A BIOCHEMICAL STUDY OF BUDBREAK AND PLANT GROWTH REGULATORS IN TABLE GRAPES

by

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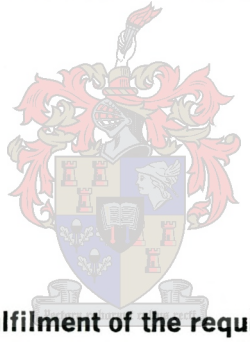

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously, in it's entirety or in part, submitted it at any university for a degree.

SUMMARY

The cultivation of table grapes in the warmer areas of South Africa, indeed worldwide, is complicated by rest breaking problems in spring due to delayed budbreak. In order to overcome these problems rest breaking agents, mainly hydrogen cyanamide, are applied. However, instead of alleviating the problem, additional problems such as uneven budbreak and reduced production are often induced. This study was initiated to further understand the physiological processes occurring during budbreak and how the application of hydrogen cyanamide influences these processes. The following aspects were investigated in this study:

a. The effect of hydrogen cyanamide on tissue cytokinin (specifically zeatin riboside) levels of Sultanina table grape vines after application at different times before natural budbreak was studied over two seasons.

In 1997, hydrogen cyanamide was applied at three weeks before induced budbreak and in 1998 at six weeks before induced budbreak. One year-old canes were sampled weekly after hydrogen cyanamide application, divided into distal and proximal sections, then further divided into buds, bark and wood tissues and the zeatin riboside (ZR) levels determined. A relatively high amount of chilling coupled to late hydrogen cyanamide application in 1997 led to a large effect on ZR release, but did not lead to significant shifting of the budbreak pattern. Zeatin riboside peaks were observed in buds, internode wood and bark of treated vines compared to control vines. The peaks were higher in distal portions compared to proximal portions in all tissues. The relatively lower chilling and earlier application of hydrogen cyanamide in 1998 had a larger effect on the budbreak pattern while the bud ZR peak was shifted earlier. The distal portion bud ZR peak was again higher than the proximal portion bud ZR peak. In 1997, as sampling was not initiated early enough, bud ZR peaks were only observed after budbreak, while in 1998 bud ZR peaks were observed before and after budbreak. The effect of these ZR increases on the development of inflorescence primordia, subsequent bunch development and ultimately production, are discussed.

b. Free xylem sap was sampled at cane and spur pruned lengths from unpruned canes of Sultanina from budswell until after budbreak in 1999 and from three table grape cultivars, i.e Sultanina, Alphonse Lavalleé and Sunred Seedless, in 2001 and ZR levels determined. The ZR levels in the buds of these three table grape cultivars, pruned to different cane lengths were also determined. One year old canes of these cultivars, were each pruned to long canes (14 buds) and short spurs (2 buds). The ZR content in buds of these canes at distal and proximal positions were determined weekly from budswell until after budbreak in 1999.

Xylary ZR peaks occurred before 50% budbreak. Spur xylary ZR levels of all three cultivars followed a similar pattern, although at lower ZR levels than that of the canes. This is similar to previous studies on xylary ZR levels of apple shoots. The high levels of free ZR found in xylem sap at the distal portions of canes support the hypothesis of a cumulative ZR build-up effect as cane length increases. Spur pruning resulted in earlier budbreak and a higher final budbreak than cane pruning. The proximal portions of shoots, whether spur pruned or the proximal portions of canes, showed elevated ZR levels in all cultivars. This difference in ZR levels in bud tissue of different portions of the cane would suggest a difference in ZR consumption or turnover.

The results of this study have important management implications for the cultivation of vines in warmer areas in which hydrogen cyanamide is used to alleviate budbreak problems.

OPSOMMING

Die verbouing van tafeldruiwe in die warmer gebiede van Suid-Afrika, soos straks wêreldwyd, word bemoeilik deur rusbreekprobleme in die lente weens vertraagde bot. Rusbreekmiddels, meestal waterstofsiaanamied, word gevolglik toegedien om hierdie probleme te probeer oorkom. In plaas van opheffing van die probleem, veroorsaak hierdie toedienings somtyds addisionele probleme soos ongelyke bot en verlaagde produksie. Hierdie studie is aangepak om die fisiologiese prosesse wat tydens bot plaasvind, beter te verstaan, asook hoe die toediening van waterstofsiaanamied hierdie prosesse beïnvloed. Die volgende is in hierdie studie ondersoek:

a. Die invloed van waterstofsiaanamied op lootweefsel sitokinien (naamlik zeatin ribosied, ZR) vlakke van 'n Sultanina tafeldruif wingerd is oor twee seisoene ondersoek na toediening op verskillende tye voor bot.

Waterstofsiaanamied is in 1997 drie weke en in 1998 ses weke voor geïnduseerde bot toegedien. Een jaar oue lote is weekliks gemonster na waterstofsiaanamied toediening, verdeel in apikale en basale gedeeltes en verder verdeel in ogies, bas en houtweefsel en die ZR vlakke bepaal. Relatief hoër vlakke koue, gekoppel met later watersiaanamied toediening het in 1997 tot 'n groot effek op ZR vlakke gelei, maar het nie die botpatroon wesentlik verskuif nie. Zeatin ribosied pieke is waargeneem in ogies, internode hout en bas van behandelde wingerd in vergelyking met kontrole wingerd. Die pieke was hoër in apikale gedeeltes in vergelyking met basale gedeeltes in alle weefsels. Die relatief laer koue en vroeër toediening van waterstofsiaanamied in 1998 het 'n groter effek op die botpatroon gehad, terwyl die ogie ZR piek vroeër geskuif is. Die apikale gedeelte se ogie ZR piek was weereens hoër as die basale gedeelte se ogie ZR piek. Monstering was nie vroeg genoeg begin in 1997 nie, aangesien ogie ZR pieke slegs na bot waargeneem is, terwyl ogie ZR pieke in 1998 voor en na bot waargeneem is. Die effek van hierdie verhoging in ZR vlakke op die ontwikkeling van blom primordia, daaropvolgende tros ontwikkeling en uiteindelik produksie, is bespreek.

b. Vry xileemsap is gemonster van langdraer en kortdraer gedeeltes van ongesnoeide lote van Sultanina vanaf ogieswel tot na bot in 1999 en vanaf drie tafeldruif kultivars, naamlik Sultanina, Alphonse Lavalleé en Sunred Seedless, in 2001 en die ZR vlakke bepaal. Die ZR vlakke van ogies van hierdie drie kultivars, gesnoei tot verskillende lootlengtes is ook bepaal. Eenjarige lote van hierdie kultivars is gesnoei tot langdraers (14 ogies) en kortdraers (2 ogies). Die ZR vlakke in ogies geleë op apikale en basale gedeeltes van hierdie lote is weekliks in 1999 bepaal vanaf ogieswel tot na bot

Xileemsap ZR pieke is waargeneem voor 50% bot. Kortdraer xileemsap ZR vlakke het 'n soortgelyke patroon as die langdraers gevolg vir al drie kultivars, alhoewel

teen laer ZR vlakke as die langdraers. Hierdie waarneming is soortgelyk aan vorige studies op xileemsap ZR vlakke van appel lote. Die hoë ZR vlakke gevind in die xileemsap van die apikale gedeeltes van lote ondersteun die hipotese van 'n kumulatiewe opbou van ZR soos die lengte van die loot toeneem. Die snoei van lote as kortdraers het gelei tot vroeër bot en 'n hoër finale bot persentasie as die snoei van langdraers. Die basale gedeeltes van lote het verhoogde ZR vlakke in al drie kultivars getoon, onafhanklik van die feit dat dit vanaf die kortdraer of die basale gedeelte van 'n langdraer was. Hierdie verskil in ZR vlakke in ogie weefsel van verskillende gedeeltes van die loot impliseer 'n verskil in ZR verbruik of omset.

The resultate van hierdie studie het belangrike bestuursimplikasies vir die verbouing van wingerd in warmer gebiede, waar waterstofsiaanamied gebruik word om botprobleme te oorkom.

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The Higher Being, without Whom nothing would have been and only through His grace this study could be completed.

"My my, hey hey
Rock and roll is here to stay
It's better to burn out
Than to fade away
My my, hey hey.

Hey hey, my my
Rock and roll can never die
There's more to the picture
Than meets the eye
Hey hey, my my."

Neil Young
From the song "My my, hey hey (Out of the blue)"
from the LP "Rust Never Sleeps", 1979

ABBREVIATIONS

Ab	antibody
ABA	abscissic acid
Ag	antigen
Ag*	radioactively labelled antigen
AMP	adenosine monophosphate
BB	budbreak
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
c	cis
°C	degrees Celsius
CBF	cytokinin binding factor
CK	cytokinin
cv	cultivar
DHZ	dihydrozeatin
ELISA	enzyme-linked immunosorbent assay
GA	gibberellic acid
GC-MS	gas chromatography-mass spectrometry
HC	hydrogen HC
HPLC	high pressure liquid chromatography
IAA	indole acetic acid
iP	isopentenyl adenine
iPP	isopentenyl pyrophosphate
LC	liquid chromatography
LTA	long-term average
max	maximum
min	minimum
MRI	nuclear magnetic resonance imaging
mRNA	messenger ribonucleic acid
MS	mass spectrometry
NSB	non-specific binding
PBS	phosphate buffer saline
PGR	plant growth regulator
PVP	polyvinylpyrrolidone
RB	rest breaking
RBA	rest breaking agent
RCU	Richardson chilling units

RIA	radioimmunoassay
t	trans
Ta	total activity
tRNA	transfer ribonucleic acid
Z	zeatin
^{3}H-ZR	tritium-labelled zeatin riboside
ZR	zeatin riboside
ZRMP	zeatin riboside monophosphate
ZCK	zeatin-type cytokinin

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Viticulture

1.1.1 Worldwide

Grapevines are the most widely planted fruit crop worldwide, cultivated on all the continents, except Antarctica (Williams *et al.* 1994). In 1998, the area under grapevines was more than 10 million hectares. The widespread distribution of grapevines is due to a large genetic diversity of available species and cultivars and low chilling requirements for the release of buds from dormancy. A single species, *Vitis vinifera* L., with currently more than 10, 000 cultivars, accounts for more than 90% of the annual production. Sixty percent of the world production of grapes (> 63 Tg) is produced in Europe. Spain, Italy and France each have more than a million hectares under grapevines. Grapes are primarily used for wine, fresh consumption (table grapes), dried fruit (raisins), juice and distilled liquors. Spain, Italy and France produce more than 50% of the world's wine, while the European countries together with the former Soviet Union countries produce 80% of the world production. The annual production of raisins is 800 Gg dried fruit, with the U.S.A. and Turkey the largest producers. The annual production of table grapes is about 7 Tg, with Italy, Turkey and the former Soviet Union countries the largest producers.

Vitis vinifera is a temperate climate species adapted to hot summers and mild winters. The suitability of a specific cultivar to a local environment is based on day length, heat summation, rainfall, length of the growing season, and minimum winter temperatures. Broadly speaking, the main grape production areas are found between 30 and 50°N and 30 and 40°S latitudes. Raisin production is limited to 30 to 39°N and 28 to 36°S latitudes, due to the fact that the best raisin cultivars, Sultanina and Zante Currant, require warm temperatures for fruit bud differentiation and maturation.

Vineyards are planted via vegetative means, such as cuttings, rootings or grafted vines. Vineyards produce a harvestable crop in the third growing season, after establishment of a root system and training the vines to fit a specific trellis system. Trellis choice depends upon the intended use of the grapes (wine, table or raisin production); methods of pruning (manual or mechanical) and harvest (manual or mechanical); climate and soil conditions.

1.1.2 South Africa

By 2001, 3 241 hectares will have been planted to Sultanina in the Lower Orange River region, comprising 54% of the total plantings in that region (ORPA – Orange River Producer's Alliance, 2001). Export of Thompson Seedless (synonym: Sultanina) amounted to 4 million 8.5 kg cartons in the 2000/2001-season, 55% of the total of 9 million cartons for the Lower Orange River region (ORPA).

1.2 Rest breaking problems

In the growth cycle of the vine, a period of dormancy is essential. In the traditional cultivation areas of the vine in Europe with its cold autumns and winters, vines are forced into dormancy. In spring, the breaking of dormancy allows growth to occur as soon as the temperature rises. In the warmer cultivation areas in the world, vines are sometimes not forced into total dormancy during autumn and subsequently cannot initiate proper spring growth, which leads to uneven and low budbreak (BB) in spring with subsequent vineyard management problems later in the growth season and, most importantly, decreased yields. Dormancy management attempts to manage these problems by adjusting pruning dates, providing additional chill units via evaporative cooling and applying rest breaking agents (RBA) in spring. Problems associated with these techniques are that pruning dates vary from year to year depending on the season, and evaporative cooling is expensive and still in the developmental stage. The drawbacks of RBAs are price; health risks; environmental concerns and they may lead to a decrease in yield and bunch quality over several seasons, especially if not applied at the correct physiological stage of the bud. Alternative cheaper and gentler RBAs are being developed worldwide.

The Lower Orange River region of South Africa is one such region, where the chilling requirement of the vines often cannot be met. This region produces 32% of table grapes in South Africa, being mainly seedless varieties of which Sultanina (synonyms Sultana, Thompson seedless) is the most important. This variety is prone to low fruitfulness and must thus be pruned to long canes (Whiting and Coombe 1984). However, this leads to uneven and delayed BB problems in spring, if RBAs are not used (Williams and Smith 1984, Smit 1985, Burnett 1985, Erez 1987, Lavee 1990). Studies have been performed in South Africa on the levels of cytokinins (CKs) of the xylem sap and the shoot tissues in apple shoots (Cook *et al.* 2001) in the Departments of Horticultural Science and Biochemistry of the University of Stellenbosch, as well as elsewhere (Tromp and Ovaa 1990). Cytokinin levels during dormancy has not yet been studied on table grape vines.

The effect of RBAs on plant growth regulators is still unclear. What is known though is that plant growth regulators (PGR) do play a role both during dormancy and BB, although the mechanism of the processes are unknown. It is proposed that the release of the reserves of PGR built up during a cold autumn stimulates BB in spring, before biosynthesis of PGR increases to sustainable levels to replace the reserves as main source of PGR. Our current hypothesis is that in warmer autumns, these reserves cannot be sufficiently formed, leading to uneven, weak BB. It is suggested that if these reserves can be boosted in autumn, BB (or the potential to) can be increased in spring.

1.3 Objectives of this study

The Agricultural Research Council was approached, through the Unifruco technical committee in 1996, by the table grape producer's organisation of the Lower Orange River region to investigate the specific RB problems of the region. The development of new, more environmentally friendly RB techniques was also requested. A presentation was made to the scientific committee of the Deciduous Fruit Producer's Trust to initiate this study as part of a larger RB project so as to obtain a better understanding of the processes occurring at RB in table grapes. Practical answers would be obtained for producers regarding various RB treatment combinations. As there is a definite RB problem in the warmer areas of South Africa, indeed worldwide, this study would give an insight into the processes happening at BB. To test our hypothesis, we investigated the following in various table grape cultivars:

a. Determination of the levels of cytokinins in Sultanina vines after application of hydrogen cyanamide (HC) at different times, before natural BB. In 1997, HC was applied at 3 weeks before expected normal BB and in 1998 at 6 weeks before expected normal BB. One-year old shoots were sampled weekly thereafter until after BB. These were then analysed for cytokinin content in buds, bark and wood in the distal and proximal portions of these shoots.

b. Determination of the levels of cytokinins in three table grape cultivars, pruned to different shoot lengths. This trial was done in 1999. Three cultivars, Sultanina, Alphonse Lavalleé and Sunred seedless were each pruned to canes (14 buds) and spurs (2 buds). The cytokinin content of these shoots at distal and proximal positions were determined weekly from budswell until after BB. Free xylem sap was also sampled at cane and spur lengths from unpruned canes of Sultanina in 1999 and from all three cultivars in 2001 during the same period and analysed for cytokinin levels.

This thesis is structured as follows: CKs and their role in plant growth and development are discussed in chapter 2. In chapter 3, dormancy and RB with specific reference to vines is discussed. The techniques used to determine CK levels in vines are described in chapter 4. The experimental design, results and discussion of the study on the effect of HC on budbreak and CK levels of Sultanina table grape canes during the 1997 and 1998 seasons are presented in article format in chapter 5. The experimental design, results and discussion of the study on the effect of different pruning systems on budbreak and CK levels in buds and free xylem sap of the table grape cultivars Sultanina, Sunred Seedless and Alphonse Lavalleé in the 1999 and 2001 seasons are presented in article format in chapter 6. The statistical analyses input files are given as an appendix.

CHAPTER 2

CYTOKININS AND THEIR ROLE IN THE PLANT

2.1 Introduction

Plant growth regulators (synonym: plant hormones) can be defined as: "compounds that stimulate metabolic activities in tissues remote from the secretory organ, normally at very low concentrations. They can have different effects in different tissues, at different concentrations and there is often complex interaction among hormones" (Mathews and Van Holde 1990). An Austrian, Gottlieb Haberlandt, demonstrated in 1913 the existence of a compound in potato tuber vascular tissue that stimulated cell division (Taiz and Zeiger 1991). The search to discover the identity of this compound came to fruition in the 1950's when Miller *et al.* (1955) found that autoclaved herring sperm-DNA formed a very active compound, named kinetin (6-furfurylaminopurine). The structure of kinetin is presented in Figure 2.1.

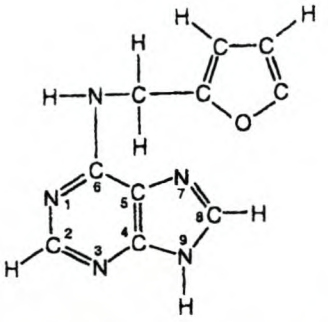


Fig. 2.1. Chemical structure of kinetin (Taiz and Zeiger 1991, p. 454).

Kinetin promoted the mitosis and cell division of tobacco callus tissue *in vitro*. Kinetin is not found in plant tissues. The first naturally occurring plant CKs was demonstrated to have similar activity as kinetin through the activity of extracts from immature endosperm of maize (*Zea mays*) by Letham in 1963. The compound was later purified and identified as 6-(4-hydroxy-3-methylbut-trans-2-enylamino) purine, which was named zeatin (Letham 1973). The structure is similar to kinetin, with different side chains, but both are adenine or aminopurine derivatives with a side chain attached to the N6 nitrogen. Zeatin can be either in the *cis* or *trans* configuration. Both forms are active, although the *trans* configuration is found only in higher plants (Letham and Palni 1983). The structure of zeatin, and related CKs, is given in Figure 2.2.

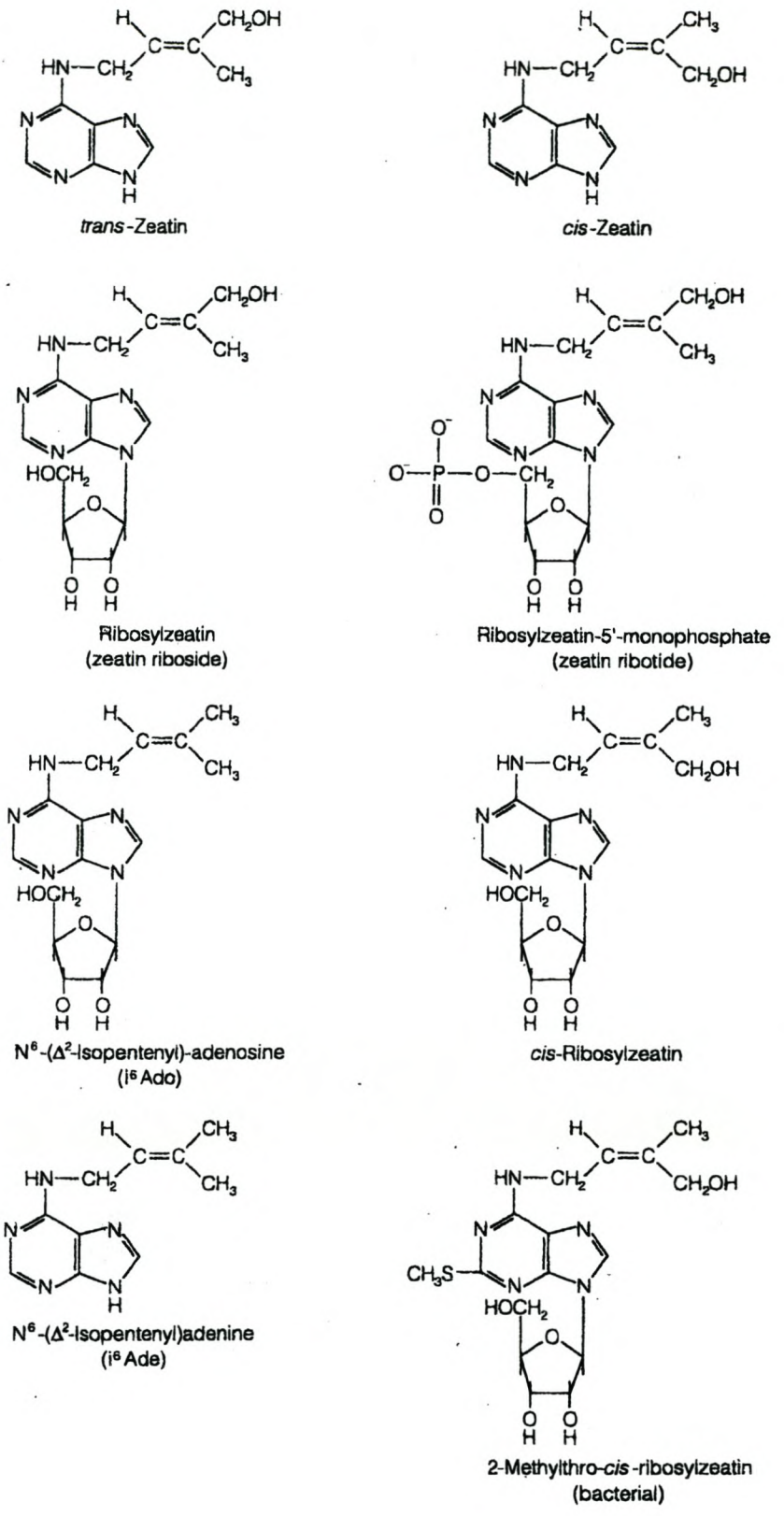


Fig. 2.2. Chemical structure of some naturally occurring cytokinins, including zeatin (Taiz and Zeiger 1991, p. 455).

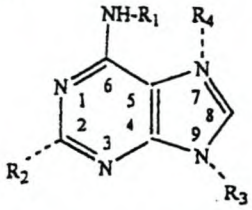
Subsequent to the discovery of zeatin in immature maize endosperm, it was found in many plants, as well as certain bacteria (Taiz and Zeiger 1991). Zeatin is the most prevalent cytokinin found in higher plants although other substituted aminopurines with cytokinin activity have been isolated from many plants and bacteria (Auer 1997). A comprehensive listing of the structures is given in Table 2.1. These differ in the type of side chain attached to the N6 nitrogen or in the attachment of a side chain to carbon 2. Each can be present as a riboside (a ribose sugar attached to the N9 nitrogen of the purine ring); a ribotide (the ribose sugar is esterified with phosphoric acid); or a glucoside (a glucose sugar molecule is attached to the N7 or N9 nitrogen of the purine ring) (Letham and Palni 1983).

Cytokinins can be defined as: "substituted adenine compounds that, in the presence of optimal auxin concentrations, promote cell division in the tobacco-pith or similar assay system grown on an optimally defined medium" (Salisbury and Ross 1992). Wareing and Phillips (1970) defined CKs "as substances which in combination with auxin stimulates cell division in plants and which interact with auxin in determining the direction differentiation of cells takes". Skoog and Armstrong (1970) defined the term cytokinin as "a generic name for substances which promote cell division and exert other growth regulatory functions in the same manner as kinetin". CKs can be detected, identified and quantified through a variety of methods, including bioassays, chemical and physical methods such as HPLC, LC, and MS immunological methods such as ELISA and RIA (Taiz and Zeiger 1991).

Synthetic CKs have been produced that mimic the effect of natural CKs (Taiz and Zeiger 1991). Analysis of these structures provides an insight into the structural requirements for activity. Most of the compounds are 6-substituted aminopurines that are similar to the naturally occurring CKs. An example of these is benzylaminopurine (BAP). However, BAP has recently been isolated as a natural cytokinin out of a number of plants (Strnad 1997) and its structure suggests a considerably different biosynthetic pathway than zeatin, as discussed by Van Staden and Crouch (1996) in a recent review. Other synthetic cytokinin-like substances, with a different structure to the 6-substituted aminopurine, are the diphenylurea compounds. An example is N-(2-chloro-4-pyridinyl)-N-phenylurea, commonly referred to as forclorfenuron (CPPU) and N-phenyl-N'-1,2,3,-thidiazol-5-urea, referred to as thidiazuron (TDZ). Both these have found application in especially commercial agriculture. CPPU has found wide use on table grapes, to improve berry size (Sugiyama *et al.* 1993, Nickell 1986), while BAP has been used as a RBA (Poll 1968). TDZ has promise both as RBA (Steffens and Stutte 1989, Wang *et al.* 1986) and

Table 2.1. Naturally-occurring cytokinins identified in green plants (Auer 1997, pp. 18-19).

Cytokinin structure, trivial name and an abbreviation as used in the text are given. Glucosyl refers to β-D-glucopyranosyl and ribosyl to β-D-ribofuranosyl. Adapted from [13, 17, 40, 88].



