or incubation in oxygen, then thermoinhibition would appear not to be regulated by the achene, but rather be induced and maintained by environmental conditions. If the initiation and maintenance of thermoinhibition appears to be regulated by endogenous hormones, then thermoinhibition would appear to be regulated by the achene, possibly to avoid unfavourable environmental conditions.

While exogenous 0.1 mg L^{-1} GA_{4+7} application did improve germination at 25 °C, no exogenous hormone applications were effective in alleviating thermoinhibition in *T. minuta* achenes. This may be due to these growth hormones not having a direct role in the breaking of thermoinhibition in this species, but rather having important roles to play in germination once thermoinhibition has been broken by another factor.

However, thermoinhibition in these achenes may be under hormonal regulation, as there is strong evidence for the role of ABA in the maintenance of dormancy and thermoinhibition of *T. minuta* achenes. High ABA levels were found in dry control samples, and the roles of ABA in the maintenance of embryo dormancy during seed development are well known. Additionally, exogenous ABA application inhibited germination, and the commencement of germination was accompanied by a decrease in endogenous ABA levels. But most interestingly, there appears to be active *de novo* biosynthesis of ABA in thermoinhibited achenes, following a marked decrease in ABA following imbibition. This active biosynthesis of ABA during thermoinhibition suggests that this phytohormone is essential in the maintenance of thermoinhibition of *T. minuta* achenes.

Thermoinhibition appears to be very rapidly imposed. Germination is rapidly inhibited following shifting to higher thermoinhibitory temperatures, even after prolonged exposure to optimal germination temperatures. This further supports the possibility of ABA-regulated thermoinhibition, as ABA metabolism and biosynthesis has been shown to be very rapidly and tightly regulated.

It thus appears that ABA is synthesized in the achenes in response to elevated temperatures that are unfavourable for germination to proceed. Unfavourable environmental conditions result in an achene-mediated inhibition of germination, which appears to be initiated and maintained by elevated levels of endogenous ABA.

Future Research

Further experiments need to be carried out to confirm the status of ABA in the regulation of thermoinhibition. Further avenues for research should include investigating ABA levels following incubation at 25 °C and subsequent shifting to 35 °C. A 12 h incubation at 25 °C would allow the initiation of germination without actual radicle emergence, during which time endogenous ABA would be expected to decrease. An increase in endogenous ABA upon shifting to 35 °C would further support the notion of ABA being involved in the regulation of thermoinhibition. Secondly, it would be interesting to note ABA levels in achenes following longer thermoinhibition at 35 °C. A continued rise in ABA to the levels observed in the dry controls would be expected, possibly followed by a plateau in ABA levels. Additionally, the application of fluridone (an ABA biosynthesis inhibitor) to thermoinhibited seeds needs to be investigated. If fluridone application alleviates thermoinhibition, with a corresponding decrease in endogenous ABA, then one could conclude with confidence that ABA is the major factor involved in the regulation of thermoinhibition. If fluridone application results in a decrease in endogenous ABA without alleviating thermoinhibition, then ABA may be only one of a number of factors involved in the regulation and maintenance of thermoinhibition. Finally, feeding experiments using radiolabeled ABA precursors need to be carried out to confirm *de novo* ABA biosynthesis during thermoinhibition. Incorporation of a high level of radioactivity in ABA extracted during thermoinhibition, following the marked decrease in endogenous ABA at 6 h, would confirm *de novo* biosynthesis of ABA during thermoinhibition.

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Appendix

Appendix 1: Basal medium for soybean callus bioassay (adapted from MILLER, 1965).

Stock solution	Chemical	g L^{-1} to make up stock	mL stock solution to make 1 L medium
Stock 1	KH_2PO_4	3.0	100
	KNO_3	10.0	
	NH_4NO_3	10.0	
	$Ca(NO_3)_2.4H_2O$	5.0	
	$MgSO_4.7H_2O$	0.715	
	KCl	0.65	
	$MnSO_4.4H_2O$	0.14	
Stock 2	NaFe.EDTA	1.32	10
	$ZnSO_4.7H_2O$	0.38	
	H_3BO_3	0.16	
	KI	0.08	
	$Cu(NO_3)_2.3H_2O$	0.035	
	$(NH_4)Mo_7O_{24}.4H_2O$	0.01	
Stock 3	Nicotinic acid	0.2	10
	Pyridoxine HCl	0.08	
	Thiamine HCl	0.08	
Stock 4	1-Naphthylacetic acid	0.2	10
Additional	Sucrose		30 g L^{-1} medium
	Myo-Inositol		0.1 g L^{-1} medium
	Agar		10 g L^{-1} medium

Adjust the pH to 5.8 using NaOH.

Kinetin was added to standards at concentrations of 1, 10 and 50 mg L^{-1}, respectively.