R_1	R_2	R_3	R_4	*Trivial Name*	*Abbreviation*
				iP-type cytokinins	
-CH2-CH=C(CH3)-CH3	H	H	–	N^6(Δ^2-isopentenyl)adenine	iP
	H	ribosyl	–	N^6(Δ^2-isopentenyl)adenosine	[9R]iP
	H	ribotide	–	N^6(Δ^2-isopentenyl)adenosine-5′-monophosphate	[9R-MP]iP
	H	H	glucosyl	N^6(Δ^2-isopentenyl)adenine-7-glucoside	[7G]iP
	H	glucosyl	–	N^6(Δ^2-isopentenyl)adenine-9-glucoside	[9G]iP
				Z-type cytokinins	
-CH2-CH=C(CH3)-CH2-OH	H	H	–	*trans*-zeatin	Z
	H	ribosyl	–	*t*-zeatin riboside	[9R]Z
	H	ribotide	–	*t*-zeatin riboside-5′-monophosphate	[9R-MP]Z
	H	H	glucosyl	*t*-zeatin-7-glucoside	[7G]Z
	H	glucosyl	–	*t*-zeatin-9-glucoside	[9G]Z
	H	alanyl	–	lupinic acid	[9Ala]Z
-CH2-CH=C(CH3)-CH2-O-glucosyl	H	H	–	*t*-zeatin-O-glucoside	(OG)Z
	H	ribosyl	–	*t*-zeatin riboside-O-glucoside	(OG)[9R]Z
-CH2-CH=C(CH3)-CH2-O-xylosyl	H	H	–	*t*-zeatin-O-xylosyl	(OX)Z
	H	ribosyl	–	*t*-zeatin riboside-O-xylosyl	(OX)[9R]Z
-CH2-CH=C(CH3)-CH2-O-acetyl	H	riboside	–	*t*-zeatin riboside-O-acetyl	(OAc)[9R]Z
				DHZ-type cytokinins	
-CH2-CH-C(CH3)-CH2-OH	H	H	–	dihydrozeatin	DHZ
	H	ribosyl	–	dihydrozeatin riboside	[9R]DHZ
	H	ribotide	–	dihydrozeatin riboside-5′-monophosphate	[9R-MP]DHZ
	H	H	glucosyl	dihydrozeatin-7-glucoside	[7G]DHZ
	H	glucosyl	–	dihydrozeatin-9-glucoside	[9G]DHZ
	H	alanyl	–	dihydrolupinic acid	[9Ala]DHZ
-CH2-CH-C(CH3)-CH2-O-glucosyl	H	H	–	dihydrozeatin-O-glucoside	(OG)DHZ
	H	ribosyl	–	dihydrozeatin riboside-O-glucoside	(OG)[9R]DHZ
-CH2-CH-C(CH3)-CH2-O-xylosyl	H	H	–	dihydrozeatin-O-xylosyl	(OX)DHZ
	H	ribosyl	–	dihydrozeatin riboside-O-xylosyl	(OX)[9R]DHZ
-CH2-CH=C(CH3)-CH2-O-acetyl	H	riboside	–	dihydrozeatin riboside-O-acetyl	(OAc)[9R]DHZ
				BA-type cytokinins	
-CH2-benzyl	H	H	–	N^6-benzyladenine	BA
	H	ribosyl	–	N^6-benzyladenosine	[9R]BA
	H	ribotide	–	N^6-benzyladenosine-5′-monophosphate	[9R-MP]BA
	H	glucosyl	–	N^6-benzyladenine-9-glucoside	[9G]BA
-CH2-(oOH)benzyl	H	H	–	N^6-(o-hydroxybenzyl)aminopurine	(2OH)BA
	H	ribosyl	–	N^6-(o-hydroxybenzyl)aminopurine riboside	(2OH)[9R]BA
-CH2-(mOH)benzyl	H	H	–	N^6-(m-hydroxybenzyl)aminopurine	(3OH)BA
	H	ribosyl	–	N^6-(m-hydroxybenzyl)aminopurine riboside	(3OH)[9R]BA

as a berry enlarging agent (personal observation by the author), although it has been used mainly to defoliate cotton plants (Arndt *et al.* 1976).

Natural CKs are found in either free or bound forms (Taiz and Zeiger 1991, Wareing and Phillips 1970). Z is the most abundant form found in higher plants, although DHZ and iP are also commonly found. Cytokinins are also found in tRNA in the cytoplasm and in the chloroplasts of plants, although these tRNA contain different CKs. When, hydrolysed and tested in bioassays, they exhibit cytokinin activity. Some bacteria also exhibit cytokinin activity, as shown by the "Witches' Broom" phenomenon as caused by *Corynebacterium fascians*. The shoots of plants look like a straw broom after a while due to the lateral buds, normally suppressed, being stimulated to grow by the bacterial CKs.

2.2 Biosynthesis

2.2.1 Free cytokinins

The 5 carbon N6 side chains of CKs are derived from melavonic acid (Taiz and Zeiger 1991). In the mevalonic acid pathway, it is enzymatically converted to mevalonic acid pyrophosphate, then decarboxylated, dehydrated and isomerised to form α^2 – isopentenyl pyrophosphate (iPP). A prenyltransferase, active in cytokinin synthesis, catalyses the transfer of the isopentenyl group from iPP to adenosine monophosphate (AMP) (Chen 1982). A schematic representation of these pathways is shown in Figure 2.3. This enzyme is known as cytokinin synthase or α^2 – isopentenyl pyrophosphate: AMP α^2 – isopentenyl transferase (Taiz and Zeiger 1991). The product, i^6 Ade, is not a major higher plant cytokinin, but active in bioassays and is readily converted into Z and other CKs, by as yet unidentified enzymes. Free CKs are likely to be the active forms of the CKs.

2.2.2 tRNA cytokinins

Synthesis of transfer ribonucleic acid (tRNA) CKs take place by a different route (Taiz and Zeiger 1991). Free CKs are not used in this synthesis. The tRNA's are made from the four conventional nucleotides (adenine, guanine, timidine and cytidine) during the transcription of the genes encoding their sequences. The first product is a tRNA, slightly larger than the final product, without any hypermodified bases. This precursor is then processed to produce the functional tRNA. During the processing, some specific adenine residues of some tRNA's are modified to the cytokinin. As with the free CKs, isopentenyl groups are transferred to the adenine molecules from iPP molecules, by a

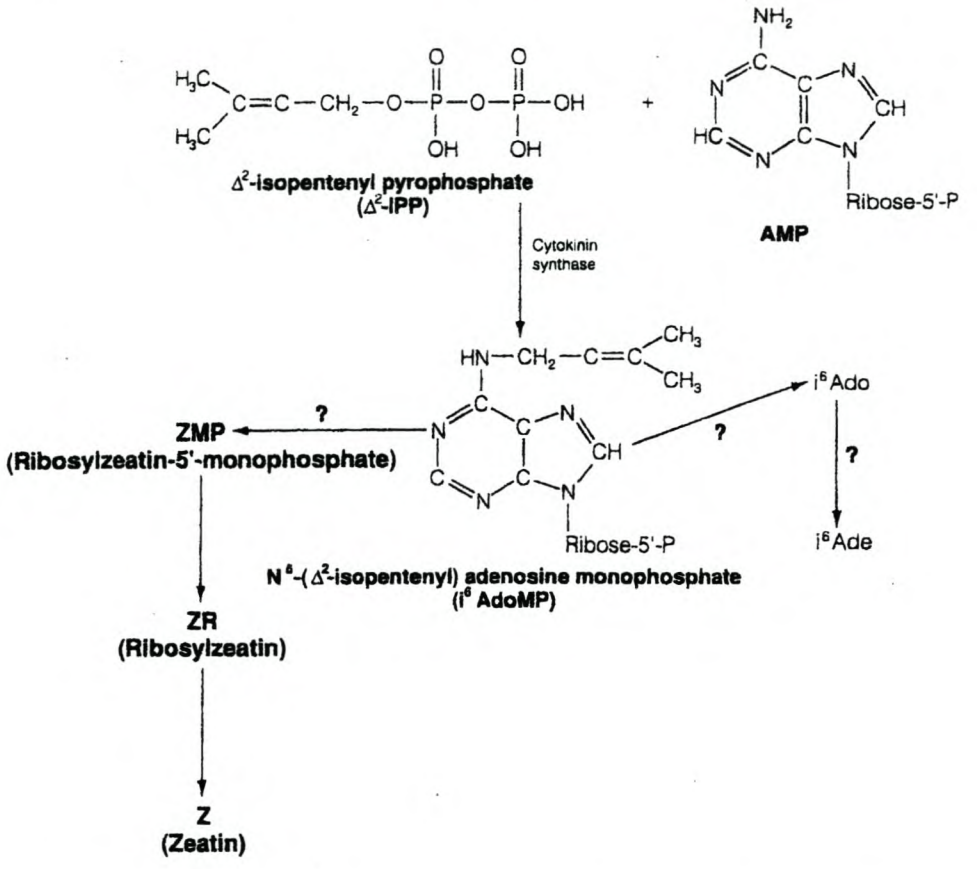


Fig. 2.3. Biosynthesis pathways for some cytokinins (Taiz and Zeiger 1991-p.459, Chen 1982).

prenyltransfertase. This transferase has to identify a specific base sequence (the CKs occur as the middle base of a triple adenine sequence) and transfer the isopentenyl group to the adenosine nearest to the 3' end of the anticodon of the tRNA. The structures of yeast tRNA's for tyrosine and serine are shown in Figure 2.4.

2.2.3 Summary

The rate of cytokinin biosynthesis, interconversion and degradation is regulated by enzymes in plant cells to maintain hormone homeostasis (Chen 1997). Cytokinins can be synthesized via direct (*de novo*) or indirect (tRNA) pathways. In the *de novo* pathway, a cytokinin nucleotide is synthesized from 5'-AMP and isopentenyl pyrophosphate. The isopentenyl side chain of cytokinin in tRNA is derived from mevalonate and turnover of cytokinin-containing tRNA may serve as a minor source of free CKs in plant cells. The enzymatic interconversion of cytokinin bases, nucleosides and nucleotides is a major

feature of cytokinin metabolism. The N6-side chain and purine moiety of CKs are often modified.

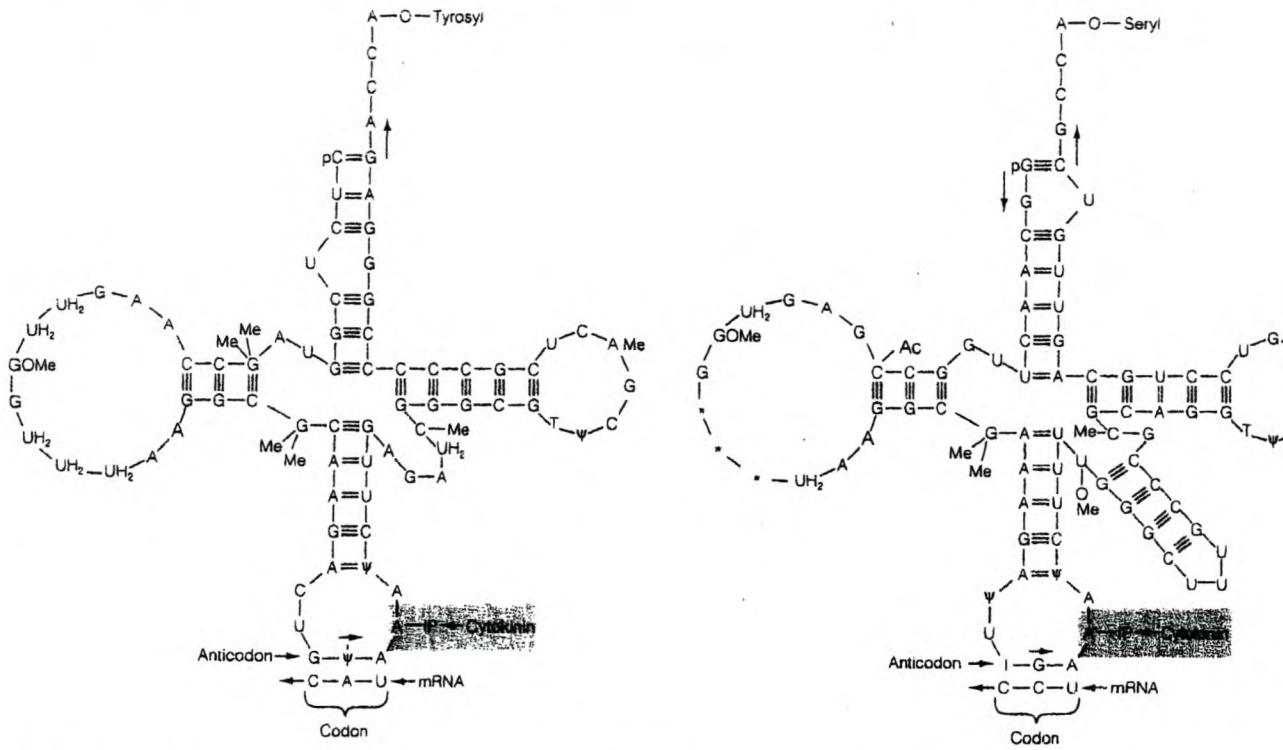


Fig. 2.4. The structures of tyrosine and serine tRNA's of yeast (Taiz and Zeiger 1991).

2.3 Metabolism

The level of active CK within plants is primarily controlled by biosynthesis, conjugation, deconjugation, degradation, transport and compartmentalization (Auer 1997).

2.3.1 Conjugation

CK conjugation, such as linkage to sugar molecules, is important because these biochemical modifications are believed to delicately regulate the active CK pool (Auer 1997). Higher land plants contain a more complex set of CKs, mainly conjugates of Z and DHZ, suggesting a more complex pattern of CK conjugation in parallel with increasing complexity of plants.

The most common conjugates contain either glucose or alanine, those that contain glucose being called cytokinin glucosides (Letham and Palni 1983). Two types are present in cell tissues, namely N-conjugated and O-conjugated glucosides. The N-types are very stable and are biologically inactive, while the O-types are biologically very active

(McGaw 1987). It is suggested that the O-types are storage forms or special transport forms, which are hydrolysed to release the biologically active form (Strnad 1997).

The cytokinin ribosides and their 5' mono-, di-and tri-phosphates are probably the most abundant natural occurring cytokinin CKs (McGaw 1987). Hydrolysis of these ribosides and ribotides yield more stable or biologically less active products, such as the N-glucosides.

The N-alanine conjugates are biologically inactive and seem to be the irreversibly formed products of cytokinin degradation (McGaw 1987).

2.3.2 Hydrolysis

The hydrolysis of CK ribosides and ribotides is the main metabolic fate of externally applied CKs. The N-glucosides and N-alanyl conjugates are extremely stable against hydrolysis, while the 0-glucosides are easily hydrolysed (McGaw 1987).

2.3.3 Reduction

DHZ derivatives are commonly found in plant cells and are more stable than Z derivatives, as they are not substrates for CK oxidase function (Letham and Palni 1983). This may be important in maintaining CK levels in an oxidative environment.

2.3.4 Oxidation

CK oxidase is the only enzyme undisputedly proven to catalyse the oxidation of specific CKs that contain an unsaturated isoprenoid side chain at the N6-position (Jones and Schreiber 1997, Kaminek *et al.* 1997). CK oxidase appears to contribute to CK homeostasis in plants. The induction of enzyme activity is rapid, transient and significant (Kaminek *et al.* 1997).

2.3.5 Possible functions of different forms

Six functions have been suggested: binding to a CK receptor (active form); transport or translocation within the plant; storage for later release of an active CK; inactivation after receptor inaction and detoxification after CK applications (Letham and Palni 1983).

2.3.6 Regulation model of cytokinin levels in the plant cell

Recently, a model has been proposed to illustrate the interaction in the regulation of cytokinin levels and it is presented in Figure 2.5 (Kaminek *et al.* 1997). In this model, the increase of CK levels may promote auto-inductive accumulation of CKs, which may function in the induction of CK-initiated physiological processes. Accumulated CKs are capable of inducing CK oxidase, decreasing CK levels. This appears to be the mechanism of re-establishment and maintenance of cytokinin homeostasis.

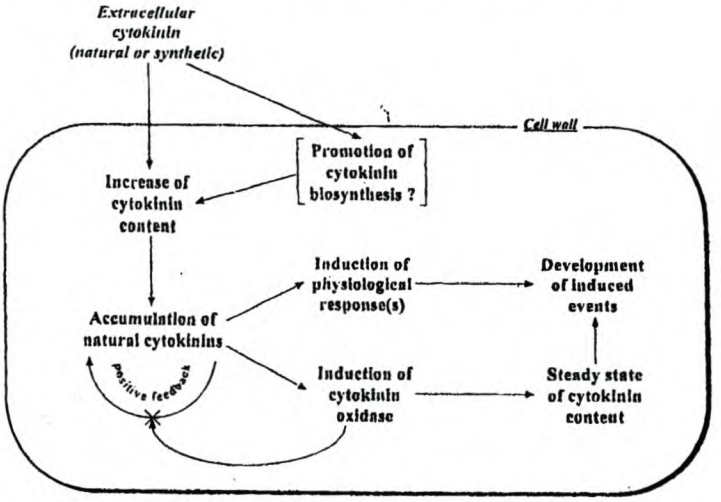


Fig. 2.5. Proposed model illustrating the interactions in the regulation of cytokinin levels in plant cells (Kaminek 1997).

2.4 Sites of synthesis, transport and storage

2.4.1 Xylem transport

Xylem sap contains large quantities of CKs that pass to the above ground parts of plants every day (Skene 1975). CKs are passively transported through the xylem, with the form found in xylem exudate mainly nucleotides, which could be the transport form (Taiz and Zeiger 1991). Once reaching their destination (e.g. leaves) CKs can then be converted to free bases or glucosides. High levels of glucosides are found in leaves. These glucosides do not appear to have high CK activity in the leaves, which could be due to compartmentalization, making them unavailable (maybe as storage forms). This could also explain the observation of movement of CKs in xylem, but when CKs are applied to intact leaves, CKs do not seem to move from the site of application. The transport of CKs in phloem of plants has been less extensively studied, due to practical sampling problems (Hoad 1995). It has been shown so far that CKs do move through the phloem of various plants, mainly CKs of the Z, ZR type and iP.

2.4.2 Shoots, main stem, leaves and buds

Synthesis of CKs in parts of the plant other than the roots has been shown in rootless tobacco plants (Chen and Petschow 1978). CKs in phloem exudates resulting most probably from storage/production in leaves of Yucca plants have also been shown (Vonk 1979). However, indications are that transport of CKs within the shoot is limited, except for delivery from the roots via the xylem (Wareing and Phillips 1970). Van Staden and Dimalla (1981) showed cytokinin production in rootless almond shoots. Production seemed to occur in the bark and CKs were utilised in the buds. Buds did not appear to have the ability to synthesise CK, but seem to be able to hydrolyse storage CKs. CK activity was also shown in stem internode sections of peas (King and Van Staden 1990). Further evidence for shoot production/release of CKs, was shown in shoots of potato, which had been without roots for up to 30 days (Wang and Wareing 1979). Buds still showed growth following decapitation of the shoot apex, suggesting shoot CK synthesis in the absence of roots. Studies on pea plants by Lee *et al.* (1974) have however suggested the ability of buds to synthesize CKs, although it could be argued that the effect they observed could also have been due to bud-mediated hydrolysis of storage forms into active free forms. Results of Skene (1975) do not support the idea of bud synthesis of CK. In studies on endogenous levels of CKs in roots, buds, stems and xylem bleeding sap of roses, the dominant CKs in buds were ZR and ZRMP, indicating that they received their CKs from the roots (Dieleman *et al.* 1997). The major translocation form in the xylem was ZR and in mature tissues the Z-type CKs were dominant (80-90%). Young leaves contained high levels of iP-type CKs were found (up to 50%), suggesting the leaves to be capable of *de novo* synthesis of CKs. Older leaves contained an unidentified CK, up to 50% of total CK. Roots also contained this substance, possibly a storage form of CK. Young stems also contained high levels of iP CK (up to 50%) suggesting either *de novo* synthesis of CK or translocation via the phloem. Studies on apple trees showed similar CK activity from xylem sap taken from shoots or from roots, suggesting that the roots are the source of shoot CK (Jones 1973). The main CK was similar to ZR and promoted growth of isolated apple shoots. Cook *et al.* (2001) found a cytokinin increase in the bark and buds of rootless apple (cultivar Granny Smith) shoots, after chilling and forcing, as growth resumed. This supports the hypothesis that shoot-derived, rather than root-derived CKs act to trigger budburst.

2.4.3 Roots

Root apical meristems are major sites of synthesis of free CKs in whole plants and transport CKs through the xylem to the rest of the plant (Taiz and Zeiger 1991, Wareing and Phillips 1970). These CKs appear to move through the xylem into the shoot, supported by circumstantial evidence. The highest levels of CKs are found in young organs (e.g. seeds, fruits and leaves) and root tips (Wareing and Phillips 1970). However, it has been shown that tomato shoots may produce much of their own CKs, raising doubts on the roots being the sole production source of CKs (Sossountzov *et al.* 1988). In studies on roses, the CK levels in bleeding sap were shown to be representative of the CK levels in xylem sap *in situ* (Dieleman *et al.* 1997). CK was also rapidly degraded in rose shoots, as the half-life of CK was found to be approximately one day. In studies on BB of roses it was found that root temperature did not affect ZR production or ZR translocation in the shoot (Dieleman *et al.* 1998, Belding and Young 1989). Root morphology was, however, markedly changed by temperature. The differences in BB caused by increasing root temperatures, could not be correlated with increasing CK translocation into the shoots.

2.5 Biological role

2.5.1 Introduction

CKs play an important role in plant growth and development, including regulation of nucleic acid metabolism and protein synthesis (Chen *et al.* 1987). However, the effects of CKs are not universal in all plants (Taiz and Zeiger 1991). CKs will affect a given response in a particular tissue at a particular stage of development. Hormonal action is also dependant on changes in tissue sensitivity for the hormone (Hare *et al.* 1997).

2.5.2 Chloroplast and leaves

As CKs can strongly stimulate the light-initiated maturation of chloroplasts, it is suggested that CKs promote the synthesis and stabilization of photosynthetic proteins (Taiz and Zeiger 1991, Wareing and Phillips 1970). It has been suggested that CKs such as Z may affect stomatal behaviour on a short-term basis on almond trees under drought conditions (Fusseder 1992). ABA had an overriding effect on these effects.

2.5.3 Cell cycle

CKs trigger cell proliferation in tissues together with an optimal amount of auxin (Taiz and Zeiger 1991, Wareing and Phillips 1970). Plant cell cycle regulation appears to be more complex than in the mammalian animal cell, and can leave the proliferative cycle either before or after the cell has replicated its DNA, suggesting two control points, namely one for initiation of DNA replication and one for initiation of mitosis. Both hormones participate in the regulation of the cell cycle, but it is suggested that auxin regulates events leading to DNA replication, while cytokinin regulates events leading to mitosis. CKs have been shown to regulate certain processes in the cell cycle of *Arabidopsis*, specifically the G1 and S-phase (Frank and Schmulling 1999, Riou-Khamlichi *et al.* 1999). This is achieved through the induction of the cyclin D3 gene by CKs and sucrose. Two roles of CK action on the cell cycle have been suggested: firstly, the well-known cell division stimulatory role and secondly, a strong inhibitory function, with the ability to arrest cells at specific points in the cell cycle (Strnad 1997). Cytokinins play an important role in vine cell growth (and other plants) and in subsequent differentiation of tendrils (vegetative) into flowers (inflorescences-reproductive) during BB and set of fruit (Srinivasan and Mullins 1978, Srinivasan and Mullins 1980). Unfortunately, our present state of knowledge is that we can rarely do more than make correlations between applications of hormones and the appearance of a given response (Taiz and Zeiger 1991).

2.5.4 Cell enlargement

CK can promote cell enlargement in certain tissues and organs of certain dicotyledonous plants (Taiz and Zeiger 1991, Wareing and Phillips 1970). This is achieved by increasing the plasticity of the cell walls without influencing the elasticity thereof.

2.5.5 Morphogenesis (auxin/cytokinin ratio)

The auxin/cytokinin ratio is important in inducing different morphological states in cultured tobacco callus tissues (Taiz and Zeiger 1991). A high auxin:kinetin ratio induced root formation, while a high kinetin:auxin ratio induced shoot formation. This indicates an important role of hormonal ratios in inducing different morphological states.

2.5.6 Senescence, nutrient metabolism and stress

CK application to leaves delay senescence (Taiz and Zeiger 1991, Wareing and Phillips 1970). Studies on the flow of nutrients between CK-treated and untreated leaves have shown that nutrients are preferentially transported to and accumulated in the CK-treated leaves. It is suggested that CKs cause a new source-sink relationship, thus leading to nutrient mobilization. The metabolism of the treated area is stimulated by the CK, so that nutrients move toward it. Levels of CKs in shoots and growth rates of leaves of wheat seedlings rapidly decreased after rapid cooling of the roots (Kudoyarova *et al.* 1998). These changes could indicate a possible connection between root temperature changes in autumn and induction into dormancy, as well as irregular growth in spring due to root temperatures not being ideal. Studies on roses and apples, however, suggest little effect of root temperature on CK levels (Dieleman *et al.* 1998, Belding and Young 1989, Young 1989). Root CKs are probably involved in senescence, which is a complex interplay between the different organs of the plant, possibly diverting CKs to fruit leading to lower levels in the leaves and their subsequent senescence (Skene 1975). Another possible mechanism how CKs may delay senescence is that they protect membranes against degradation (Wareing and Phillips 1970), probably by preventing oxidation of unsaturated fatty acids in membranes. The process is as follows: CKs protect membrane oxidation because CKs inhibit formation and speed breakdown of free radicals (e.g. superoxides and hydroxy radicals) that would otherwise oxidise membrane lipids (Wareing and Phillips 1970, Leshem 1988). CKs can act both as direct scavengers and as incipient preventatives of free radical formation. As direct scavengers CKs, that are amines, are converted into amides. CKs have also been implicated in plant response to stress and seem to have an influence on methylation reactions (Nir *et al.* 1986). Methyl group transfers are apparently important in biochemical acclimation to environmental stress.

2.5.7 Seasonal changes and budbreak

In studies on axillary buds of pea cotylodons it has been suggested that release from apical dominance is a selective process, resulting from the ability of the buds to utilize and or synthesize certain growth regulators within a certain time interval (Prochazka and Jacobs 1984). It was proposed that CK involvement was not only from root sources, but could also be from other close tissues or from bud synthesis itself. Studies on apple trees showed that t-ZR levels in the xylem peaked before BB, declining afterwards (Belding and Young 1989). Root and shoot temperatures had no effect on the ZR levels, but did have an effect on BB. As root temperature had no effect on ZR levels, it was

suggested that the initial ZR source was from a storage form. The role of ZR as a dormancy-breaking agent was suggested not to be the initial causasive agent, but involved in the subsequent BB and shoot growth. Chilling, although a necessity for dormancy release of apple trees, is not necessary to increase the CK (specifically t-ZR) levels, as levels increased after forcing at higher temperatures, regardless of chilling received (Young 1989). Cytokinin is also involved in the BB of the buds, as levels decreased rapidly after BB of chilled trees, but not in buds of unchilled trees (i.e. buds not bursting). These results support Saure (1985) in that CKs probably play a supplementary role in dormancy release, becoming involved after other dormancy changes have been induced. Studies on willow showed that main CK activity in xylem sap was due to a ZR-like compound (Strnad 1997). Growth in spring was preceded by an increase in CK activity and a decrease in ABA activity, while at onset of dormancy, low levels of CK and high levels of ABA were observed.

Studies on xylem sap of mature apple trees by Tromp and Ovaa (1990) showed in general that Z and ZR accounted for more than 80% of CKs. Z was dominant over the ZR during most of the year, but ZR dominated in early spring. The CK levels also showed a marked rise in late winter, at bud swell, before declining later in spring. Due to the fact that transpiration is still low, it is suggested that the increased levels were mainly due to mobilization of reserves or synthesis in for example bark or cambium (Skene 1972), rather than root supply. Whether this suggests that ZR (in a storage form of for example glucoside/nucleotide) is the main storage form of CK or whether it is formed by *de novo* synthesis in the bark or cambium is unclear. As CK concentrations start to increase in late winter, before visible bud swell, it is suggested that this increase marks the end of winter (possibly endodormancy) dormancy. The hydrolysis of storage proteins also happens at the same time. The observed reduction of CK levels in summer is suggested to be due to accumulation (possibly storage) of CK in perennial tissues (stem, wood, bark).

2.6 Mechanism of action

2.6.1 Introduction

It is generally assumed that plant hormones interact with specific receptors that reside either on the cell surface or within the cytoplasm (Taiz and Zeiger 1991). The process would then follow a similar course to that of animal hormones, with the hormone binding to a specific receptor on the target cell surface. This binding then stimulates the formation of a second messenger in the cytoplasm that alters some aspect of cellular

metabolism or forms an active hormone-receptor complex that then enters the cell nucleus and leads to the expression of specific genes. However, very little direct evidence has been found that CK action follows either of these two models.

2.6.2 Cytokinin-binding proteins

CK-binding proteins and CK-binding sites have been identified from certain plants (Taiz and Zeiger 1991, Napier and Venis 1990). The best characterized cytokinin binding factors (CBFs) are from wheat and other cereal crops. A protein factor called CBF-1 has been isolated from barley and its function is suggested to involve protein synthesis (Wareing and Phillips 1970). Another possible function of CBF could be as an inactivation protein, binding to and regulating the CK concentration in the plant cell (Napier and Venis 1990).

2.6.3 Protein synthesis

There is good evidence that CKs play a role in regulating protein synthesis (Taiz and Zeiger 1991, Wareing and Phillips 1970). Polyribosomes are the protein synthetic machinery and this levels increase when CK is added to cultured soybean cells. CK can also change the spectrum of proteins made by plant tissues. The specific nature of the proteins whose synthesis is enhanced or suppressed by CK is not yet known. CK seems to act on the level of translation (Wareing and Phillips 1970). Furthermore, it is thought that t-RNA CKs may play a regulatory role in protein synthesis by influencing the binding of t-RNA to mRNA, in view of the fact that the CK components are situated directly adjacent to the anticodon (Taiz and Zeiger 1991). Turnover of CK from the tRNA CKs can also contribute to the total CK levels in the plant.

2.6.4 Calcium in cytosol

Plants are both axial and polar structures (Taiz and Zeiger 1991). An axial structure is symmetrically arranged around an axis, while in a polar structure, the opposite ends of the axis are different. Axiality and polarity are key attributes of plant development. Hormones play an important role in this development. In studies on tip-growing cells *in vitro*, Weisenseel and Kicherer (1981) showed the earliest formation of polarity is by means of a transcellular current largely induced by calcium ions. This occurs before any morphological sign of polarity. CKs regulate calcium concentration in the cytosol, leading to increased uptake of Ca^{2+} (Taiz and Zeiger 1991, Hepler and Wayne 1985). The regulatory protein, calmodulin, has four calcium binding sites and the calmodulin-

calcium complex can bind to and activate a number of enzymes, including protein kinases, involved with protein synthesis. Thus, the calmodulin-calcium complex could act as a master switch to regulate alternative metabolic pathways in the cell. Whether CKs act on the plasma membrane and by transduction lead to enhanced levels of calmodulin-calcium, thus affecting other processes, is as yet unresolved (Wareing and Phillips 1970), although support for the calmodulin-calcium role has been noted (Elliott *et al.* 1983, Vallon *et al.* 1989). Further support for the role of calcium as mediating the CK effect, has been suggested by Poovaiah (1988).

CHAPTER 3

DORMANCY WITH SPECIFIC REFERENCE TO VINE DORMANCY

3.1 Definition of dormancy

Bachelard (1980) stated that "We need to define carefully the state of bud dormancy at the time of examination and relate the internal structure of the zones within the bud to these states of dormancy." Lang *et al.* (1987) provided an answer to this question. They defined dormancy as follows: "Dormancy is a temporary suspension of visible growth of any plant structure containing a meristem". They also suggested the following descriptions of dormancy states: *ecodormancy* for dormancy imposed by environmental factors, e.g. temperatures, nutrient, water (previously 'imposed dormancy', 'quiescence'); *paradormancy* for dormancy imposed by physiological factors outside the affected structure/organ, e.g. apical dominance, photoperiod (previously 'summer dormancy', 'correlative inhibition') and *endodormancy* for dormancy imposed by physiological factors inside the affected structure/organ, e.g. chilling, photoperiod (previously 'winter dormancy', 'rest'). Lavee and May (1997) suggested a subdivision of the description of vine bud dormancy as follows: pre-dormancy; entry into dormancy; dormancy; lifting of dormancy; post dormancy. They also considered that grapevine buds pass from one phase to the next in such a diffuse way that it is unwise to relate the name of each phase to it's physiology as suggested by Lang *et al.* (1987).

3.2 History of dormancy study

Fennell (1948) reported on the observation that all European and North American grapes, in spite of being grown in subtropical and tropical climates, had a definite low temperate rest requirement. With some cultivars, spring growth was inhibited if the winter temperatures did not fall far enough. Other cultivars exhibited abnormal, delayed spring growth from fully dormant canes or uneven BB with the development of only a number of dormant buds. As almost all table grape cultivars grown commercially worldwide are derivatives of European and North American grape cultivars and are often grown in warm, arid conditions, producers world-wide have a problem with RB. In temperate climates, starch hydrolysis in dormant tissues is aided by cold weather, while in warmer climates this does not occur. Tropical grape species are adapted to achieve this by other means, but the temperate climate vines are not adapted for the warmer climates, leading to considerable dormancy problems. In South Africa, Blommaert (1956) already reported problems experienced in the deciduous fruit industry, including

table grapes, with inadequate chilling and delayed foliation. He already raised the need to fully understand the biochemistry of the dormancy process and that adequate dormancy control measures can only be implemented once the basic mechanism of winter dormancy was fully understood. Doorenbos thoroughly reviewed literature on bud dormancy of woody plants in 1953, giving a good overview of the work done up to then. The fact that cold was found to be necessary to break so-called winter dormancy was apparently discovered by Knight in 1801. Samish (1954) and Vegis (1964) also thoroughly reviewed dormancy in woody, higher plants. In 1971, Perry gave a general overview of dormancy of trees with special reference to variables that interact to control leaf fall and the other dormancy phenomena. The current general consensus is that the best method to prevent delayed foliation is to breed varieties that require little cold, rather than having to resort to chemical RB techniques. Unfortunately, the current situation has not improved much as many high-chill requirement cultivars are still cultivated and chemical RB will thus be needed for a while yet.

3.3 Stages of dormancy induction

The sequence of events leading to bud dormancy can be described as follows: leaf senescence, leaf fall, wood ripening/maturity, bud dormancy and BB.

This natural, controlled process of leaf senescence happens in response to short, bright, cool days (Wood 2000). Included is the decline of photosynthesis, with a loss of chlorophyll, appearance of yellow and orange pigments and synthesis of anthocyanins. Various compounds such as nitrogenous compounds, soluble carbohydrates and various ions such as potassium are remobilised from leaves into storage in permanent structures (roots and main stem). Sugars produced in leaves after autumn are converted to starch in roots and wood and is the first carbohydrate used by new shoots in the following spring.

Leaf fall occurs after the first autumn frosts and marks the end of the vine growth cycle (Wood 2000). Vine vigour influences the levels of carbohydrates – being lower in overly vigorous and devigourated vines, than in well-balanced vines. Premature senescence can negatively influence the levels of these reserves.

Wood ripening/maturity occurs prior to the onset of dormancy and involves the transition of the shoots into mature canes (Wood 2000). In contrast to many plants, the phloem of above ground parts of *Vitis spp.* remain functional for more than one season. At this time of wood ripening, corky bark appears on the surface of canes. These changes are

accompanied by a colour change (brown), beginning at the base and progressively moving along the shoot. Wood ripening involves an accumulation of carbohydrates and an accompanying reduction in water content of both the canes and dormant buds. A small amount of carbohydrates is utilised for maintenance respiration throughout the dormant period. Glucose and fructose accumulate and act as an osmiticum to provide protection to the cells against low temperatures (Lavee and May 1997).

During bud dormancy, the dormant bud of grapevines is a compound bud consisting of three growing points – the primary, secondary and tertiary buds (Wood 2000). At the woolly bud stage, when the bud swells and is still enclosed in it's brown protective scales, it can withstand temperatures down to -3.5°C. The primary bud is the only one that normally bursts, only when this bud dies or is damaged, will the secondary and then the tertiary buds develop. The secondary bud only produces about a third of the bunches of the primary bud, while the tertiary bud normally does not have any bunches at all. Fully dormant buds have a hardened scale that protects them; shoots do not 'bleed' when cut; starch grains become prominent and moisture content drops from 80 to 60%.

A period of chilling is required to terminate bud dormancy and allow normal BB (Wood 2000). When air temperatures increase in spring, the previous year's phloem cells within the cane at the base of each swelling bud resumes their transport to the growing bud tissues. The reactivation spreads down the cane, down the main stem and into the roots.

The degree of dormancy can have an impact on propagation of vines (by means of vegetative cuttings) in terms of the amount of carbohydrates available for root development (Wood 2000). For successful propagation, the wood needs to be properly hardened. Improper hardening can also affect the survival of cuttings and young rooted cuttings during the necessary hot water treatment.

Sugars appear to increase cold hardiness by accumulating in the vacuoles and decreasing the formation of intercellular ice (supercooling effect) (Wood 2000). This causes changes in the cell that increase tolerance to freeze-induced dehydration and diluting compounds, such as electrolytes that may damage cellular membranes.

Timing of the application of fertilisers and irrigation in autumn is crucial in building up the nutrient store prior to dormancy (Wood 2000). The vine is still active during dormancy and metabolic activity continues albeit at a much slower rate than during the growing season. The nutritional status of the vine is of great importance when it enters dormancy, as it will impact on the growth of the vine in the following spring. Firstly, as

active root growth does not peak until close to flowering, the initial growth draws mainly on stored nutrients. Secondly, carbohydrates accumulated and stored in autumn, are used in maintenance respiration during the dormant season. The nutritional status also appears to influence the maturity of the wood, which is necessary for cold hardiness and propagation. It has also been noted in certain wine grape vines, that the amount of young inflorescence primordia can be reduced, if nutrients are withdrawn by the rest of the shoot before BB (Jako 1981). Also, it was found that in vine cuttings, the inflorescence primordia were reduced through atrophy, when BB preceded the development of adventitious roots (Mullins 1968). This reduction could be due to a failure to compete with the other sinks (leaves, shoots) for nutrients and also due to a lack of CKs, as when roots developed before BB, the bunches did not atrophy. CKs were thus necessary for inflorescence retention.

3.4 Physiology during dormancy

Studies on the changes of the metabolic status of buds of the cultivar Pinot Noir showed a pattern suggesting a major change in the metabolic pathways between the ecodormant bud stage and bud swelling (Gardea *et al.* 1994). This correlates with the expected initiation of growth and activation of various metabolic processes before actual bud growth in spring. Faust and Wang (1993) drew the following conclusions on resumption of growth of temperate fruit trees:

1. Budbreak is a distinct phenomenon which can be triggered at will in paradormant buds;
2. The signal to stimulate BB in artificially induced conditions, is CK (cytokinin), but the signal in orchard conditions is not yet known, although CKs probably play a role;
3. The resumption reaction of buds is the same whether natural or artificially induced;
4. After receiving the BB signal, there is an enzyme activity burst for 2-8 days, depending on the enzyme;
5. None of the known theories explain conditions that could be regarded as a process breaking dormancy, initiating BB or signalling the resumption of growth (i.e. colloquially referred to as the "silver bullet/magic starter key").

3.4.1 Growth regulators

An increase in growth promoting substances in early spring has been reported in fruit trees (Walker and Seeley 1973). Lavee (1990) stated that the dormancy of resting buds

of trees is governed by environmental factors affecting the level of regulating substances which, in turn, control the metabolic changes leading to the onset of, or release from, dormancy. On studies on trees it was proposed that bud dormancy is regulated by an interaction between endogenous inhibitor and promotor substances (Eagles and Wareing 1964). Studying grapevine buds, the presence of inhibitory substances was reported during winter, which disappeared at the start of shoot growth with stimulatory activity increase (Weaver *et al.* 1968).

Auxins are obligatory for active growth, but their role in the control of bud dormancy seems to be rather limited (Lavee and May 1997). It is thought that auxins are not involved in dormancy release or dormancy control, but rather in bud growth. Furthermore, exogenous application of auxins had no effect on bud opening on deciduous fruit trees. However, there is no doubt that auxin does have an effect in promoting correlative inhibition during paradormancy as well as endodormancy (Faust *et al.* 1997).

Regarding abscisic acid (ABA), Broquedis and Bouard (1993) found in studies on buds of the cultivar Merlot that the highest ABA levels were in winter when the water content of the buds was low. Just as soon as the water content of the buds increased, with ending of dormancy, the ABA levels dropped rapidly. Recent studies by Or *et al.* (2000) who tried to use levels of endogenous ABA in buds as an indicator or marker of dormancy release in the table grape cultivar Perlette grown in the Jordan Valley, were not successful. However, they did correlate the deepest levels of bud dormancy with the highest levels of endogenous ABA in the buds. There was no correlation between the decrease in ABA level and dormancy release – the sharp ABA decline began well before dormancy release. Other studies on buds of the winegrape cultivar Merlot showed the ABA maximum level was during winter, for leaves during the onset of dormancy and for internodes at leaf fall (Koussa *et al.* 1998). Other studies on the winegrape cultivar Riesling confirmed this peak of ABA levels during winter dormancy and decline in spring (During and Bachmann 1975). Studies on grapevine callus cultures showed higher ABA levels in winter and a reduction in ABA after a chilling period (Jimenez and Bangerth 2000). However, there have been results that question the role of ABA as dormancy inhibitor, although a reduction of ABA content was noted during chilling of grapevine buds (Emmerson and Powell 1978). Changes in ABA levels were found to be in the bud apex, while bud scales stayed low and constant (Lavee and May 1997). From this it is considered that the involvement of ABA is more an active one related to the metabolic state of the buds. However, the general relationship between ABA and bud dormancy in grapevines has so

far neither been proven nor disproven, due to the limited knowledge of the biochemical processes leading into and out of bud dormancy.

In the grapevine, application of gibberellic acid (GA) during the previous growing season will delay and even completely inhibit bud opening in the following growing season (Lavee and May 1997). There are reports that in many cultivars, GA applications during flowering in one season lead to complete failure of buds to burst in the next one. This can partially explain many of the difficulties experienced with RB in the cultivar Sultanina in South Africa, as GA is routinely applied during flowering for bunch thinning as well as for berry sizing.

Ethylene is ineffective in releasing grapevine buds from dormancy, the ethylene releasing compound ethephon even delays budbreak (Lavee and May 1997). Ethephon was also found to delay opening of pre-dormant buds and subsequent elongation growth. The role of ethylene *per se* in controlling bud opening during the pre-dormant season is therefore questionable.

In studies on the table grape cultivars, Flame seedless and Superior seedless, endogenous levels of phytohormones in buds varied as follows: ABA peaked in winter and declined rapidly at BB, while GA and auxin peaked at the BB stage (Chekol 1994). This would suggest an inhibitory role of ABA in winter while the GA and auxin would be associated with growth in spring. However, the ratio between the different hormones was also deemed to be important. Recent studies showed an inhibiting effect by auxins and gibberellins manufactured in leaves on subjacent buds of grapevines (Ezzili and Bejaoui 2000, Ezzili and Bejaoui 2001). Application of the cytokinin, BAP, on the buds stimulated BB. The cytokinin-auxin interaction was important, as the CK seemed to attenuate or lessen the inhibitory effect of the auxin. The levels of IAA were twice as high after chilling, compared to normal samples, suggesting a possible role of IAA during dormancy release. Cook *et al.* (2001) demonstrated a cytokinin (ZR) increase in the bark and buds of rootless apple (cultivar Granny Smith) shoots, after chilling and forcing, as growth resumed. This supports the hypothesis that shoot-derived, rather than root-derived CKs act to trigger budburst. The presence of roots may have a promoting effect on the readiness of buds to burst due to the CK produced in the roots (Lavee and May 1997). CKs trigger metabolic activities that are involved with growth, including DNA, RNA and protein synthesis, an increase in energy metabolism and a decrease in pathways important in dormant tissues (Faust *et al.* 1997). Chemicals used to partially replace chilling, such as DNOC-oil and hydrogen cyanamide (HC), increased xylem concentration of CKs five weeks before it happened in the control (Cutting *et al.* 1991). The CK levels

peaked at approximately two weeks before budbreak in the treated trees. Skene (1972) showed that dormant canes of vines possess higher levels of CK in xylem sap after months of cold storage than dormant canes that were freshly harvested, suggesting cambium storage/processing of CKs. This would concur with the thoughts on initial BB occuring on this 'storage' CK, before root CK supply increases. In his comprehensive review of dormancy in deciduous fruit trees, Saure (1985) stated that CK probably has some supplementary function in dormancy release, but is not the cause. The main effect of CK apparently is to hasten the development of buds that have been at least partially released from dormancy.

In summary, the consensus of opinion is that PGRs are not the primary factor controlling the timing of dormancy and post-dormancy, but rather play a role in the control of subsequent growth (Lavee and May 1997).

3.4.2 Membranes and fatty acids

Fatty acids are an important component of cell membranes and the lipoproteins that make up the membrane's growth (Lavee and May 1997). With the resumption of growth, after dormancy, there is a change in membrane composition, allowing increased permeability of solutes and water to the cytoplasm Faust *et al.* 1997). Marquat *et al.* (1999) studied the absorption of sucrose and sorbitol by the bud and stem during the rest period in peaches (*Prunus persica* L.). In this study, it was assumed that the sink capacities of tissues depend on their potential to absorb carbohydrates by active transport. During dormancy the bud exhibited a low absorption potential and increased it's sucrose potential by starch hydrolysis. During dormancy release, the bud was able to absorb carbohydrates, allowing carbon storage. During dormancy, the bud showed a low absorption potential for nutrients, hydrolizing starch reserves and increasing freeze protection by sucrose synthesis. During dormancy release, the bud exhibited important sink strength by active transport and accumulated carbon reserves (sorbitol, stachyose, raffinose and starch) used for growth metabolism, inducing BB.

Studies on apple (*Pyrus malus* L.) embryos showed that anaerobic treatments affected membrane permeability (Barthe and Bulard 1983). Various studies by Wang and Faust and various co-workers were done on changes in fatty acid composition of membranes of buds during RB (Wang and Faust 1988, Wang and Faust 1990, Wang *et al.* 1991). They observed that an accumulation of unsaturated polar membrane fatty acids started after a treatment with thidiazuron (TDZ), a RBA, which also led to a decrease in the relative amount of free sterols and suggested that these changes may alter the molecular

structure and physiological functions of the membrane. The enzymatic activity peaked when buds were in the green tip stage (Coombe 1995). An increase in membrane polar lipids was associated with BB and an increase of sitosterol and other sterols decrease in sitosteryl esters and a decline in the ratio of free sterols to phospholipids. During the dormant period major changes take place in membrane composition, especially the phospholipids, with linoleic acid increasing to a maximum during dormancy, while linolenic acid levels stay quite constant (Faust *et al.* 1997). The phospholipid ratio between linolenic acid (18:3) to linoleic acid (18:2) is about 1. With chilling requirement satisfied, the ratio changes, as the levels of linoleic acid decreases, with an increase in linolenic acid. By the time of BB, the ratio 18:3/18:2 is about 2. This could be an important tool to determine dormancy states in the bud and is discussed in a later section. During chilling there is also a large increase in total phospholipids per bud dry weight. The importance of membrane changes in dormancy and dormancy release and the involvement of ratios between sterols and lipids from the above is clear (Lavee and May 1997).

3.4.3 Anabolic potential of buds

The "French school" of dormancy approach sees the development of dormancy due to the loss of potential competition with other plant tissues (Faust *et al.* 1997). Dormancy release is considered as improved competing bud power with it's neighbouring tissues. A communication block develops gradually between the bud and the adjacent tissue during the transition to dormancy. This can be described as a transition from the long-distance effect of correlative inhibition to a short-distance inhibition by a barrier between the bud and adjacent tissue. This approach to dormancy can also be seen in the work of Marquat *et al.* (1999), who studied the absorption of sucrose and sorbitol by the bud and stem during the rest period in peach (*Prunus persica* L.). It was assumed that the sink capacities of tissues depend on their potential to absorb carbohydrates by active transport. During dormancy the bud exhibited a low absorption potential and increased it's sucrose potential by starch hydrolysis. During dormancy release, the bud was able to absorb carbohydrates, allowing carbon storage. During dormancy, the bud showed a low absorption potential for nutrients, hydrolizing the starch reserves and increasing freeze protection by sucrose synthesis. During dormancy release, the bud exhibited important sink strength by active transport and accumulated carbon reserves (sorbitol, stachyose, raffinose and starch) used for growth metabolism, inducing BB. A highly significant correlation was found between the pH of cells and the ability of the tissue to compete with other sinks in the tree (Faust *et al.* 1997). By reversing the competing power of the different tissues, the bud may overcome the block to it's development. The internal bud

cell pH is rising and higher than the surrounding stem and receptacle cells when dormancy is over, while the opposite is true during dormancy. The rise is due to increased plasmalemma ATPase activity in the cell membranes and proton pumping, a measure of active metabolic activity.

3.4.4 Thiols

The involvement of thiols in the onset and also release from dormancy has been shown in certain plants (Lavee and May 1997). Free glutathione in reduced (GSH) and oxidised (GSSG) form was found at low levels during dormancy and increased considerably during BB. In grapes, their level was shown to increase with the onset of dormancy and to increase again during dormancy release. Thus any involvement of glutathione in the metabolism of dormancy might involve various pathways.

3.4.5 Amino acids and proteins

Similar seasonal fluctuations in the levels of proteins in grapevine buds occur, with definite differences in levels between varieties (Lavee and May 1997). The protein content peaked before dormancy, dropped in winter and peaked again during BB and the growth period. This has led to suggestions that specific dormancy-inducing proteins and sprouting-inducing proteins are involved in the initiation and release of dormancy. These proteins still need to be conclusively isolated and identified.

3.4.6 Nucleic acids

So far no information is available for DNA and RNA content in grapevine buds (Lavee and May 1997). In apple shoots the RNA content of the bark was found to be lower during dormancy than during growth as could be expected. It was shown that short days promoted the synthesis of RNA, leading to the production of bark storage proteins.

3.5 Release from dormancy

According to Faust *et. al.* (1997) the four major biological factors that change the intensity of dormancy are:

1. Hormone balance in the bud of tree;
2. State of water within the bud;
3. Structure of membranes affecting cold resistance;
4. Anabolic potential of buds.

3.5.1 Chilling

It is generally accepted that chilling is essential to terminate grapevine bud dormancy and allow normal budburst (Lavee and May 1997). However, it is suggested that the requirement for chilling may not be obligatory for breaking dormancy in all grapevine varieties. The chilling rather leads to improved BB, but is not a necessity for BB. In its absence and without any other RB treatments, grapevine buds will show limited, uneven and delayed BB. The chilling requirement for grapevines is low compared to that of peaches. In studies on maize seedlings it was observed that a period of acclimation made the seedlings able to withstand much lower temperatures (Nir *et al.* 1984). It is suggested that during the initial chilling acclimation hydrogen peroxide is released, which signal the release of antioxidant (e.g. catalase) enzymes which then protect the plant from hydrogen peroxidase released at lower temperatures. This helps the plant to overcome chilling induced stress. Studies on apricots showed a catalase activity increase at the end of winter (Viti and Bartolini 1998). In studies on grapevines, a decrease in catalase activity was found during exposure to cold temperatures and dormancy breaking Nir *et al.* (1986). This decrease leads to an increase in hydrogen peroxide levels, favouring a shift from the Embden-Meyerhof Parnas system to the pentose phosphate pathway, which leads to an increase in reduced nucleotide production, which is essential for intensified metabolism (Nir *et al.* 1984). The role of the pentose phosphate pathway and dormancy of seeds has been thoroughly discussed by Roberts and Smith (1977). However, studies on nectarine seeds have questioned the role of the pentose phosphate pathway and catalase activity in dormancy control (Hu and Couvillon 1990). It appears that high temperatures may either prevent the onset of dormancy (for example the tropics), or replace the effect of chilling by activating an alternative pathway (Lavee and May 1997).

3.5.2 Water states in the cell

As stated before, the water content of grapevine buds drop from about 80% to 60% as they enter dormancy (Wood 2000). Cold hardiness of grapevine buds and canes increases with a decrease in water content (Bell 1997, Faust *et al.* 1997, Wood 2000). At the end of dormancy, when buds start growing again, the water content rises rapidly to 80% again. Faust *et al.* (1991) found that MRI could distinguish between bound and free water in dormant apple buds. The bound water was correlated with endodormancy, while eco-and paradormancy could not be distinguished, as both had free water. This was a way to determine the state of bud dormancy non-destructively in intact buds. MRI was

also shown to be able to determine between chilled (i.e. ecodormant) and dormant (endodormant) blueberry (*Vaccinium corymbosum* L.) flower buds (Rowland *et al.* 1992). The chilled buds had free water and the dormant buds bound water, as shown by MRI. Fennell *et al.* (1996) showed the same in studies on buds of *Vitis raparia* L. grapes, with bound water been observed in more dormant buds and free water in less dormant buds. However, recent studies by Erez *et al.* (1997), has questioned the role of bound versus free water as the method to control induction and release from dormancy suggesting rather that it plays a role with the cold resistance mechanism, rather than the dormancy per se of the bud. Later Erez suggested rather a controlling role by lipids in bud cell membranes (Erez 2000) with the basic mechanism being the activation of two membrane-bound enzymes, oleate desaturase (OD) and linoleate desaturase (LD). These two enzyme activities are influenced by temperature, with the OD being more active at low temperatures and the LD at higher temperatures. This combination ensures that processes, as yet not fully clarified, can occur slowly during the dormancy period enabling swift resumption of growth in spring, as temperatures rise. As stated previously, studies by Broquedis and Bouard in 1993 found highest ABA levels in winter when the water content of the buds was low. Just as soon as the water content of the buds increased (possibly due to more available free water), with ending of dormancy, the ABA levels dropped very quickly. Dehydrin proteins are heat-stable hydrophilic proteins that are induced during cold stress and/or dehydration (Faust *et al.* 1997). These proteins, due to their high hydrophilicity, are possible candidates to bind water. ABA may play a role in controlling the levels of these proteins and thus indirectly, have an effect on dormancy. As yet, dehydrin formation has only been correlated with cold hardiness, but its role in the control of bud dormancy cannot be excluded.

3.5.3 Evaporative cooling

There is some controversy regarding the absolute chilling requirement of grapevines, but practises like evaporative cooling in autumn and winter are used to achieve more rapid and uniform BB in many warm growing areas (Nir *et al.* 1988, Williams *et al.* 1994, Lavee and May 1997). Evaporative cooling on Sultanina and Perlette table grape vines in the Jordan Valley decreased the temperature of buds exposed to direct sunlight from 30 to 16°C and that of shaded buds from 25 to 13°C (Nir *et al.* 1988). This resulted in earlier, even BB, higher yields and earlier fruit maturation. Studies on apples and pears showed that winter rainfall or laboratory soaking reduced the time required for breaking endodormancy (winter rest) (Westwood and Bjornstad 1978). They suggested that possibly a water-soluble inhibitor was leached from the buds.

3.5.4 Desiccation and anaerobia

Limited desiccation and placement under anaerobic conditions caused dormancy to be broken (Lavee and May 1997). However, application of mineral oils, an effective BB treatment in most deciduous plants, caused an inhibition of BB in the cultivars Sultanina, Alphonse Lavalleé and Perlette. This was probably due to anaerobiosis.

3.5.5 Bud scale removal and other manipulations

Removal of bud scales from grapevine buds hastens BB (Iwasaki and Weaver 1977, Emmerson and Powell 1978). It has been assumed, though not proven, that this effect is related to ABA present in the scales (Lavee and May 1997). Removal of bud scales also hastened BB on apple (Swartz *et al.* 1984) and Japanese pear (Yotsuka *et al.* 1984). Wounding, by notching or girdling, directly above buds of apple shoots also induced BB (Paiva and Robitaille 1978). Bending Japanese pear shoots at a 45° angle led to an increase in Z-type CKs in lateral buds over vertical shoots (Ito *et al.* 1999). This led to accelerated flower development, possibly by reducing competition between buds and other organs by altering hormone levels.

3.5.6 Rest breaking agents

Hydrogen cyanamide is the most effective RBA at this stage on table grapes grown in warm, semi-desert or tropical conditions (Shulman *et al.* 1983, Pires *et al.* 1993, Siller-Cepeda *et al.* 1994, Lavee and May 1997, Dokoozlian *et al.* 1998, Or *et al.* 1999). Also, locally in South Africa, HC is used to improve BB of grapevines (Burnett 1985, Smit 1985). The best effect is found with applications applied some weeks before natural BB – applications too soon, may have no, or weak RB effect, while applications too close to BB may be harmful to the bud, as the bud's resistance to the chemical declines rapidly upon release from endodormancy (Shulman *et al.* 1983, Siller-Cepeda *et al.* 1992, Pires *et al.* 1993, Siller-Cepeda *et al.* 1994, Dokoozlian *et al.* 1995, Lavee and May 1997, Or *et al.* 1999). The degree of response is usually related to the grape variety and the depth of dormancy (Lavee and May 1997). Under warm subtropical conditions, early HC application advanced fruit maturity but decreased yield. Also, HC applications after the vines have been through sufficient chilling to break dormancy does not result in significantly increased BB over untreated vines (Iwasaki and Weaver 1977). The mode of action of HC has not yet entirely been established (Lavee and May 1997). However, HC was found in grapevines to inhibit catalase activity, as well as result in a reduction in the

free sulphydryl groups of glutatione. Catalase activity in grapevine buds is high in autumn and decreases to a minimum level when the buds are ready to BB.

Producers generally apply HC at a fixed date every year (Or *et al.* 1999). The risk of serious bud damage exists if the application proves to have been mistimed in early or later seasons. Or *et al.* (1999), thus evaluated several HC application times over a number of seasons. No differences in level and uniformity of BB was found between the different application timings, but major differences in cluster size, number and yield was found. Too early application led to the greatest negative effects. The yield loss observed with early HC application cannot be explained by lower BB, as BB was similar for both early and late applications. These results point to specific negative influences induced by early application of HC on the reproductive part of the bud. Early pruning *per se* did not have a negative influence on the reproductive meristem (yield), so the conclusion is that the early HC application led to an actual loss of clusters that would otherwise have developed well. They suggested a number of possible explanations:

1. HC might act indirectly, forcing early BB, so that the partially developed reproductive meristem is forced to emerge, resulting in abortion of floral primordial or clusters that are only partially developed. Anatomical studies of buds are planned to clarify this possibility;
2. By inducing early BB, the HC can expose the floral primordial to unfavourable temperatures for further development, leading to cluster abscission or poor development. Similar results have been observed with cultivars, e.g. Sultanina grown in a cool climate. However, the weather in the Jordan Valley never reached such low temperatures, which would deem this hypothesis unlikely;
3. With the early HC application, there may have been a specific phytotoxic effect on the reproductive meristem. Other deciduous trees have floral buds with lower chilling requirements than the vegetative buds. Grapevines are, however, viewed to have quite well protected reproductive buds. The hypothesis is that earlier, rather than too late applications can damage the floral bud, as early, the floral buds are not yet so well developed to cover and protect the floral bud, leading to damage with earlier applications. There is as yet no data to prove this hypothesis;
4. Vines have a compound bud that consists of two secondary buds and a primary floral bud in the middle, which produces the main clusters. The vegetative buds have only weakly developed clusters. If the early HC application induces the secondary buds to develop rather than the primary bud, it would explain the loss in yield, as these shoots have small clusters, if any.

Whiting and Coombe (1984) also reported similar observations on Sultanina, that HC stimulated more shoots from latent (secondary) buds. None of the previous possibilities can be excluded, except the temperature effect on flowering, to explain the loss in yield induced by early HC application (Or *et al.* 1999). The early application was effectively given 9-10 weeks before natural BB and the late application at 5-6 weeks before natural BB. Harvest of the early application was 12 days earlier, but the loss in yield may not be made up by the possibly higher prices achieved with earlier fruit. Other studies on Sultanina also showed that early application of HC did not result in dramatically earlier harvesting, only a number of days earlier (Williams and Smith 1984). Hydrogen cyanamide had no effect on the rate of growth of shoots after BB (Lavee and May 1997). The results suggest that the environment controls the phenology (growth and development) of the grapevine, even after early application of HC, and that too early application with lots of cold weather ahead, will not really be beneficial to earlier harvesting. The environment must thus also contribute favourable conditions for growth. It was also reported that fruit set in grapes is inhibited by high temperature Williams *et al.* 1994). Eventually, only when we understand the physiological nature of dormancy induction and release, will we be able to effectively manipulate this physiological event in warm regions (Bell 1997). Then application of RBAs can be at a specific state of dormancy for optimum BB and yield.

Nitrogen-containing products (e.g. KNO_3 and organic nitrogen products) are also used, although to a relatively lesser effectiveness. Normally these agents only work satisfactory in climates where winter rainfall and high chilling units are accumulated, as in the Mediterranean climate of the Western Cape region of South Africa.

3.5.7 Schools of thought on dormancy

There are two basic schools of thought on dormancy and dormancy release (Dennis 1994): the 'classical school' and the 'French school'. The classical school prefer simple hypotheses based on the assumption that dormancy is controlled by relatively few factors. Many of these hypotheses have been obtained with seed dormancy research and assume that chilling removes an inhibitor; stimulates promoters, or both, leading to growth resumption. The French school have looked mainly at bud dormancy studies. They view hormones with a certain amount of scepticism – they may play a secondary role in dormancy, but they are not the key factors. Dormancy is too complicated a process to be controlled by one or two factors, as it is the last stage of a cascade of correlative inhibitions, beginning with apical dominance, gradually extending from control by the apical bud alone to control by tissue immediately subjacent to the

meristem of the lateral bud, and finally to control within the meristem. The French school considers growth to be a very complex process comprised of many sub processes that fluctuate in intensity and are interrelated. Dormancy is but one phase of rhythmic growth. Until environmental and correlative control of each of the many processes involved in growth has been characterized, the search for "silver bullets" (i.e. hormones) that control dormancy is premature. Reweaves (1986) stated that the notion of controlling complex developmental processes by a single chemical is not a tenable proposition. He then argued for substituting a concept of sensitivity to control in a complex network for one of limiting factors. This will tie in with the French school of thought. Faust *et al.* (1997) concluded that a multi-faceted control exists for dormancy. The classical school is looking for "silver bullets" and work is focussing on genes that may control dormancy (Dennis 1994). Simple systems should yield the quickest answers and here *Arabidopsis* is the current favourite.

3.5.8 Summary

A unifying concept for describing dormancy has been suggested by Faust *et al.* (1997). The depth of dormancy changes during dormancy. They suggested further dividing the endodormant period into deep dormancy (d-endodormancy), where even RB treatments will not yield satisfactory results and shallow endodormancy (s-endodormancy), the stage where endodormancy can be overcome by artificial means, as most of the cold-induced membrane changes have taken place. All stages of dormancy are considered to overlap, due to the variation in dormancy levels between buds. Long distance influences are thought to be involved in the beginning of dormancy, similar to correlative inhibition. Later short distance influences work in on the bud and cause communication blocks between the bud and the adjacent tissues, mainly due to permeability barriers. Membranes react to the cold by increasing resistance to the low-temperature winter period. The membranes become more fluid, allowing functionality under colder conditions and also become less rigid, and highly permeable for transport of solutes. With the freeing of water in the bud, the bud enters the latter stage of endodormancy and then become sensitive to CKs and RB treatments. With sufficiently high temperatures, growth resumes and the energy metabolism shifts from the pentose pathway to the tricarboxylic acid pathway. It is suspected that a longer chilling period results in more unsaturated (fluid) membranes, allowing for more intensive or explosive BB.

A two-stage approach to dormancy should be seen: firstly, the hormonal component, involved with correlative inhibition (auxins), environmental stimuli for dormancy induction (ABA) and dormancy release (CKs); and secondly, the processes driven by

cellular responses to freezing resistance, that also affects dormancy (membranes, water state). It should, however, be remembered that most of the work has been done on apples and peaches and not on vines.

3.6 Budbreak date models

Modelling of rest and rest completion for deciduous fruit trees has been a long-standing research problem worldwide. A number of temperature-based models have been developed. The Richardson (Richardson *et al.* 1974) model for rest completion on peaches is a well-known model based on environmental temperatures. Kobayashi *et al.* (1982) used the Degree Stage Model and environmental temperatures to describe and quantify the annual growth cycle and rest development of red-osier dogwood (*Cornus sericea*). In most fruit trees, high temperatures (above 20°C) following chilling reduces and even reverses the chilling effect (Erez *et al.* 1988). The subsequent development of the Dynamic Model of Erez *et al.* (1988) for rest completion of peaches in warmer areas was a further step forward. However, it has not yet been established whether this model is also relevant for the unique nature of dormancy in the grapevine (Lavee and May 1997). A model for predicting the date of bud burst of grapevines under South African conditions was developed by Swanepoel *et al.* (1990), based on winter environmental temperatures and cultivar coefficients, determined from normal BB dates. This has the advantage that pruning can be planned more efficiently (e.g. best pruning time, about 2-3 weeks before budburst). Dokoozlian (1998) recently developed a practical model for predicting rest completion in table grapes, using simple relative chill accumulation and chill negation temperature ratios that showed good correlation with actual BB, providing a practical tool for the researcher and producer to manage dormancy. Recently, Martin and Dunn (2000) investigated the effect of pruning time and HC application on budburst of the wine grape cultivar Cabernet Sauvignon. Delaying HC application by six weeks, only delayed budburst, anthesis, véraison and maturity by five days. HC caused more 'extra' shoots to burst, especially at the base of spurs and on old wood. Primary shoots burst earlier than extra shoots, especially if it had more bunches. They suggested that temperature-based models designed to predict the timing of phenological events in grapevines may be improved by including parameters that take account of pruning time and the reproductive potential of classes of buds.

3.7 Future of dormancy research

3.7.1 Genetic basis of dormancy

With the modern techniques available to the researcher nowadays the search for the dormancy mechanism is taking more molecular routes (Fennell 1999). Roughly two approaches are followed: a. analysis of differential gene expression in search of unidentified genes or cDNAs that are correlated with dormancy processes, and b. searching for specific gene expression based on ultrastructural, physiological or biochemical information or genes identified in other systems. Examples of genetic model systems used to study molecular and genetic regulation of bud dormancy are: low-chill vs. high-chill blueberry (*Vaccinium* sp.); Poplar (*Populus* sp.) bud dormancy and more recently *Arabidopsis thaliana* (Koornneef *et al.* 2000). It seems that specific proteins take part in both the induction into and release from dormancy (Lavee and May 1997). The specificity of these proteins has to be verified and the controlling genes characterised. As many different factors and chemicals are active in the induction and release from dormancy, a multi-gene system seems likely to be involved in dormancy control. Identification and characterisation of these proteins at the various stages of dormancy could lead to the identification via RNA and thereafter to the control and manipulation of specific DNA in the different genes that control the process of dormancy.

3.7.2 Problems in dormancy interpretation

Lavender and Silim (1988) mentioned that one of the main problems in interpreting PGR results and the resulting contradictions and discrepancies on forest trees is *inter alia* the lack of a clear, physiologically valid definition of the term 'dormancy'. Also, the fact that the research accent is on the effects of levels of PGR's on plant response, excluding the possible effects of changes in tissue sensitivity or of the flux of PGR being investigated, as also mentioned by Trawavas (1986). This is very important if one takes the latest information on membrane composition and permeability during dormancy release into account. Maybe the membranes being more permeable also changes the affinity to the PGR's. Seeley (1994) said that the problem with dormancy research is that although many physiological studies have been done on endodormancy, we still do not know the basic biochemistry of endodormancy induction, transition or completion. We know more about endodormancy phenology, and models available of endodormancy release give few clues to the basic mechanisms involved. This is the challenge for the future.

CHAPTER 4

METHODOLOGY

4.1 Removal of interfering compounds from plant samples

4.1.1 Introduction

A major obstacle in the study of plant growth regulators (PGRs) is that they occur in very low levels in plant tissues. In addition, they also occur in an environment together with numerous closely related compounds so that the specificity of any PGR detection method is also very important. Methods that have been developed to measure and quantify these levels include bioassays, physical-chemical methods and immunological methods (Weiler *et al.* 1986). Historically, the first method used for the detection of PGRs were the so-called bioassays. In these assays an indicator biological system, e.g. maize coleoptiles, were used to indicate the presence of plant growth regulators in a sample. Bioassays were found not to be suitable for the quantification of PGRs, due to limited sensitivity and selectivity, although they are useful for qualitative detection of PGRs (Horgan 1987). Subsequently, combined physical-chemical methods, specifically gas chromatography – mass spectrometry were developed and these are considered to be the best technique for quantifying certain PGRs. However, this technique requires extensive pre-cleaning of the plant extracts to remove interfering compounds that can lead to a considerable loss of the PGRs (Weiler *et al.* 1986).

Immunoassays also have been developed for the quantification of PGRs. They have the advantage that they exploit the natural selectivity of an antibody for its antigen (Chard 1982). Antibody binding can then be detected by means of labelled antigens with very high specificity and sensitivity. This means that low PGR levels can be detected in relatively crude plant extracts. However, large-scale contamination of plant samples with interfering compounds necessitates some purification before immunoassays can be used successfully. Thus cytokinin immunoassays have been applied to detect cytokinin levels in a variety of plant tissues, often with some purification of the sample prior to immunoassay. Cytokinins have been quantified in e.g. xylem sap of *Leucadendron rubrum* (De Kock *et al.* 1994) and *Urtica dioica* (Fusseder *et al.* 1988); shoots of wheat seedlings (Kudoyarova *et al.* 1998) and apple shoot xylem sap, buds, wood and bark (Cook *et al.* 2000). Sometimes combined HPLC for the separation and RIA for the subsequent quantification of combinations of CKs in plant extracts up to the picogram level, have been used (MacDonald *et al.* 1981).

As a rule, a plant extract can be considered to be immunologically pure, if besides the hormone (s) to be analysed, no other cross-reacting material is present and secondly, if the hormone-antibody interaction is not affected by other factors present in the sample (Weiler *et al.* 1986). Thus plant extracts need to be purified to various purities before unbiased measurements can be made.

Methods often employed to purify plant samples include ion exchange chromatography, solvent partitioning and paper and thin layer chromatography (Horgan 1987). Bohinski (1987) described chromatography as follows: "Sample substances, dissolved in a mobile phase, move through a stationary phase, then move through a stationary medium, which, by interacting to varying degrees with individual substances, impedes the flow of these substances to different degrees. The result is that different substances will migrate at different rates". In this study, interfering contaminants were removed from plant samples using reverse phase hydrophobic interaction chromatography prior to quantification of CKs by immunoassay. Thus, the plant extracts were first passed through a C18 reverse phase packing material column to remove lipids and pigments. Lipids, pigments and PGR were retained on the column (Weiler 1986). Cytokinins were then eluted with methanol, while the lipids and pigments were retained.

4.1.2 Extraction of cytokinins from vine shoots

In this study, xylem sap, wood, bark and bud CKs were extracted and quantified as described by Cook *et al.* (2000). This procedure is described in the following sections.

An amount of 0.5 g freeze-dried sample was taken (or the whole sample, if less than 0.5 g), 10 ml extraction solution (80% methanol, butylated hydroxytoluene (BHT) and ascorbic acid) was added and extracted for 24 hours at 4°C while stirring. The antioxidant capacity of the extraction solution is important to prevent any oxidative damage to the CKs. After extraction, the sample was centrifuged at 12 100 xg for 20 minutes at 5°C in a refrigerated centrifuge. The supernatant was then decanted into glass vials. About 2 ml of extraction solution (80% methanol, BHT and ascorbic acid) was added to the pellet, mixed and then centrifuged again at 12 100 xg at 5°C for 10 minutes. This supernatant was added to the previous supernatant. Finally, the sample was dried down in a Savant Speedvac concentrator and kept at –80°C until analysed.

4.1.3 Polyvinylpyrrolidone pre-cleaning step

The dried sample was taken up in 5 ml 0.01 M ammonium acetate buffer pH 8.2 by shaking for one hour at room temperature. The pH of the sample was adjusted to 8.2, to ensure that the compounds to be removed had the correct charge for the purification step with polyvinylpyrrolidone (PVP). PVP was used to bind phenolic compounds, chlorophyll and organic acids (Glenn *et al.* 1972). This enables the removal of possible interfering compounds. PVP (0.4 g) was added to the pH adjusted sample and left to stand on the workbench for at least 30 minutes. Thereafter the sample was filtered through a glass microfibre filter.

4.1.4 Reverse-phase chromatography

Mini columns were packed with bulk pack preparative C18 reverse phase, Waters, 125 Å, 55-105 µm packing material in a 20 ml plastic column. The packing material consisted of dimethyloctadecylsilyl-bonded amorphous silica (Horgan 1987). These columns were pre-conditioned as follows: 10 ml 100% methanol, followed by 10 ml distilled water and finally by 0.01 M ammonium acetate pH 8.2 was run through the columns.

The cytokinin containing plant sample filtrate was then subsequently taken over a preconditioned C18 reverse phase mini-column. This enabled non-polar extraction of the sample, as polar compounds elute before non-polar compounds (including CKs). Then 10 ml 0.01 M ammonium acetate pH 8.2 was allowed to run through the column followed by 10 ml of distilled water. By washing the column with a polar solvent such as ammonium acetate, high polarity compounds like organic acids were eluted from the column. The CKs were eluted with 10 ml of methanol. A less polar solvent such as methanol removed the CKs, which are less polar as they contain non-polar functional groups (aromatic rings), while lipids and pigments were retained on the column. This allowed separation of CKs from the more polar solutions of water and ammonium acetate buffer. The column was then run dry and the eluate dried down in a Savant Speedvac concentrator. The sample was kept at -80°C and was now ready for immunoassay.

4.1.5 Purification recovery and verification

To assess the recovery of hormones during the extraction and clean-up procedures, radioactively labelled hormone internal standards were used. In this study, ^{3}H-labelled ZR was used as internal standard. This was added to bud, wood and bark samples before extraction. These were then purified and the radioactivity of the collected fractions

determined with a liquid scintillation counter. Recovery was found to be 58.8% for buds; 51.2% for wood and 47.9% for bark. All relevant values obtained in the course of this study were subsequently adjusted for these recoveries. Xylem sap was not subjected to any purification procedures, as such samples were found to be relatively free of interfering compounds (Cutting *et al.* 1991).

A measure of the absence of interfering compounds in plant samples is the use of so-called parallelity. Parallelity implies that samples should show additivity, i.e. different volumes of the same samples, should give similar RIA values when all are converted to the same volume. Parallelity of the different types of samples, i.e. wood, bark and buds, was tested and results presented in Table 4.1. The bark and wood were found to be additive, but buds gave more varying results. The variations were still deemed to be acceptable for the purposes of this study.

Table 4.1. Parallelity of wood, bark and bud samples, as tested at different sample volumes.

Sample type (three separate samples)	RIA ng ZR values of different sample volumes (adjusted to 100 µl)			
	25 µl	50 µl	75 µl	100 µl
Wood 1	8.064	7.598	7.298	7.032
Wood 2	5.944	5.144	5.414	5.199
Wood 3	3.248	3.220	3.214	3.432
Bark 1	12.608	13.152	12.900	12.121
Bark 2	7.152	7.500	7.904	7.547
Bark 3	3.896	4.424	4.829	4.452
Buds 1	1.724	1.128	0.953	0.749
Buds 2	1.808	1.324	1.056	0.838
Buds 3	2.176	1.480	1.356	1.240
*Com Buds 1	2.293 (100)	2.015 (150)	1.934 (200)	1.978 (250)
*Com Buds 2	2.088 (100)	2.012 (150)	1.565 (200)	1.527 (250)
*Com Buds 3	1.723 (100)	1.258 (150)	1.076 (200)	1.047 (250)

* Three different bud samples were combined to produce a "combined bud sample". The volumes were: 100, 150, 200 and 250 µl and all results were adjusted to 250 µl.

4.2 Determination of zeatin riboside by radioimmunoassay

4.2.1 Introduction

Immunoassay is based on the competition of a known amount of labelled antigen and an unknown amount of sample antigen for a limited number of high-affinity antibody binding sites (Weiler 1984). Radioimmunoassay (RIA) was developed by Berson and Yalow in 1959, but Fuchs and his co-workers were the first in 1969 to try to detect and quantify PGRs with the use of antibodies (Weiler 1984).

As they combine specificity of the antibody-antigen interaction with sensitive tracer techniques, immunoassays can be used to detect very low concentrations of metabolites in relatively impure solutions and in small samples. Crucial to the development of these assays are: availability of specific antibodies; availability of a labelled antigen; an efficient way of separating antibody-bound from free antigen; immunogen synthesis; immunization; tracer (labelled hormone) synthesis and antibody selection (Weiler 1982).

The basic principle of immunoassay can be described as follows: the assay uses the reversible interaction of an antibody (Ab) with its antigen (Ag), as shown below (Chard 1982):

$$Ag\text{-}Ab + Ag^* \leftrightarrows Ag + Ab + Ag^* \leftrightarrows Ag^*\text{-}Ab + Ag$$

By incubating an Ab with a labelled antigen (Ag*), both at low concentrations, the unknown concentration of Ag can be determined from a reference dose-response curve of known standard Ag concentrations (Chard 1982). Competition between the labelled and unlabelled Ag for the Ab occurs and an equilibrium state is reached as shown in the reaction above. As mentioned before, three crucial factors for the development of immunoassays are: the availability of Ag-specific Ab's obtained by immunizing selected animals with Ag-protein conjugates (immunogens); availability of a labelled Ag with similar affinity to the Ab as the unlabelled Ag; and an efficient way of separating Ab-bound Ag from free Ag without disturbing the reaction equilibrium.

For the application of RIA to determine ZR levels, the following terminology applies: antibodies (i.e. anti-trans-ZR) are used as binder and antigen (i.e. trans-ZR) is used as ligand (Chard 1982). Ag is a substance which will react with an antibody, but is not

necessary immunogenic on it's own. An immunogen is a substance that will stimulate an immune response to form antibodies. Cytokinins are haptens, i.e. they cannot elicit an antibody response on their own. They therefore need to be covalently linked to a large protein such as bovine serum albumin (BSA), ovalbumin or hemocyanin, to render them immunogenic. Chard (1982) stated the basic principle of all binding (i.e. in this studies case, antibody as binder) assays as: "Given an unvarying quantity of binder of fixed K-value, the ratio of bound to free ligand (i.e. antigen) at equilibrium will be quantitatively related to the total amount of ligand present." The law of mass action states that, at equilibrium, the ratio of the products of the concentrations on both sides of the equation will be constant, and designated as K, the affinity constant. This is shown as: [AgAb]/[Ag][Ab] = K.

The development of antibodies against the non-immunogenic CKs came about from an interesting sequence of events in the 1960's. Khym (1963) in a reaction of methylamine with periodate, oxidized AMP and formed a cyclic, hemialdal type of structure of the ribose component. This showed that periodate oxidation can be used to couple amino groups to ring structures. This concept was used by Butler and Chen (1967) in coupling a *Digitalis* cardiac glycoside, digoxin, to BSA. They oxidized the terminal digitoxose with periodate and the two resultant free aldehyde groups could then react with free amino groups of the carrier protein. The resultant stable conjugate was then used as an antigen to induce formation of Ab with digoxin specificity. This approach was used to couple CKs which have similar ring structures to the digitoxose used in the *Digitalis* experiment, to link them to larger proteins, thus rendering them immunogenic (Weiler 1990). The procedure was as follows: the cytokinin-9-riboside was oxidised with periodate to form a di-aldehyde. It was then allowed to react with an amino group of the carrier protein (BSA) under basic conditions (pH>9) to form a Schiff base. This secondary amine was then stabilized by reduction with sodiumborohydride. The stable product was then immunogenic and could be used to immunize rabbits or mice to form specific cytokinin Ab.

Monoclonal antibodies can be used to eliminate the inherent variability of polyclonal antibodies, showing both high affinity and specificity (Weiler 1984). Cytokinins are low molecular weight compounds and the synthesis of cytokinin-protein conjugates are necessary for an immune response, as described earlier. Regarding CKs, the assays use either ^{3}H (tritium) or ^{125}-I labelled cytokinin derivatives and can detect from 30 - 200 femtomol of the specific CKs. Sensitivity of RIA for CKs is approximately $3x10^{-14}$ M, compared to MS with $3x10^{-12}$ M (Weiler 1982). The future potential of cytokinin immunoassay is based on its combination of intrinsic specificity, sensitivity, high

reproducibility and ease of operation (Weiler 1982, Weiler 1984). This allows for the convenient analysis of rather unpure samples for CKs. RIA at least matches and mainly exceeds the sensitivity of other techniques and can be applied to unprocessed plant extracts. Another advantage is the large sample capacity, economical costs and speed, without expensive heavily equipped laboratories. However, the potential of immunoassay should not be overestimated and a range of internal controls should always be used.

4.2.2 Methodology

4.2.2.1 Introduction

Weiler developed a number of sensitive RIA's for the detection of various PGR's in the 1970's and 1980's, including *trans*-ZR (t-ZR) and ABA (Weiler and Ziegler 1981, Weiler 1980a, Weiler 1980b). Antisera produced against BSA conjugates of t-ZR had a high affinity for ZR and Z, but negligible cross-reaction to iP and *cis*-ZR (c-ZR) and low cross reactivity to DHZ. The detection limit of these assays employing ^{3}H-labelled (tritiated) tracer, was 40 femtomol with a high assay reproducibility. Due to the high specificity, crude extracts could be used for analysis. Weiler and Ziegler (1981) applied the RIA to identify and quantify phytohormones in phloem exudates of various tree species. The hormones were ABA, IAA, GA_3, GA_7, various zeatin CKs and iP CKs. The RIA was specific enough to distinguish between t-Z CKs and iP CKs, but could not differentiate between CKs linked to free bases, ribosides or glucosides.

The development of high affinity monoclonal antibodies against ZR and DHZR by Eberle *et al.* (1986) led to the refinement of the RIA, removing the specificity problems associated with the polyclonal antibodies used previously. Mice were hyperimmunized with ZR and DHZR bovine serum albumin conjugates respectively. The antibodies allowed detection of femtomol amounts of free Z and DHZ, as well as their ribosides and ribotides in plant extracts. For this study, monoclonal t-ZR mouse antibodies were kindly donated by Prof. E.W. Weiler, Department of Plant Physiology, Rühr-Universität, Bochum, Germany.

4.2.2.2 Standard curve

In this study, samples were separated, extracted and the levels of ZR determined by RIA as described by Cook *et al.* (2001). The basic procedures for the RIA were as follows: To each Greiner tube constant amounts of t-ZR Ab (binder) and tritium-t-ZR radioactive tracer (Ag, ligand) was added. To this was added increasing amounts of ZR standards (Ag, ligand) to produce a standard curve, and in the case of the trial samples, unknown

amounts of ZR (Ag, ligand). These were incubated for 30 minutes at 37°C for binding to occur. Ammonium sulphate was added to precipitate the antibody-antigen protein complexes and centrifuged. The excess ligand (tracer, standard and unknown) was then washed off. The precipitate was then taken up in scintillation fluid (PicoFluor). Radioactivity was measured on a liquid scintillation counter and the levels of ZR determined by means of the Securia RIA data reduction and quality control program (Packard Instrument Company: SecuRia 2000CA option RIA/QC Software Package 1986).

All the standards and unknown samples were triplicated as follows: Greiner tubes were labelled 0-9, Ta (total activity) and NSB (non-specific binding). In tube 0, 100 µl ethanol was pipetted. ZR standards in 100 µl ethanol were pipetted into tubes 1-9 in the following amounts: (1) 0.1 ng; (2) 0.25 ng; (3) 0.5 ng; (4) 1 ng; (5) 2.5 ng; (6) 5 ng; (7) 10 ng; (8) 25 ng; (9) 50 ng. Tubes 0-9 were dried in a Speedvac concentrator. After drying 100 µl radioactive ZR PGR tracer was added to all tubes including Ta and NSB. Then 100 µl t-ZR antibody was added to tubes 0-9 (kindly donated by Prof. E.W. Weiler, Department of Plant Physiology, Rühr-Universität, Bochum, Germany). Subsequently 0.5 ml bovine serum was added to tubes 0-9 and NSB, except Ta and mixed, except Ta. The tubes were then incubated for 30 minutes at 37°C. Thereafter 0.85 ml 90% ammonium sulphate solution was added to all tubes, except Ta, mixed and allowed to stand for at least 20 minutes at room temperature. The samples were then centrifuged at 4000 xg for 10 minutes, decanted and drained briefly on paper towels. Subsequently, 1.5 ml 50% ammonium sulphate was added to each of the tubes, except Ta, mixed and centrifuged at 4000 xg for 10 minutes. The tubes were then decanted, drained briefly on paper towels and the sides of the tubes carefully wiped to remove excess fluid. Thereafter 0.25 ml water was added to all tubes (including Ta) and mixed. Subsequently, 2 ml PicoFluor Scintillation Fluid was added to all tubes, capped, mixed and radioactivity determined in a liquid scintillation counter.

An example of a typical ZR standard curve as used in this study is shown in Figure 4.1. A binding percentage of 50% was deemed as ideal. As the procedure is a "competitive" procedure, it follows that the percentage binding will reduce with more unlabelled (standard or unknown) ZR being present in the sample. As a fixed amount of ZR Ab is added to the mixture and a fixed amount of ZR tracer it follows that as the amount of unlabelled ZR increases, there will be less binding sites left for the labelled ZR, thus resulting in a lower binding % or cpm. The levels of bound labelled ZR, at equilibrium, is thus inversely proportional to the levels of the Ag (unlabelled/unknown ZR) with which it

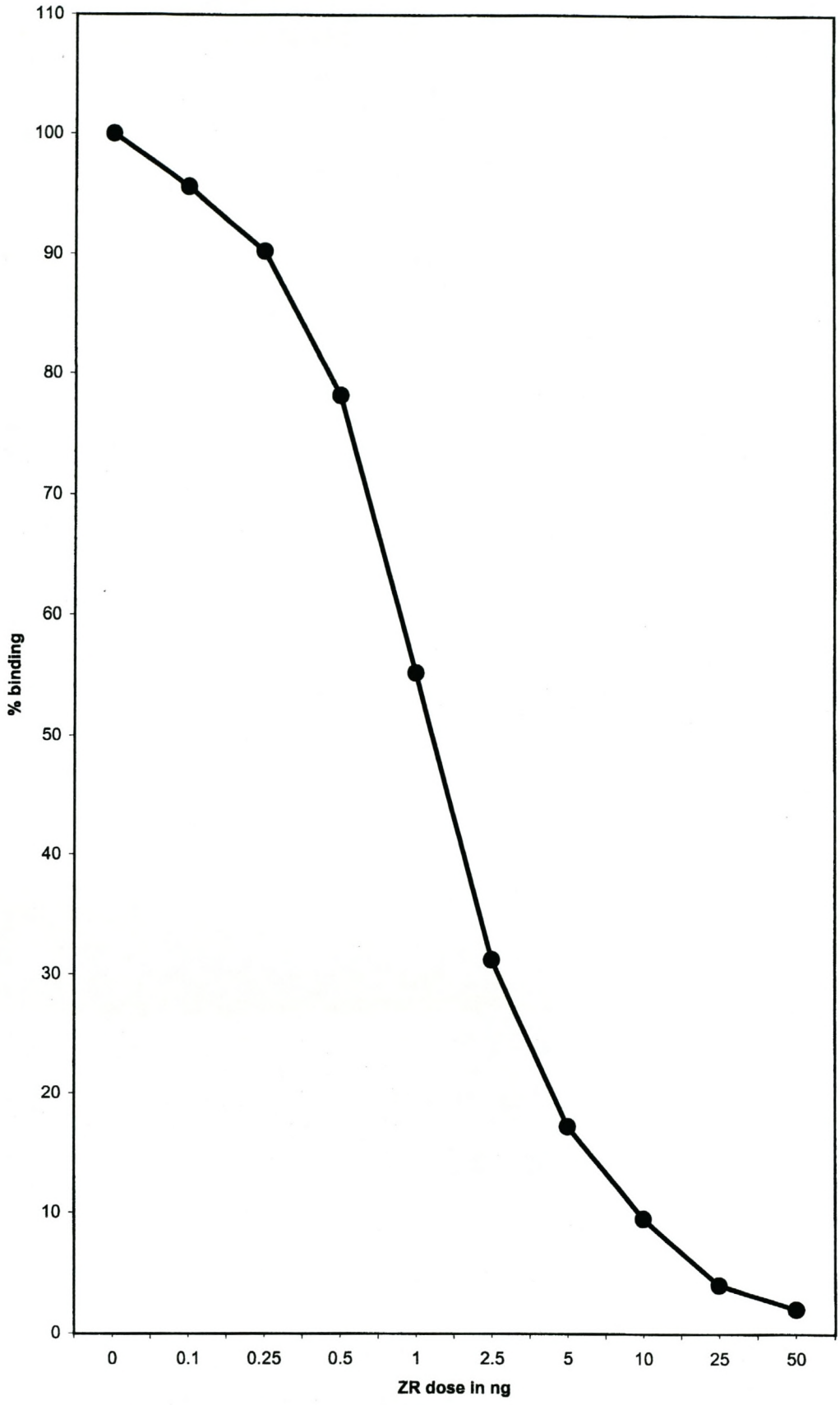


Fig. 4.1 Typical RIA ZR standard curve as used for this study, plotted as ng ZR standard versus labelled antibody binding percentage.

competes in the incubation medium. Thus, if one plots the known ZR levels on a curve, a sigmoidal curve results, from which the unknown ZR levels can then be calculated.

4.2.2.3 Unknown samples

The purified dried-down samples were taken up in 2 ml 100% methanol and shaken for one hour at room temperature to dissolve. If the sample was not clear, it was centrifuged at 4000 xg for 10 minutes and 100 µl samples were then pipetted in triplicate into Greiner tubes. These samples were dried down in a Speedvac concentrator. After drying, the same procedures were followed as for the standard curve from after the drying step when samples were first pipetted into the tubes.

The results of the two different trials, using these techniques, are presented in article format in the following two chapters.

CHAPTER 5

THE EFFECT OF HYDROGEN CYANAMIDE ON BUDBREAK AND CYTOKININ LEVELS OF SULTANINA VINES

5.1 Introduction

The study was carried out over two seasons, 1997 and 1998. As so little is known of the effect of HC on ZCK levels it was decided to do an initial trial to see if any effects of HC on ZCK levels could be observed. Thus, in 1997, HC was applied at the recommended dosage of 2.5% v/v, at three weeks before induced BB (6 weeks before BB in controls) and ZCK levels monitored from time of application until nine weeks thereafter, giving a total of 10 sampling times. The canes were then divided into distal and proximal sections and these were further divided into different tissues, namely buds, bark and wood, processed and the ZR levels determined. To contrast the trial to the previous year and to observe the effect of early HC application, as is commonly done commercially it was decided to do a trial with earlier HC application than in 1997. For this reason, in 1998 the standard HC treatment was applied at six weeks before induced BB (8 weeks before BB in controls). One year-old canes were sampled weekly from time of application until eleven weeks after application, giving a total of twelve sampling times. Again, canes were divided into distal and proximal sections and bud tissue only was isolated, processed and ZR levels determined. The results are presented in article format in the following section.

5.2 Manuscript: "The effect of hydrogen cyanamide application on budbreak and cytokinin levels of Sultanina vines"

The following manuscript represents original work by the author and co-authors. All experimental work was done by the main author, including clean-up procedures and RIA's. The main author compiled the manuscript and the contributions of the co-authors were as follows:

a. Prof. Dirk U. Bellstedt, associate professor of Biochemistry at the University of Stellenbosch is the promotor of this thesis. In this capacity, he was involved in the conceptual development of and practical execution of all aspects of this study. He promoted collaboration with the Horticultural Science Department and facilitated all logistical aspects within the Department of Biochemistry. He made contributions to

interpretation of the data and suggested some changes to the text of the manuscript that were subsequently incorporated.

b. Dr. Nigel C. Cook, visiting lecturer of Horticultural Science at the University of Stellenbosch and Katolieke Universiteit Leuven, Belgium, is the co-promotor of this thesis. In this capacity, he was involved in the conceptual development of and practical execution of all aspects of this study. He made contributions to the presentation and interpretation of the data as well as the structure of the thesis.

"The effect of hydrogen cyanamide application on budbreak and cytokinin levels of Sultanina vines"

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ABSTRACT

Hydrogen cyanamide is the most commonly used rest breaking agent for Sultanina vines in the Lower Orange River region of South Africa and other semi-arid areas of the world. The effect of hydrogen cyanamide on cane tissue cytokinin (specifically zeatin riboside, ZR) levels of table grape vines was studied over two seasons, 1997 and 1998. In 1997, hydrogen cyanamide was applied three weeks before induced budbreak. One year-old canes were sampled weekly after hydrogen cyanamide application, divided into distal and proximal sections, then further divided into buds, bark and wood tissues and the ZR levels determined. A relatively high amount of chilling coupled to late hydrogen cyanamide application in 1997 led to a large effect on ZR release, but did not lead to significant shifting of the budbreak pattern. Zeatin riboside peaks were observed in buds, internode wood and bark of treated vines compared to control vines. The peaks were higher in distal portions compared to proximal portions in all tissues. In 1998, hydrogen cyanamide was applied six weeks before induced budbreak. The relatively lower chilling and earlier application of hydrogen cyanamide in 1998 had a larger effect on the budbreak pattern while the bud ZR peak was shifted earlier. The distal portion bud ZR peak was again higher than the proximal portion bud ZR peak. In 1997, as sampling was not initiated early enough, bud ZR peaks were only observed after budbreak, while in 1998 bud ZR peaks were observed before and after budbreak. The effect of these ZR increases on the development of inflorescence primordia, subsequent bunch development and ultimately production, are discussed.

ABBREVIATIONS

BB, budbreak; CK, cytokinin; HC, hydrogen cyanamide; LTA, longterm average; PGR, plant growth regulator; RB, rest breaking; RBA, rest breaking agent; RIA, radioimmunoassay; t-ZR, trans zeatin riboside; Z, zeatin; ZR, zeatin riboside; ZCK, zeatin-type cytokinin

KEY WORDS

zeatin riboside, radioimmunoassay, South Africa, table grapes, rest breaking

INTRODUCTION

The Lower Orange River region is one of the early maturing regions of the South African table grape industry, due to the low occurrence of early spring frost and warm spring and summer temperatures. It comprises approximately 32% of the total area under table grapes and produces most of the local seedless grapes (Unifruco Ltd. 1996) and has an abundance of available water and good soil. This region has the potential to realise high prices with early maturing grapes in northern hemisphere markets. The ripening period of these grapes can be advanced even further by using rest breaking (RB) techniques such as early pruning, together with hydrogen cyanamide (HC) application, to induce early and/or more even budbreak (BB) (Burnett 1985, Smit 1985, Dry 1992). When producing early ripening table grapes in arid areas, HC is presently the most commonly used rest breaking agent (RBA), both locally and in other table grape producing countries and is indispensable (Shulman *et al.* 1983, McColl 1986, Erez 1987, George *et al.* 1988, Zelleke and Kliewer 1989, George and Nissen 1990, Lavee 1990, Pires *et al.* 1993, Dokoozlian *et al.* 1995). Without treatment with HC, very late, uneven and low BB levels are experienced. Due to low fruitfulness, Sultanina table grape vines are pruned to long canes (fourteen buds and more), which favours apical dominance thereby exacerbating the BB problem (Whiting and Coombe 1984, Dry and Gregory 1988). Problems that are generally experienced with the use of HC on vines include difficulty in predicting expected BB and therefore application time. As a result of this, uneven BB due to too early application or bud damage if applied too close to BB or also if applied too early, can be caused (Or *et al.* 1999). The absence of HC as a RBA, however, could significantly decrease profits in this region due to later harvesting periods and lower yields.

The effect of HC on the physiology of the vine has been previously investigated (Shulman *et al.* 1983, Nir *et al.* 1986, Lavee and May 1997), but the effect on the tissue endogenous Z-type cytokinins (ZCKs) is unclear and has not been investigated before. What is known is that they have a direct influence on the bud itself, which leads to other processes that ultimately stimulate BB. These processes are still unclear. During

seasons with sufficient chilling in the dormant stage of the vine, BB in spring is normal. However, as South Africa's vineyards are sometimes planted in marginal climatic zones, such as the Lower Orange River region, the chill requirement is not always satisfied.

Cytokinins (CKs) play an important central role in BB of deciduous fruit trees (Cook *et al.* 1998) and vines (Lavee and May 1997) in spring. In spring, endogenous CK levels in xylem sap of apple shoots increase prior to BB (Jones 1973, Young 1989, Tromp & Ovaa 1990, Cutting *et al.* 1991). The increase starts just before BB, increases rapidly from bud swell and peaks approximately two weeks before BB (Tromp & Ovaa 1990, Cutting *et al.* 1991) and is thought to originate from the shoot, as shown by ZR increases in rootless shoots, after chilling and bud forcing (Skene 1972, Hewett and Wareing 1973). Skene (1972) showed that dormant canes of vines possess higher levels of ZCK in xylem sap after months of cold storage than dormant canes that were freshly harvested, suggesting cambium storage/processing of ZCK. This would concur with the hypothesis of initial BB being initiated by this 'storage' ZCK, before root ZCK supply increases. The stems of woody plants could be significant sources of CKs, especially when root supply is limited, as at the end of winter (Skene 1975). In studies on poplars, it was observed that CK levels in buds of excised stems in late winter increased prior to BB independently of a root system (Skene 1975). The CK levels in xylem sap peaked three weeks before levels peaked in the buds themselves, suggesting accumulation from the stem. Also, HC applications after vines have been through sufficient chilling to break dormancy does not result in significantly increased BB over untreated vines (Iwasaki and Weaver 1977). Cook *et al.* (2001) also showed higher CK peaks in the distal portions relative to the proximal portions of apple shoots.

A number of studies have been initiated locally on a diverse number of plants, to determine CK levels associated with various metabolic states, including BB. Plants studied have varied from fynbos shrub (De Kock *et al.* 1994) to plums (Cook *et al.* 1998), with most work been done on apples (Cutting *et al.* 1991, Cook *et al.* 2001). The work by Cutting *et al.* (1991) in which xylem sap ZR levels were correlated with HC treated and untreated apple shoots, showed that the CK peak was moved forward by three weeks before the control peak. Both treated and untreated control shoots had ZR peak levels at approximately one week before 50% BB. However, similar studies on CK levels of table grapes have not been undertaken. Cook *et al.* (2001) observed a CK increase in the bark and buds of rootless apple (cultivar Granny Smith) shoots after chilling and forcing as growth resumed. This supports the hypothesis that shoot-derived, rather than root-derived CKs act to trigger BB and it was decided to use the approach of Cook *et al.* (2001) as basis for this study on Sultanina table grapes.

In this study, the effect of HC application to Sultanina vines at different times before BB on BB and ZCK levels was investigated. This study was initiated to determine the differences in ZR levels in cane tissues with HC applications at different times before BB. Thus ZR levels were determined in various tissues of canes of Sultanina vines after HC application at three weeks before normal induced BB until after BB in 1997. In 1998, ZR levels were determined in buds of Sultanina canes after HC application at six weeks before induced BB in 1998 until after BB.

MATERIALS AND METHODS

Vineyard A Sultanina clone H5 vineyard grafted onto Richter 99 rootstock and situated in the Kakamas area of the Lower Orange River region of South Africa, was used. The vines were planted in 1990, (seven years old in the 1997/98 season), trained to a 3.3 m X 2.0 m gable trellis and pruned to sixteen canes with fourteen buds and fourteen spurs with three buds respectively. The longer canes supply the current season's fruiting branches, while the shorter spurs are left to provide the next seasons fruiting branches. The vineyard was irrigated using microjet systems with irrigation cycles scheduled according to standard water usage calculations. The vineyard was further treated as recommended for the production of export Sultanina grapes in the Lower Orange River region (Van der Merwe *et al.* 1991).

Meteorological measurements and chillings units Maximum and minimum temperatures were measured daily in the vineyard area and monthly rainfall was determined. These were used to calculate the number of Richardson chilling units (RCU) (Richardson *et al.* 1974).

Treatments In 1997, a standard RB treatment of 2.5% HC was applied at 3 weeks before normal induced BB. It was applied at a spray volume of 900 litres per hectare using pressurised backpack sprayers. Untreated vines served as a control. Canes were sampled weekly from the date of RB treatment, i.e. 3 weeks before normal induced BB until 6 weeks after BB, giving a total of 10 weekly sampling times. In 1998, an identical HC treatment was applied, except that the HC treatment was applied at 6 weeks before normal induced BB. Canes were sampled from this application as in the previous year, until 8 weeks after BB, giving a total of 15 weekly sampling times.

Experimental design and sampling The experimental design was a split plot: a two treatments (control and HC) by 10 weekly sampling times factorial (2 X 10). The sub-plot or sampling plots were one year-old canes that were sampled, separated and ZR levels

analysed. These fourteen bud long canes were separated into groups of two nodes, including the internodes taken from two positions, namely distal (synonym: top) (buds 13, 14) and proximal (synonym: bottom) (buds 1, 2). Each treatment was replicated twice. In 1997, a total of 40 canes were sampled (total for the two treatments: HC, and control, including the two repetitions) over a period of ten weeks. The distal and proximal sections were further divided into different tissues, namely buds, bark and wood. These gave a total of 40 canes X 2 position sections X 3 tissue subsections = 240 samples. Each sample was freeze-dried, ground (samples that were too small were ground by hand using a mortar and pestle, under liquid nitrogen). These 240 samples were extracted, purified and ZR levels quantified by RIA.

In 1998 the design was similar, except that the experimental plot contained an extra vine and that two canes were sampled from two vines and pooled for further analyses. There were also two extra sampling times, giving a sampling period of 12 weeks. A total of 48 canes were sampled (total for the two treatments: HC and control, including the two repetitions). Each sample was processed as in 1997, except that only buds were analysed, freeze-dried and ground (samples that were too small were ground by hand using a mortar and pestle, under liquid nitrogen). The canes were divided into distal and proximal sections and these were further divided into buds. These gave a total of 48 canes X 2 sections (distal and proximal) X 1 subsection (buds) = 96 samples. The 96 samples were extracted, purified and ZR levels quantified by RIA.

Monitoring of budbreak Buds from different grapevine varieties vary in their appearance during the transition from dormant bud to shoot and this creates problems in defining the day of BB in a uniform way (Lavee and May 1997). In Sultanina table grapes, a "green tip" appears at an early stage. This "green tip" stage 4 of the modified E-L-system coincides with the "woolly bud" stage 3 of Coombe (1995). In this study, the "green tip" stage was taken as the standard indication of bud opening for Sultanina. Budbreak was monitored at each sampling time on two canes between the monitor vines, on the same side, height and orientation of the cane to be sampled on the cytokinin-sampling vine. The week of 50% BB and total BB was determined.

Sample preparation and cytokinin determination Canes were cut off and frozen within two hours at -80 °C. The canes were subsequently separated into two sections, distal (top) and proximal (bottom) positions of the cane and three subsections: internode wood, internode bark and buds, freeze-dried and milled. Buds, too small for machine milling were hand-milled with a mortar and pestle under liquid nitrogen. Extractions were made of CKs, purified, dried down and frozen for quantifying by radioimmunoassay (RIA)

as described by Cook *et al.* (2001). Zeatin-type cytokinins (ZCK) was assayed by RIA as described by Cutting *et al.* (1991) and Cook *et al.* (2001), using monoclonal ZR specific antibodies (Eberle *et al.* 1986, De Kock *et al.* 1994) and expressed as zeatin riboside equivalents. The recovery of ZR from the different sections, i.e. wood, bark and buds, was determined as described by Cook *et al.* (1998) and was found to be 51.2% for wood, 47.9% for bark and 58.8% for the buds, respectively. The levels of ZR were expressed as ng ZR per gram dry material and adjusted for recovery.

Apparatus Samples were centrifuged on a Sorvall Refrigerated Superspeed centrifuge Model RC-5B (Du Pont Instruments), using a SS-34 rotor. Samples were dried on a Savant Speedvac Vacuum concentrator Model SVL 200, with a refrigerated condensation trap. Radioactivity was determined with a Packard Tri-Carb Model 1900 CA scintillation counter.

Statistical analyses All data on BB and ZR levels were subjected to statistical analysis by the ARC-Agrimetric division. Standard factorial analysis of variance was performed for each year separately, using GLM (General Linear Models) Procedure of SAS statistical software version 6.12 (SAS Institute Inc., Cary, NC, U.S.A.).

RESULTS

Meteorological observations (The meso climate of the Kakamas location, Lower Orange River region, 1997/98 and 1998/99)

The 1997/98 season had a cool autumn period, followed by a warm growth period until harvest in December (Figs. 1 and 2). In comparison, the 1998/99 season was characterised by a warm autumn and winter, followed by a cooler growth period until harvest.

Rainfall The corresponding total monthly rainfall figures (Fig. 2) showed variation between the two seasons, with 1997 recording lower levels than the LTA while the 1998 levels were lower than both 1997 and the LTA. Whereas very little rainfall was recorded in 1998, 1997 received rain in the first half of the year, with the second half being both dry and warm. However, the vineyards were at no stage exposed to drought stress, as they were irrigated.

Chilling units More chilling (RCU, Table 1) had been accumulated in 1997 at the time of pruning and applications of the RB treatments than in 1998. This was conducive to earlier BB in 1997 and an early harvest date, about 10 days earlier than normal. In 1998 BB was normal despite lower levels of chilling received and had a normal harvest date. According to these measurements, it is clear that in 1997 the vines received a higher number of chilling units than in 1998 during early autumn (April to May). The

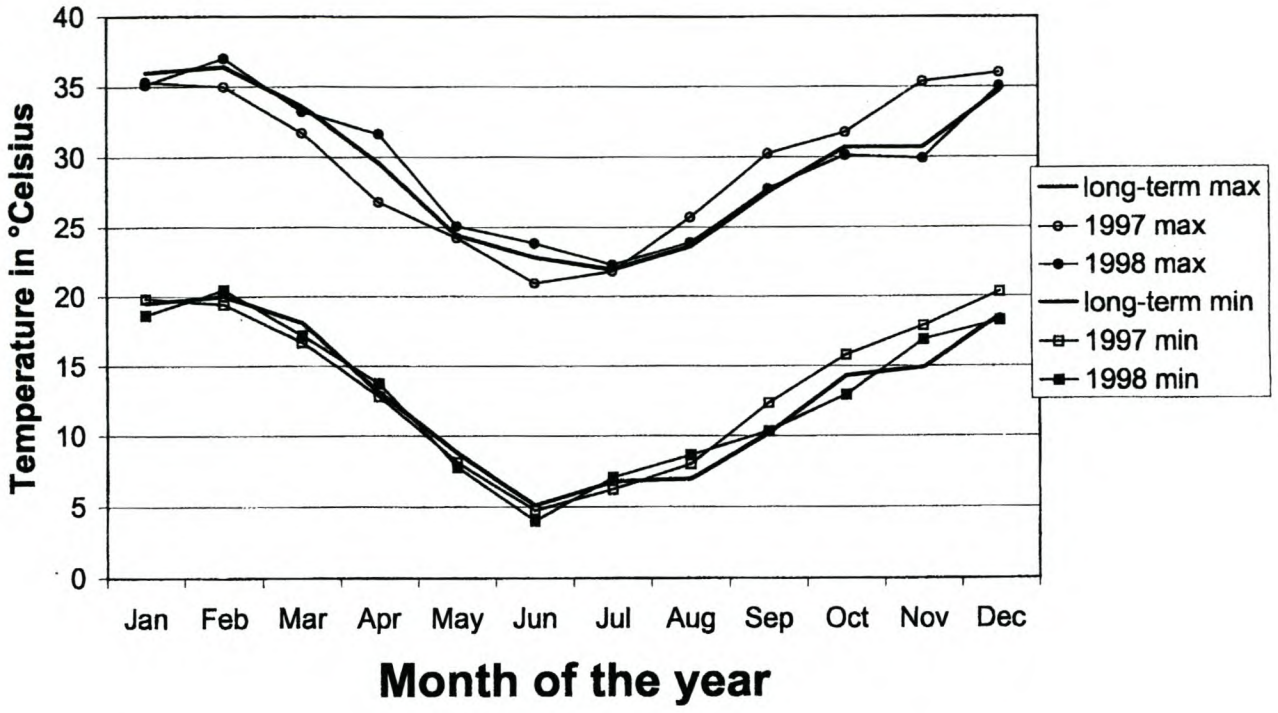

Fig. 1. Average monthly temperatures and maximum temperatures of Kakamas, South Africa, 1997 and 1998.

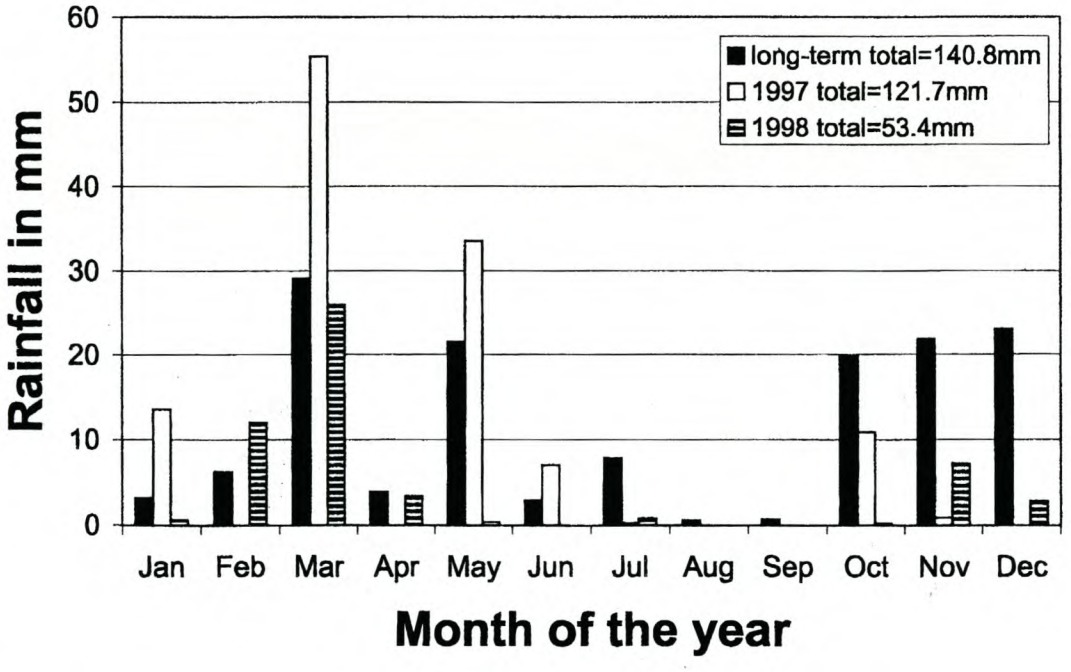

Fig. 2. Total monthly rainfall for Kakamas, South Africa, 1997 and 1998.

Table 1. Month to month accumulated Richardson Chilling Units for the period April to August, Kakamas, Lower Orange River Region, 1997 and 1998.

Month of year	Richardson Chilling Units		
	1997	1998	long-term average
April	- 162	- 548	- 506.4
May	- 115.5	- 180.5	- 228.6
June	142	78.5	15.3
July	30	- 46.5	43.6
August	-197	- 176.5	-78.8
Total positive RCU	**172**	**78.5**	**58.9**

early, warm spring weather of 1997 (from August onwards), which was warmer than 1998, then lead to rapid and even BB. The autumn of 1998 was normal, but combined with cooler weather during BB resulted in some problems with uneven and delayed BB.

Effect of hydrogen cyanamide application on ZR levels

1997 season (application of hydrogen cyanamide 3 weeks prior to budbreak)

The cumulative BB of the untreated vines compared to the treated vines is shown in Fig. 3. The vines that were treated in week 31 (28/07/1997) had a more even (steeper), pronounced BB than the control. They reached 10% BB at week 33.4 compared to the control, which reached 10% BB in week 34.1. The total BB of the treated vines was also slightly higher than the untreated vines. Both treated and untreated vines took about two weeks to reach 50% BB from 10% BB, showing that the HC treatment did not have a significant effect on the BB dynamics (Fig. 3). The free ZR levels of the buds are shown in Fig. 4 a, the internode wood in Fig 4 b and the internode bark in Fig. 4 c.

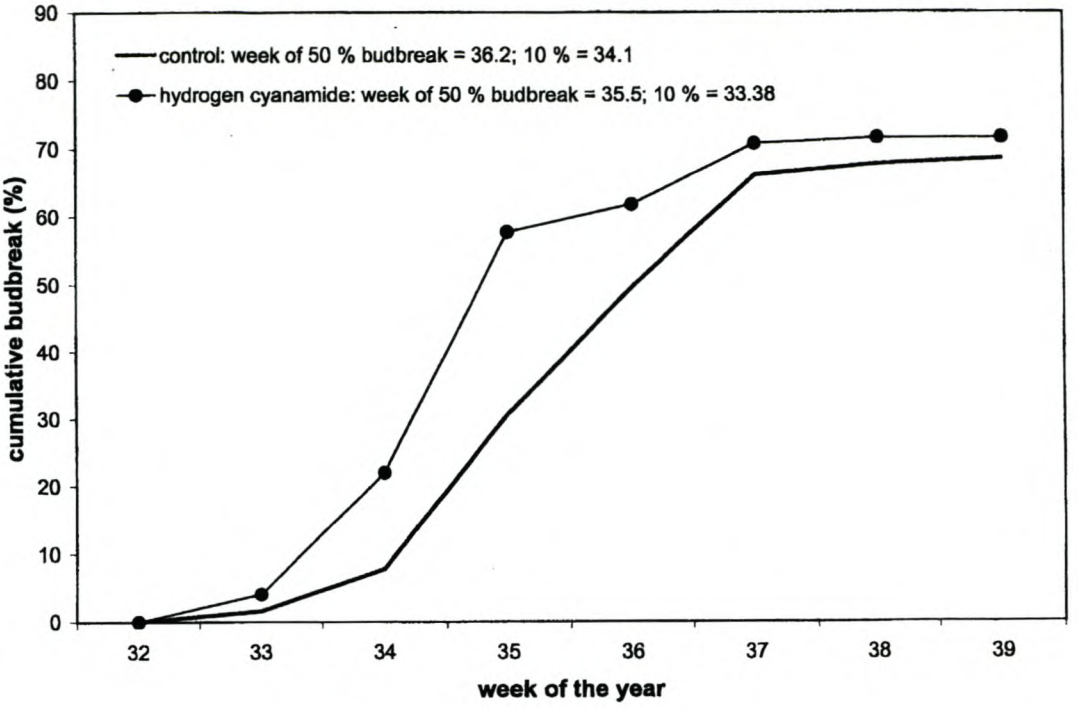

Fig. 3. Cumulative budbreak of Sultanina vines, Lower Orange River region, South Africa, 1997. Hydrogen cyanamide applied at week 31 (28/07/1997).

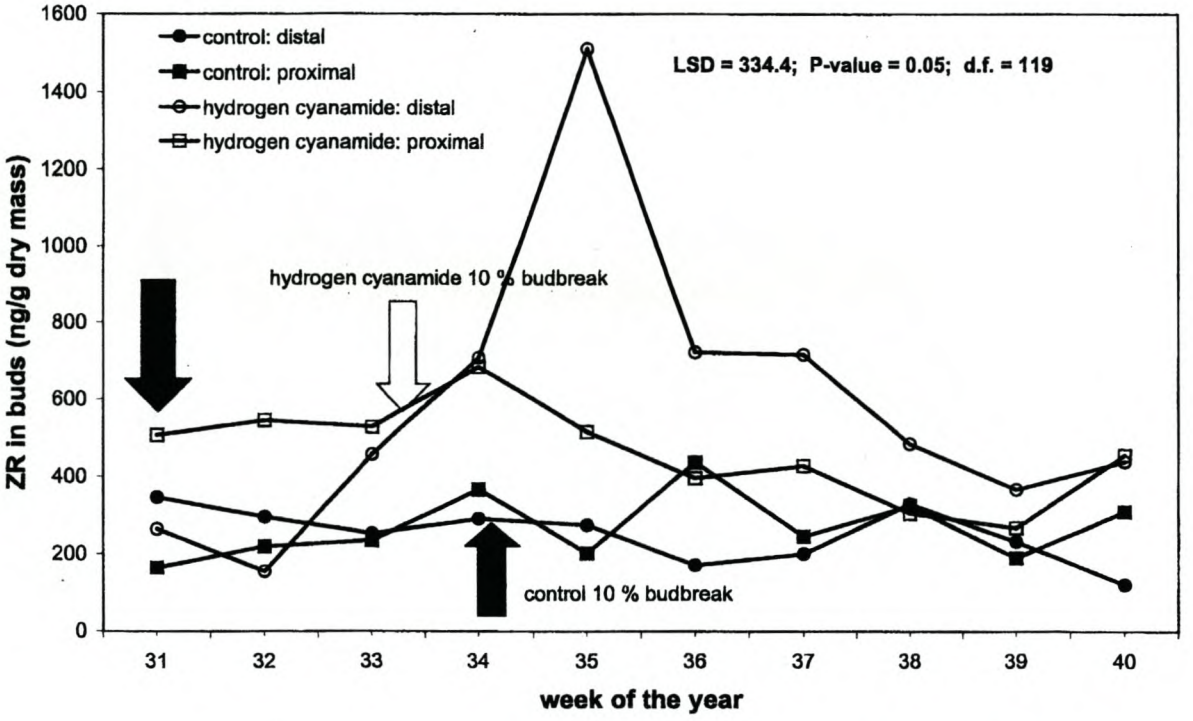

Fig. 4 a. Free zeatin riboside (ZR) levels in buds of canes of Sultanina vines, Lower Orange River region, South Africa, 1997. Hydrogen cyanamide applied at week 31 (28/07/1997) (solid arrow)

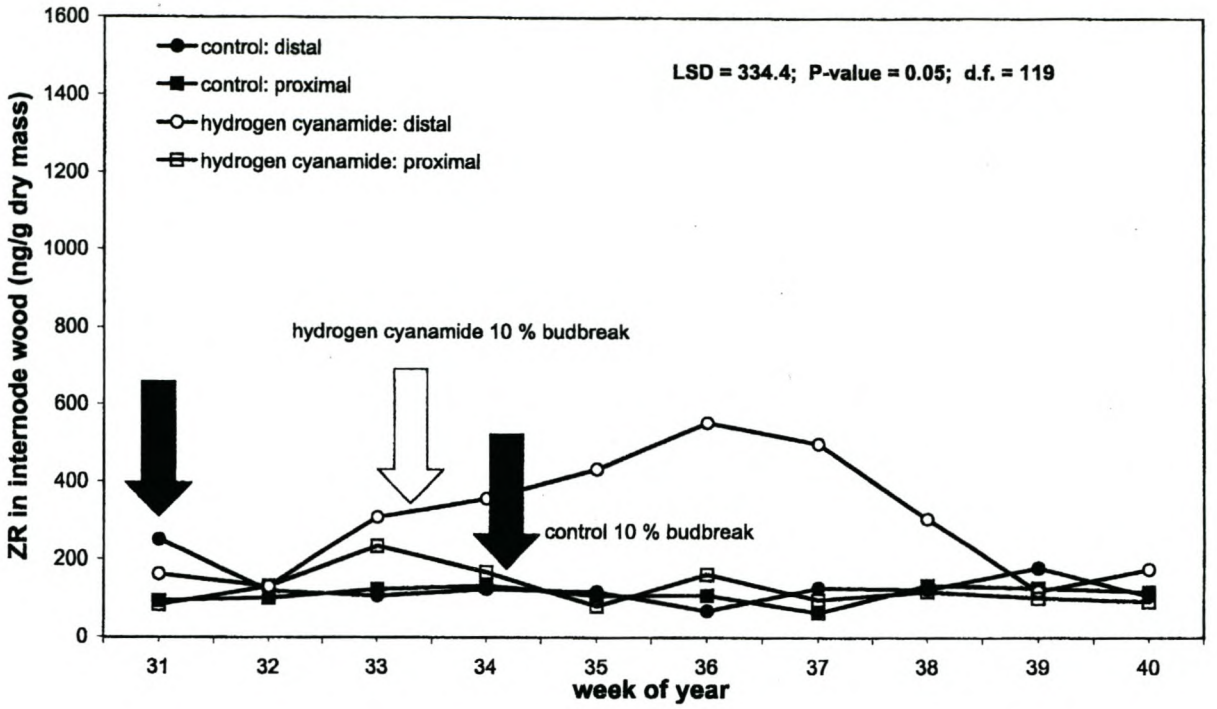

Fig. 4 b. Internode wood.

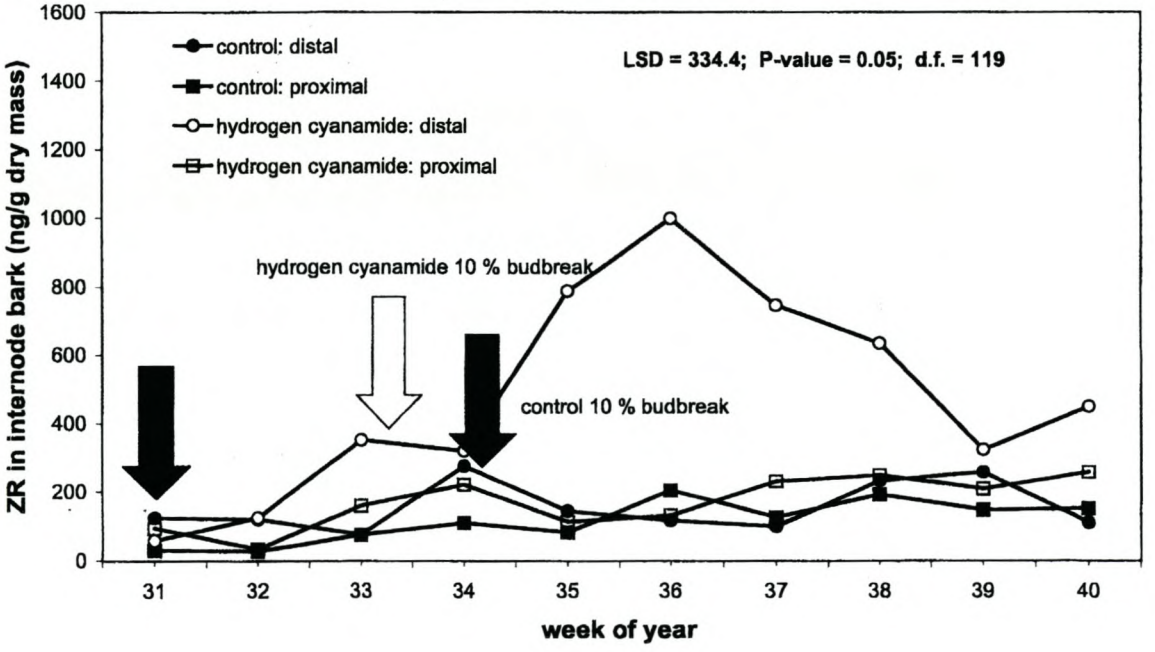

Fig. 4 c. Internode bark.

ZR levels in the buds of canes of treated vines showed a significant increase in comparison to untreated vines (Fig. 4 a). Distal buds of treated vines produced a significantly higher ZR peak than the proximal buds approximately two weeks after 10% BB was reached and four weeks after HC application. Distal and proximal buds of untreated vines did not show large ZR peaks or variation two weeks after 10% BB.

ZR levels in the internode wood of canes of treated vines showed a significant increase after HC application, while wood from untreated vines had constant low levels of ZR (Fig. 4 b). As in the buds, distal sections of treated wood produced a significantly higher ZR peak than the treated proximal wood sections. This ZR peak was also significantly higher than the untreated wood controls. This was approximately 2.5 weeks after 10% BB was reached and 5 weeks after HC was applied. Proximal and distal wood sections of untreated vines did not show much variation during the sampling period and stayed constant at low levels.

ZR levels in the internode bark of canes of treated vines showed a significant increase after HC application, while bark from untreated vines had much lower ZR levels (Fig. 4 c). As with the bud and wood tissues, the distal portions of treated canes produced a much higher ZR peak than the proximal treated portions as well as the untreated controls about two weeks after 10% BB was reached and 4 weeks after HC application. Proximal and distal portions of untreated control vines did not show any significant variation over the sampling period.

1998 season (application of hydrogen cyanamide 6 weeks prior to budbreak)

The cumulative BB of the untreated vines compared to the treated vines is shown in Fig. 5. The vines that were treated in week 26 (30/06/1998) had a much earlier BB than the control and the evenness ('steepness') was very similar to the untreated vines. The total BB was slightly higher in the untreated vines. The treated vines reached 10% BB much earlier at week 31.5 compared to the control, which reached 10% BB at week 35.6. However, treated vines took longer (approximately 6.1 weeks) to reach 50% BB from 10% BB than the controls (approximately 3.5 weeks). Only the free ZR levels in the buds were determined as shown in Fig. 6.

ZR levels in the buds of canes of treated vines showed an earlier, higher peak compared to canes of untreated vines (Fig. 6). ZR levels in the buds of untreated canes also showed an increase, but this was delayed by two to three weeks in comparison to treated vines. There was no significant difference in the bud ZR peak between the distal and proximal portions of treated canes in comparison to untreated controls at week 29, three weeks

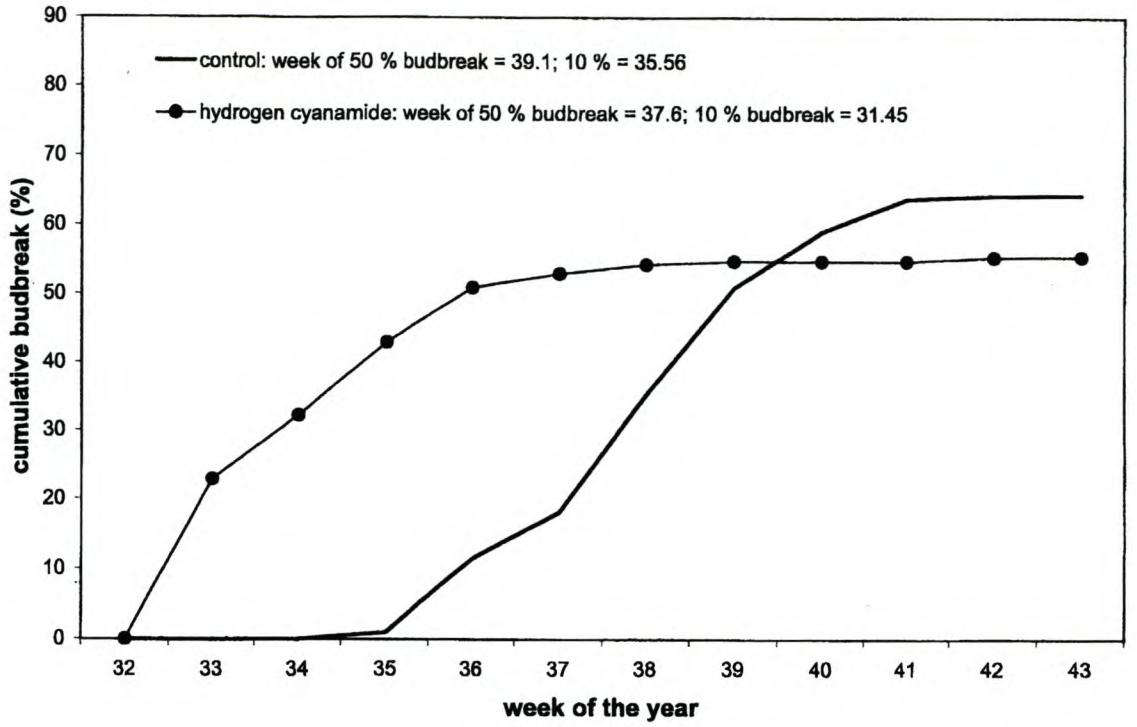

Fig. 5. Cumulative budbreak of Sultanina, Lower Orange River region, South Africa, 1998. Hydrogen cyanamide applied at week of year 26 (30/06/1998).

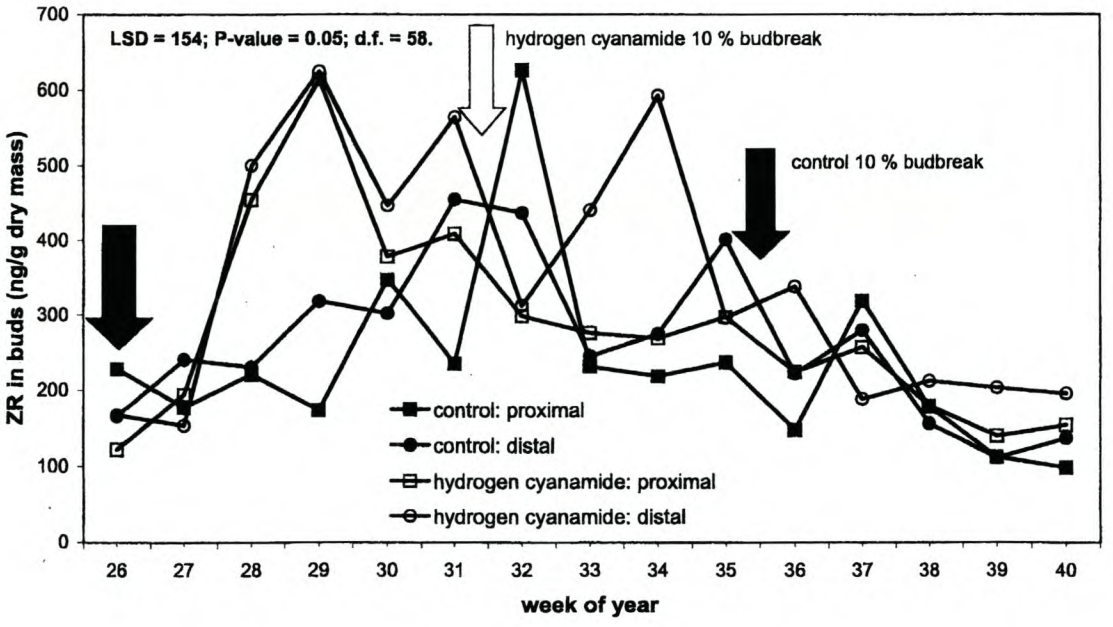

Fig. 6. Free zeatin riboside (ZR) levels in buds from the distal and proximal portions of canes of Sultanina vines, Lower Orange River region, South Africa, 1998. Hydrogen cyanamide applied at week of year 26 (30/06/1998) (solid arrow).

after HC application and about two weeks before 10% budbreak. Later, about three weeks after 10% budbreak at week 34, ZR levels in the buds of the distal portion peaked at a similar level than before, significantly higher than the proximal portion and both the untreated portions.

Some differences between the bud ZR levels of the proximal and distal portions of untreated canes were observed, although they were generally not significant. The greatest variation was seen at week 32 with proximal portion levels that were significantly higher, approximately 3.5 weeks before 10% BB. There was also a significant ZR peak in proximal buds of untreated canes at week 37 and in distal buds at week 35. Thus a similar pattern of bud ZR peaks (peaks in distal and proximal buds prior to BB and a peak in the distal bud after BB) observed in the HC treated vines were also observed in the control vines, although these peaks were delayed in comparison to the HC treated vines.

DISCUSSION

The results obtained in this study show that HC application has a profound effect on ZR levels in the tissues of Sultanina vines. However, the results obtained in these trials should be interpreted in relation to the amount of winter chilling received and the timing of the HC application.

In 1997 the vines in the trial had received a relatively high amount of chilling and HC was applied at three weeks before BB. This led to a minimal shifting of the BB pattern, compared to the untreated vines, but there was a large effect on the release of ZR. The HC treated vines showed ZR peaks in buds, internode wood and internode bark shoot tissues, whilst no peaks in the respective tissues were observed in control vines. ZR levels in the distal portions of the HC treated vines were higher than proximal portions in all the tissues analysed. The same pattern that was observed in the buds was observed in the internode wood and internode bark, although it was a little delayed in the internode wood and internode bark. It was therefore decided not to repeat the ZR determinations in wood and bark tissue in 1998, as the bud ZR levels gave a good indication of the pattern of ZR release during BB.

By contrast, in 1998 the vines in the trial had received relatively lower amounts of chilling and the HC was applied at six weeks before BB. This caused a larger effect on BB of the treated vines compared to the untreated vines, which had later BB. However, HC treated vines took twice as long to reach 50% BB from 10% BB than the untreated controls. There was also an effect on the bud ZR levels. The ZR peak shifted earlier,

before 10% BB for the HC treated vines, although there were no differences between the distal and proximal portions. However, about three weeks after 10% BB, there was a significantly higher distal portion ZR peak compared to the proximal portion of the HC treated vines. No significant differences between distal and proximal portions of controls were observed except at about six weeks before 10% BB. The bud analyses gave a good indication of ZR levels before, during and after BB.

In 1997, bud ZR peaks were observed after BB only, which could suggest that sampling may have been started too late to observe an earlier peak. In 1998, bud ZR peaks were observed before BB and again after BB. The untreated vines also showed a bud ZR peak before BB and again after BB, but these peaks were delayed in comparison to the HC treated vines. These results are comparable to those obtained by Cutting *et al.* (1991) on xylem sap ZR levels of apple shoots, who found that the application of RBA resulted in an earlier xylary ZR peak followed by BB one week thereafter. No further xylary ZR peaks were detected after BB and another increase in xylary ZR levels was only found in autumn in apples (Tromp and Ovaa 1990). However, the peaks observed in the buds after BB of HC treated vines in this study may reflect the vigourous growth in distal buds at that time.

It is likely that the increased ZR levels in the buds of HC treated vines have a profound effect on the development of inflorescence primordia and hence bunch development and ultimately production. It has been shown that the application of CKs to vine buds can increase inflorescence formation and reduce tendril formation (Srinivasan and Mullins 1978, Srinivasan and Mullins 1980). Thus, it is likely that the increase in fruit set as a result of HC application, may be explained in this way. However, the timing of the HC application is critical to the success of increased fruit production and can also have detrimental effects as is well documented (Siller-Cepeda *et al.* 1994, Or *et al.* 1999). 'Correctly' induced dormancy and subsequent BB are obviously very finely coordinated with bud and consequent bunch development.

Thus, to understand and successfully manipulate these processes, further studies into the physiological processes during this period will have to be undertaken in future.

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CHAPTER 6

THE EFFECT OF DIFFERENT PRUNING SYSTEMS ON BUDBREAK AND CYTOKININ LEVELS IN THE TABLE GRAPE CULTIVARS SULTANINA, SUNRED SEEDLESS AND ALPHONSE LAVALLEÉ

6.1 Introduction

Cytokinins (CKs) play an important role in vine cell growth and in subsequent development of flowers from inflorescence primordia during BB and fruit set (Srinivasan and Mullins 1978, Srinivasan and Mullins 1980). The question is whether a difference in Z-type CKs (ZCKs) in parts of the shoot at BB due to the genetic differences between table grape cultivars, can be the cause of variability of fruit set. These differences would then require different pruning systems or shoot lengths to ensure sufficient amounts of fruit.

To test this hypothesis, the levels of ZCKs were determined in three table grape cultivars, namely Sultanina, a traditionally cane pruned cultivar, Sunred Seedless, a traditionally half cane pruned cultivar and Alphonse Lavalleé, a traditionally spur pruned cultivar. The free xylem sap ZR levels of unpruned canes of Sultanina vines that were pruned to long cane and short spur lenghts were determined from budswell for five weeks in 1999 and for all three these cultivars from budswell for eight weeks in 2001 in order to compare these results with those obtained by Cutting *et al.* (1991) and Tromp and Ovaa (1990) on apple shoot xylem sap. No RB treatment was used with any of these studies.

In addition, canes of these three cultivars were pruned to canes and spurs for comparitive purposes and the levels of ZCKs in the tissues of canes were followed using the approach employed by Cook *et al.* (2001) on apples. One year-old canes were sampled weekly from budswell, before BB, until a number of weeks after BB. The canes were then divided into distal and proximal sections and these were further divided into bud, bark and wood tissues. Buds were subsequently processed further and the ZR levels determined. The results are presented in article format in the following section.

6.2 Manuscript: "The effect of different pruning systems on budbreak and cytokinin levels in the table grape cultivars Sultanina, Sunred Seedless and Alphonse Lavalleé"

All experimental work for the following manuscript was carried out by the main author. These included trial layout, sampling, processing and laboratory work, including RIA's.

The main author compiled the manuscript and the contributions of the co-authors were as follows:

a. Prof. Dirk U. Bellstedt, associate professor of Biochemistry at the University of Stellenbosch is the promotor of this thesis. In this capacity, he was involved in the conceptual development of and practical execution of all aspects of this study. He promoted collaboration with the Horticultural Science Department and facilitated all logistical aspects within the Department of Biochemistry. He made contributions to interpretation of the data and suggested changes to the text of the manuscript that were subsequently incorporated.

b. Dr. Nigel C. Cook, visiting lecturer of Horticultural Science at the University of Stellenbosch and Katolieke Universiteit Leuven, Belgium, is the co-promotor of this thesis. In this capacity, he was involved in the conceptual development of and practical execution of all aspects of this study. He made contributions to the presentation and interpretation of the data as well as the structure of the thesis.

"The effect of different pruning systems on budbreak and cytokinin levels in the table grape cultivars Sultanina, Sunred Seedless and Alphonse Lavalleé"

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ABSTRACT

Cytokinins play an important role in vine cell growth and in subsequent development of flowers from inflorescence primordia during budbreak and fruit set. In this study, free xylary ZR levels were determined in Sultanina in 1999 and in three table grape cultivars, Sultanina, Sunred Seedless and Alphonse Lavalleé, in 2001. ZR peaks occurred before 50% budbreak. Spur xylary ZR levels of all three cultivars followed a similar pattern, although at lower ZR levels than that of the canes. This is similar to previous studies on xylary ZR levels of apple shoots. The high levels of free ZR found in xylem sap at the distal portions of canes support the hypothesis of a cumulative ZR build-up effect as cane length increases. In addition, in 1999 all three cultivars were pruned to long canes and short spurs and sampled weekly from budswell. The zeatin riboside (ZR) levels in buds at distal and proximal positions of these one year old canes and spurs were then determined. Spur pruning resulted in earlier budbreak and a higher final budbreak than cane pruning. The proximal portions of shoots, whether spur pruned or the proximal portions of canes, showed elevated ZR levels in all cultivars. This difference in ZR levels in bud tissue of different portions of the cane would suggest a difference in ZR consumption or turnover.

ABBREVIATIONS

BB, budbreak; CK, cytokinin; LTA, longterm average; PGR, plant growth regulator; RB, rest breaking; RBA, rest breaking agent; RIA, radioimmunoassay; t-ZR, trans zeatin riboside; Z, zeatin; ZCK, zeatin-type cytokinin; ZR, zeatin riboside

KEY WORDS

zeatin riboside, radioimmunoassay, South Africa, Sultanina, Sunred Seedless, Alphonse Lavalleé, cane, half-cane, spur, table grapes, budswell, xylem sap, buds

INTRODUCTION

Increases in cytokinin levels in xylem sap before and during budbreak (BB) have been shown in a number of fruit and tree types (Faust *et al.* 1997). Studies on shoot xylem sap of mature apple trees showed that in general zeatin (Z) and zeatin riboside (ZR) accounted for more than 80% of zeatin-type cytokinins (ZCK) (Tromp and Ovaa 1990). Z was dominant over the ZR during most of the year, but ZR dominated in early spring. Zeatin-type cytokinin (ZCK) levels showed a marked rise in late winter, at budswell, before declining later in spring. Due to the fact that transpiration is still low at this stage, it was suggested that the increased levels were mainly due to mobilization of reserves or synthesis in tissue, e.g. bark or cambium (Skene 1972), rather than root supply. Whether this suggests that ZR (in a storage form of, for example glucoside or nucleotide) is the main storage form of cytokinin (CK) or it is formed by *de novo* synthesis in the bark or cambium is unclear. Cytokinin concentration in xylem sap started to increase in late winter, before visible budswell and it is suggested that this increase marks the end of winter dormancy (endodormancy, Lang *et al.* 1987). This rise in xylem CK levels before BB was also observed in other deciduous trees. In studies on poplars, it was observed that CK levels in buds of excised stems in late winter increased prior to BB independently of a root system (Skene 1975). The CK levels in xylem sap peaked three weeks before levels peaked in the buds themselves, suggesting accumulation from the stem.

Cook *et al.* (2001) demonstrated a ZR increase in the bark and buds of rootless apple (Granny Smith) shoots as growth resumed after chilling and forcing. This supported the hypothesis that shoot-derived, rather than root-derived CKs act to trigger BB. Furthermore, higher ZR peaks were observed in the distal shoot portions relative to the proximal shoot portions. Cook *et al.* (1998) observed differences in the ZR levels between distal and proximal portions of plum shoots before BB.

A number of studies have been initiated locally on a number of diverse plants to determine ZR levels associated with various metabolic states, including BB. Plants

studied included fynbos shrubs (De Kock *et al.* 1994) and plums (Cook *et al.* 1998), with most work being done on apples (Cutting *et al.* 1991, Cook *et al.* 2001). The work by Cutting *et al.* (1991) in which xylem sap ZR levels were correlated with HC treated and untreated apple shoots, showed that the ZR peak of treated shoots was moved forward by three weeks ahead of the control peak and both treated and untreated control ZR levels peaked approximately one week before BB. However, similar studies on ZCK levels of different table grape cultivars and pruning systems have not been undertaken.

In South Africa, various pruning systems are used for table grape cultivars, namely spur (2-3 buds), half-cane (6-8 buds) and cane (14-16 buds) systems, depending on the cultivar and region. The reason for a specific pruning system can be necessitated by a variety of factors: genetic factors, in that the specific cultivar is unfruitful, or bears fruit on buds distal on the cane (as in Sultanina) or very fruitful, when spur pruning is adequate (Alphonse Lavalleé). The trellis system to be used also influences the pruning system and this can be gable, factory or double gable systems. The pruning system can also be used to control excessive vigour of vines, to thus create more balanced growth and as a result, better quality fruit. Cytokinins have been shown to play an important role in development of flowers from inflorescence primordia during BB and during fruit set (Srinivasan and Mullins 1978, Srinivasan and Mullins 1980). This study was initiated with a view to determine whether differences in xylem sap ZCK levels during BB could be correlated with the fruitfulness of the cultivar. Sultanina and Sunred Seedless are both seedless cultivars, with Sultanina quite unfruitful (Whiting and Coombe 1984, Dry and Gregory 1988, Van der Merwe *et al.* 1991), requiring cane pruning and Sunred Seedless fruitful, requiring spur or half-cane pruning (Van der Merwe *et al.* 1991). Alphonse Lavalleé is a seeded cultivar that is highly fruitful, requiring only spur pruning. Thus ZCK levels in xylem sap and buds prior to and during BB were determined in the cultivars Sultanina, Sunred Seedless and Alphonse Lavalleé as representatives of the three pruning types. In view of the fact that cane length has a profound effect on fruitfulness, ZCK levels in the xylem sap and buds of distal and proximal sections of the cane, as well as in spurs, were investigated in all three cultivars.

MATERIALS AND METHODS

Vineyard Trials were conducted in three different table grape cultivar vineyards in close proximity to one another on the same commercial farm in the Wellington area of the Berg River Valley region, South Africa. These were a traditional cane-pruned cultivar Sultanina (synonyms Sultana, Thompson Seedless), traditional half-cane pruned cultivar Sunred Seedless and a traditional spur-pruned cultivar Alphonse Lavalleé (synonym Ribier). All three cultivars were grafted onto Ramsey rootstock and trained to 4 m X 2 m

gable trellises. To facilitate comparison in ZR levels between the cultivars, they were all pruned to 12 canes with 14 buds and 12 spurs with 3 buds respectively. The longer canes supply the current season's fruiting branches, while the shorter spurs are left to provide the next seasons fruiting branches. All vineyards were irrigated using drip systems with irrigation cycles scheduled using standard water usage calculations to ensure that the vines were not subjected to any water stress. Vineyards were further treated as recommended for the production of export quality grapes in the Berg River Valley region of South Africa (Van der Merwe *et al.* 1991).

Meteorological measurements and chillings units Maximum and minimum temperatures were measured daily in the vineyard area and monthly rainfall was determined. These were used to calculate the number of Richardson chilling units (RCU) (Richardson *et al.* 1974).

Trials

Cytokinin levels in the free xylem sap of unpruned canes of different cultivars This trial was initiated in 1999 on the Sultanina vineyard to determine the levels of free xylem ZR levels at different positions on unpruned one year old Sultanina canes. Vines to be sampled were not pruned at all until sampling and no chemical RB treatments were applied. Sampling was performed weekly from budswell for five weeks (five total sampling times). A 14 bud (long) and a 3 bud (spur) cane was then pruned at the same relative height and position per sampling vine and the free flow xylem sap of each immediately collected (one sampling vine per sampling time). A combined total of 40 (20 cane and 20 spur) free xylem sap samples were collected over five weeks of sampling, including four replicates. To compare the free xylem sap ZR levels of the different cultivars with the pruning trial, this trial was repeated in 2001 on the same Sultanina vineyard, as well as vineyards of the cultivars Alphonse Lavalleé and Sunred Seedless. To ensure that all possible ZR peaks could be detected, weekly samples were taken from just before budswell for eight weeks (eight total sampling times). Thus, a combined total of 144 (72 cane and 72 spur) samples were collected over eight weeks of sampling from all three cultivars. These included three replicates. The samples were frozen at –80 °C until further analyses. The trial was laid out as a completely randomised block design in 1999 and 2001, with the cane and spur-pruned unpruned shoots as sub-plots.

The effect of pruning method on tissue cytokinin levels during budbreak of different cultivars This trial was initiated in 1999 to determine the effect of long and spur pruning on ZCK levels of different cultivars, as well as the genetic effect of seedless and seeded cultivars on these ZCK levels during BB. The cultivars and vineyards

were as described in the "Vineyard" section above. The trial was laid out as a randomised block design, with the sampled canes and spurs as sub-plots. One year old shoots of the vines were pruned as follows one month before sampling: two 14 bud (long) canes and two 3 bud (spur) canes were pruned at the same relative height and position of each sampling vine. No chemical RB treatments were applied. The duplicate canes and spurs were sampled on a weekly basis from budswell for six weeks to give a total of six sampling times. The treatments were replicated four times. A total of 24 canes and 24 spurs were sampled per cultivar (including four repetitions) over the six sampling times (a total of 72 canes and 72 spurs for all three cultivars) and samples were frozen at –80 °C.

Radioimmunoassay of cytokinins

Free xylem sap (unpruned shoot trial) Xylem sap was frozen at –80 °C as soon as possible after sampling. The levels of free ZR were determined by means of radioimmunoassay (RIA), without any additional cleaning up steps. Zeatin-type cytokinins (ZCK) was assayed by RIA as described by Cutting *et al.* (1991) and Cook *et al.* (2001), using monoclonal ZR specific antibodies (Eberle *et al.* 1986, De Kock *et al.* 1994) and expressed as zeatin riboside equivalents. The levels of ZR were expressed as ng ZR per 100 µl free bleeding xylem sap.

Pruning system (cane versus spur trial) Duplicate cane and spur samples were pooled. The canes were separated into two sections (distal and proximal), while the spur was sampled as one section. The sections were further separated into one subsection, namely buds, giving a total of [(1 cane X 2 sections X 1 subsection) + (1 spur X 1 section X 1 subsection)] X 6 sample times X 4 repititions = 72 samples per cultivar (a total of 216 samples for all three cultivars). These were then freeze-dried and milled. The buds were hand-milled with a mortar and pestle under liquid nitrogen. Extractions were made of CKs, purified, dried down and frozen for quantifying by RIA as described by Cook *et al.* (2001). Zeatin-type cytokinins (ZCK) was assayed by RIA as described by Cutting *et al.* (1991) and Cook *et al.* (2001), using monoclonal ZR specific antibodies (Eberle *et al.* 1986, De Kock *et al.* 1994) and expressed as zeatin riboside equivalents. The percentage recovery of the buds was determined as decribed by Cook *et al.* (1998) and gave a recovery of 58.8%. The levels of ZR were expressed as ng ZR per gram dry material and adjusted for recovery.

Apparatus Centrifugation was done on a Sorvall Refrigerated Superspeed centrifuge Model RC-5B (Du Pont Instruments), using a SS-34 rotor. Samples were dried on a Savant Speedvac Vacuum concentrator Model SVL 200, with a refrigerated

condensation trap. Radioactivity was determined with a Packard Tri-Carb Model 1900 CA scintillation counter.

Monitoring of budbreak Buds from different grapevine varieties vary in their appearance during the transition from dormant bud to shoot and this creates problems in defining the day of BB in a uniform way (Lavee and May 1997). A uniform and generally measurable protocol for standardising the visual sign of bud opening, as well as the environmental conditions of bud opening, is essential for meaningful comparisons of research results. In Sultanina table grapes, a "green tip" appears at an early stage. This "green tip" stage 4 of the modified E-L-system coincides with the "woolly bud" stage 3 of Coombe (1995). In this study, the "green tip" stage was taken as the standard indication of bud opening for all three cultivars. The week of 50% BB and total BB was determined.

Free xylem sap trial Unpruned monitor canes were monitored in each vineyard at each sampling time to determine the natural BB of the different cultivars.

Pruning system trial Budbreak was monitored at each sampling time on two canes and two spurs between the monitor vines, on the same side, height and orientation of the cane to be sampled on the cytokinin-sampling vine.

Statistical analyses All data on BB and ZR levels was subjected to statistical analysis by the ARC Agrimetric division. Standard factorial analysis of variance was performed for each cultivar separately, using the GLM (General Linear Models) Procedure of SAS statistical software version 6.12 (SAS Institute Inc., Cary, NC, U.S.A.).

RESULTS

Meteorological Observations (The meso climate of the Wellington area, Berg River Valley Valley region in 1998, 1999 and 2000, 2001)

The 1998 season was characterised by a warm autumn and normal winter, followed by a warm spring growth period until harvest (Figs. 1 and 2). In comparison, the 1999 season was much warmer than average throughout the year, with just September in spring being cooler than average, followed by a warm growth period until harvest. The 2000 season was a warmer than average season, especially during the winter months, with a normal spring and summer period. The temperatures, however, varied considerably lot with high maximums and low minimums recorded during winter. The 2001 season was a more average season with average temperatures recorded, slightly lower than the long-term average.

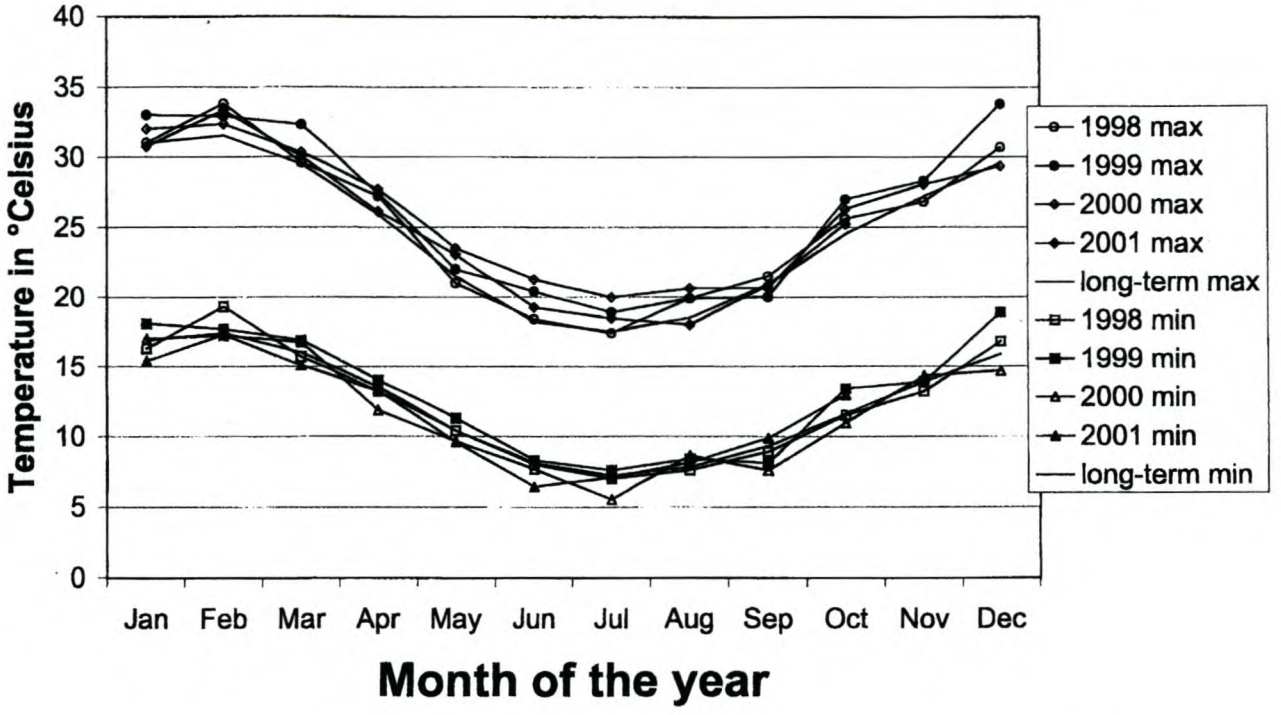

Fig. 1. Average monthly minimum and maximum temperatures of Wellington, South Africa, 1998 to October 2001.

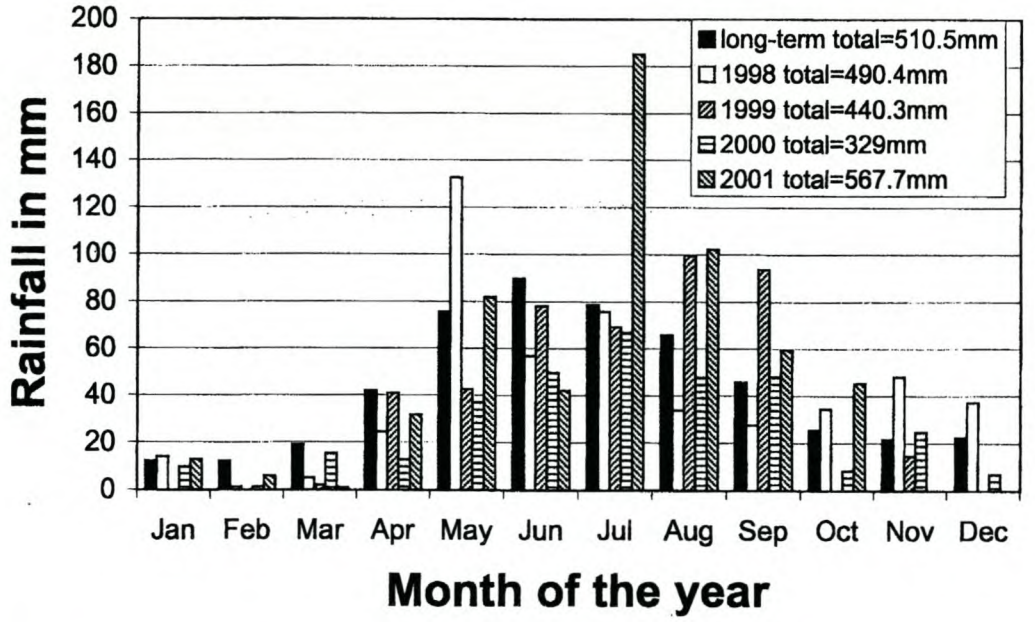

Fig. 2. Total monthly rainfall for Wellington, South Africa, 1998 to October 2001.

Rainfall The corresponding total monthly rainfall figures (Fig. 2) showed variation between the seasons, with 1999 recording lower levels than in 1998, but both years were much lower than the LTA. The time of rainfall also differed a bit between the years, with 1998 receiving higher than average rainfall in autumn and then again just before harvest, while 1999 received higher than average rainfall in spring and very low levels in summer before harvest. The 2000 season was dry, with much lower than average rainfall. In contrast, the 2001 season was very wet, with record rainfall levels recorded in July and August and an above-average total.

Chilling units The accumulation of RCU is shown in Table 1. In 1998, the vines had accumulated much more chilling at the time of pruning and applications of the RB treatments than in 1999, which was also much lower than the LTA. The accumulation of RCU was also later than the LTA, with units still being accumulated in September, which had an effect on the quality of BB. All this was conducive to normal BB in 1998, whereas 1999 required application of RB agents to ensure even BB. The 2000 season was an average season regarding RCU accumulation, while the 2001 season was exceptional, with much higher than average RCU recorded. The high chilling levels of 2001 resulted in even, strong BB of the vines.

Cytokinin levels in the free xylem sap of unpruned canes of different cultivars

Sultanina: 1999 The ZR levels of free bleeding xylem sap of cane (long) and spur (short) sampled unpruned Sultanina shoots from budswell until after BB in 1999 is presented in Fig. 3. A significant increase in xylary ZR levels (11.5 ng ZR per 100 µl free bleeding xylem sap) was observed in the cane pruned shoots at 0.5 weeks before 50% BB. A significant yet lower ZR peak (6.4 ng ZR per 100 µl free bleeding xylem sap), approximately half of the cane pruned shoots' xylary ZR maximum, was observed in the spur pruned shoots 0.5 weeks after 50% BB.

Sultanina: 2001 The free ZR levels of free bleeding xylem sap of cane and spur-sampled unpruned Sultanina shoots from before budswell until after BB in 2001 are presented in Fig. 4 a. A significant ZR peak (13.1 ng ZR per 100 µl free bleeding xylem sap) was observed with the cane pruned shoots at week of year 34.3, 19 days (2.5 weeks) before 50% BB of unpruned shoots. Lower ZR peaks were observed for the spur pruned shoots at both week of year 31.4 (6.4 ng ZR per 100 µl free bleeding xylem sap) and 35.3 (6.9 ng ZR per 100 µl free bleeding xylem sap), approximately 12 days before 50% BB of unpruned shoots. Compared to the 1999 Sultanina results (Fig. 3), the 2001 results show a similar pattern of ZR levels in cane and spur pruned shoots.

Table 1. Month to month accumulated Richardson Chilling Units (Richardson *et al.* 1974) for the period April to September, Wellington, Berg River Valley Region, South Africa, 1998-2001.

Month of year	Accumulated Richardson Chilling Units				
	1998	1999	2000	2001	long-term average
April	- 449	- 458	- 368.5	- 402	- 424.4
May	- 152	-231.5	- 137	-51	-159.4
June	76.5	- 6	88.5	169	85.3
July	187.5	83.5	212.5	211.5	162.2
August	52	23.5	11	169.5	89
September	-80	11	56.5	- 46.5	- 80
Total positive RCU	316	118	368.5	550	336.5

Sunred Seedless: 2001 The free ZR levels of free bleeding xylem sap of cane and spur-pruned Sunred Seedless shoots from before budswell until after BB in 2001 are presented in Fig. 4 b. A significant xylary ZR peak (8.4 ng ZR per 100 µl free bleeding xylem sap) was observed in cane pruned shoots at week of year 33.7, two weeks before 50% BB. The ZR levels of spur pruned shoots increased at lower levels than the cane pruned shoots to peak (3.7 ng ZR per 100 µl free bleeding xylem sap) five days after 50% BB, at week of year 36.4.

Alphonse Lavalleé: 2001 The free ZR levels of free bleeding xylem sap of cane and spur-pruned Alphonse Lavalleé shoots from before budswell until after BB in 2001

are presented in Fig. 4 c. A significant xylary ZR peak (13.7 ng ZR per 100 µl free bleeding xylem sap) was observed in cane pruned shoots at week of year 36.4, approximately two weeks before 50% BB. The ZR levels of spur pruned shoots increased steadily, at lower levels than the cane pruned shoots, to reach a maximum (4.9 ng ZR per 100 µl free bleeding xylem sap) at week of year 37.4, approximately a week before 50% BB, when sampling was discontinued. The ZR levels of the canes were again significantly higher than the ZR levels of the spurs, from a week after budswell until just before 50% BB at week of the year 38.3.

Alphonse Lavalleé had the highest ZR peak (13.7 ng ZR per 100 µl xylem sap), followed by Sultanina (13.1 ng ZR per 100 µl xylem sap) and Sunred Seedless (8.4 ng ZR per 100 µl xylem sap). Both Sultanina and Alphonse Lavalleé had significantly higher peaks than Sunred Seedless. All these peaks were recorded with cane pruned shoots.

Comparisons between cultivars

All three cultivars showed the same pattern of xylary ZR peaks, i.e. a significant peak in cane pruned xylary ZR levels prior to BB and a much reduced xylary ZR peak in spur pruned vines.

The effect of pruning method on tissue cytokinin levels during budbreak of different cultivars

Sultanina The BB of the canes and spurs is presented in Fig. 5 a. Spurs had more rapid, even BB, reached 50% BB a week earlier and had a higher final BB than the canes. The graphs of the bud ZR levels of spurs and canes are presented in Fig. 5 b. A significant increase in bud ZR levels (726.3 ng ZR per g dry bud mass) from basal levels was observed in the spurs at week 39, approximately 3.5 weeks after spur 50% BB. A similar significant ZR peak (666 ng ZR per g dry bud mass), just lower than the highest level of the spurs was also observed in the proximal cane portions at week 39, approximately 2.5 weeks after cane 50% BB. The distal cane portions showed a tendency to increase in ZR levels from 217.7 ng ZR per g dry bud mass at week 35 to a high of 430.1 ng ZR per g dry bud mass at week 40, although this increase was not significant.

Sunred Seedless The BB of the canes and spurs is presented in Fig. 6 a. Spurs had more rapid, even BB, reached 50% BB a week earlier and had a higher final BB than the canes. The ZR graphs of the buds of spurs and canes are presented in Fig. 6 b. A significant increase (compared to both the distal and proximal cane portions) in ZR levels (526.4 ng ZR per g dry bud mass) was observed in the spur at week 36,

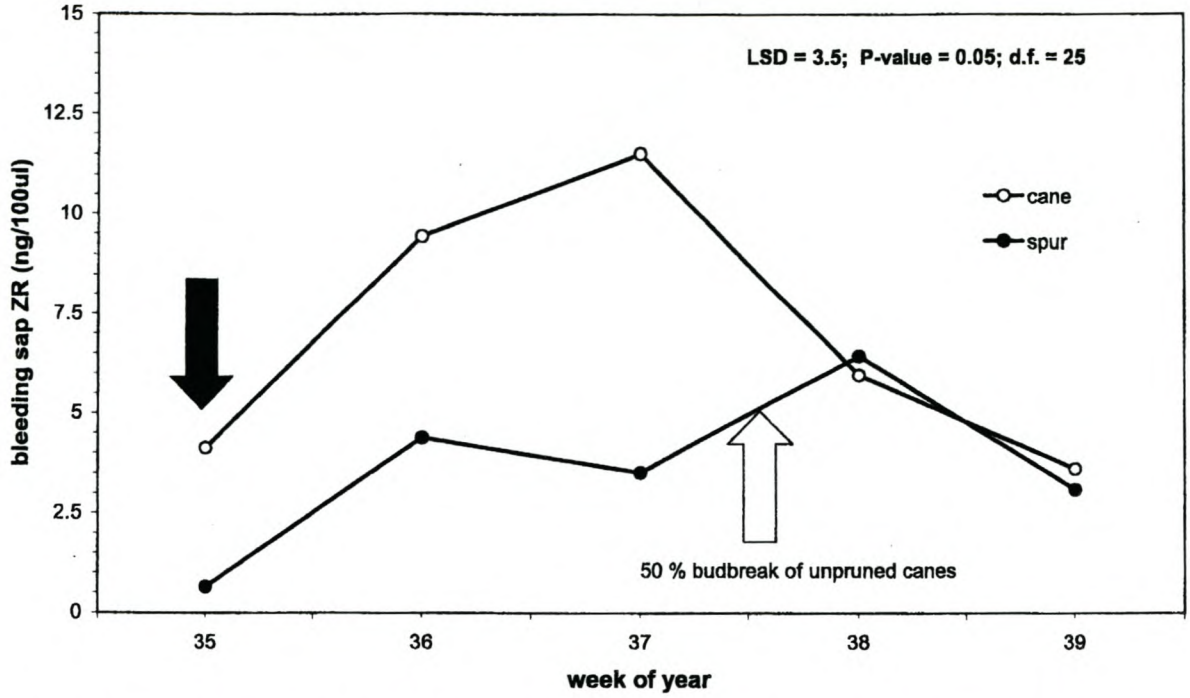

Fig. 3. Free bleeding xylem sap zeatin riboside (ZR) levels of freshly-pruned spurs and canes, sampled from budswell (solid arrow) of Sultanina vines, Wellington, South Africa, 1999. Week of year 50% budbreak = 37.6.

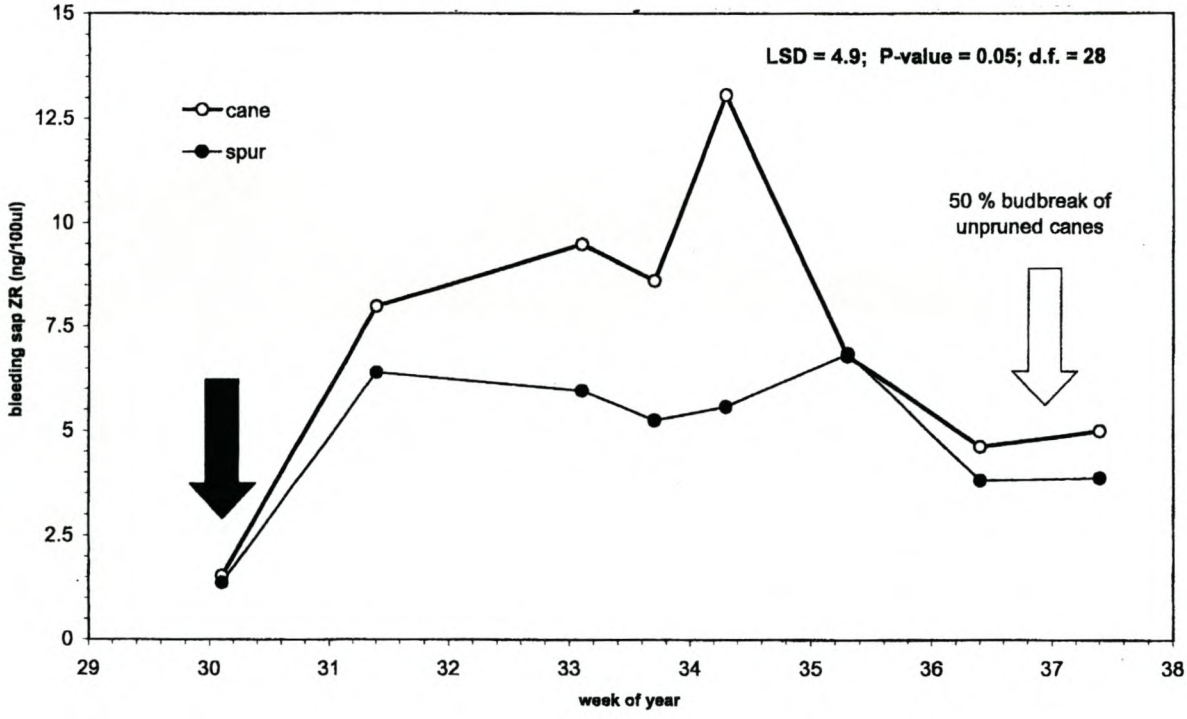

Fig. 4 a. Free bleeding xylem sap zeatin riboside (ZR) levels of freshly-pruned spurs and canes, sampled from before budswell (solid arrow) of Sultanina vines, Wellington, South Africa, 2001. Week of year 50% budbreak = 37.

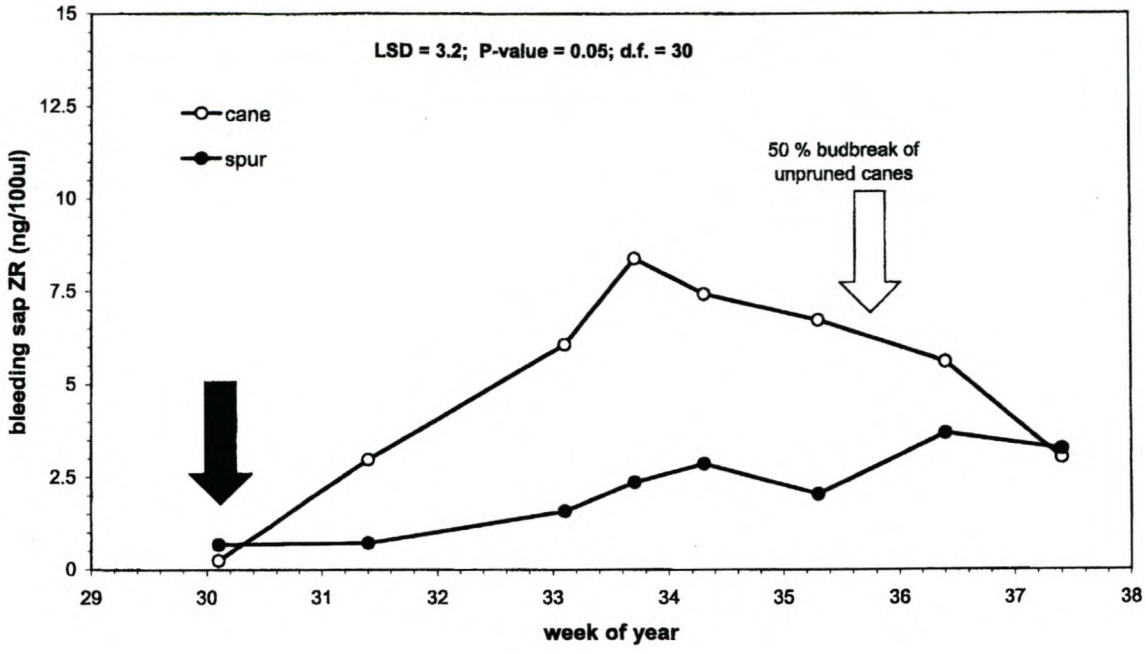

Fig. 4 b. Free bleeding xylem sap zeatin riboside (ZR) levels of freshly-pruned spurs and canes, sampled from before budswell (solid arrow) of Sunred Seedless vines, Wellington, South Africa, 2001. Week of year 50% budbreak = 35.7.

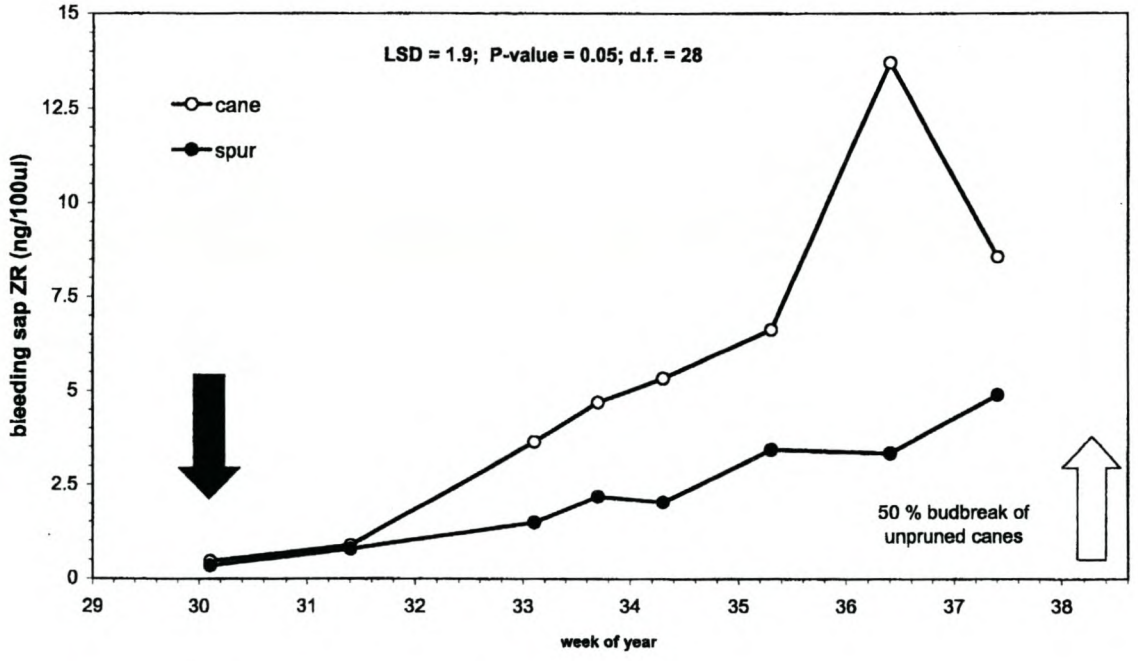

Fig. 4 c. Free bleeding xylem sap zeatin riboside (ZR) levels of freshly-pruned spurs and canes, sampled from before budswell (solid arrow) of Alphonse Lavalleé vines, Wellington, South Africa, 2001. Week of year 50% budbreak = 38.3.

approximately 0.5 weeks after 50% BB. The ZR levels of both proximal and distal cane portions fluctuated non-significantly between approximately 200 and 350 ng ZR per g dry bud mass in this period.

Alphonse Lavalleé The BB of the canes and spurs is presented in Fig. 7 a. Spurs showed more rapid, even BB and reached 50% BB two weeks before the canes and had a higher final BB. The ZR level graphs of buds of spurs and canes are presented in Fig. 7 b. A significant decrease in ZR levels (1496.0 ng ZR per g dry bud mass) was observed in spurs from week 35 to week 36, approximately 0.5 weeks before 50% BB. A second lower but significant ZR peak (817.2 ng ZR per g dry bud mass) was observed at week 38, about 2.5 weeks after 50% BB. A significant decrease in bud ZR levels (694 ng ZR per g dry bud mass) was observed in proximal portions of canes at week 35, approximately 2.5 weeks before 50% BB. A second significant ZR peak (770.9 ng ZR per g dry bud mass) was observed at week 37. A significant increase in bud ZR levels (1021.3 ng ZR per g dry bud mass) was observed in the proximal cane portion at week 39, approximately 1.5 weeks after 50% BB. The ZR levels of the distal cane portions remained relatively constant throughout the sampling period at levels of approximately 350 ng ZR per gram dry bud mass. At week 35, the bud ZR levels in spurs differed significantly from the distal and proximal canes. At week 39, the bud ZR levels in the proximal canes differed significantly from the spurs and distal canes.

Comparisons between cultivars

All three cultivars showed earlier, more even BB when pruned to short spurs as opposed to longer canes. Pruning to spur length of all three cultivars resulted in a significant increase in bud ZR levels after BB. A significant peak in bud ZR levels in proximal canes was found in Sultanina and Alphonse Lavalleé after BB. A similar but non-significant bud ZR peak was observed in Sunred Seedless after BB. Bud ZR levels did not vary significantly in the distal portions of pruned canes of all three cultivars.

DISCUSSION

Sultanina in 1999 and all three cultivars in 2001 showed similar xylary ZR peaks in that cane pruned canes had the highest ZR peaks and that these peaks occurred before 50% BB. Xylary ZR levels of spurs of all three cultivars followed a similar pattern, although at lower ZR levels than the canes. Spur pruned canes did show a xylary ZR peak, although at lower levels and after 50% BB. However, in the pruning system trial, the distal positions' buds themselves had lower ZR levels. This could be due to active growth in the vines. These peaks consistently occurred at 2 to 2.5 weeks before 50% BB. These are

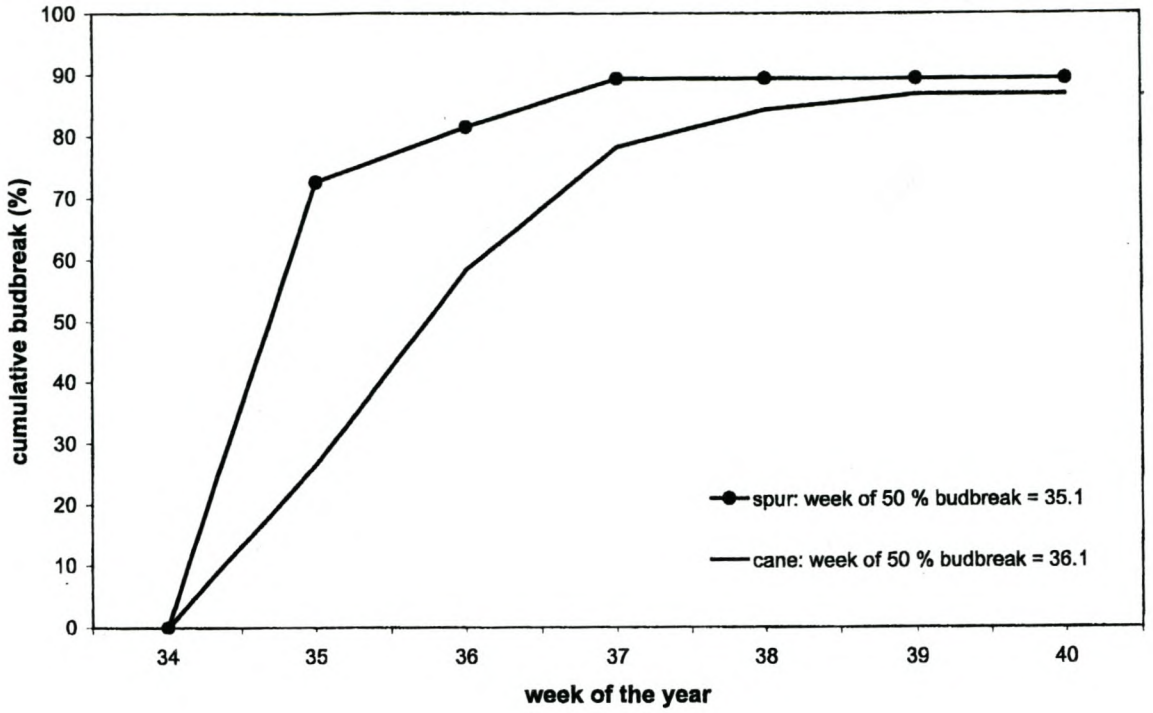

Fig. 5 a. Cumulative budbreak of Sultanina vines, Wellington, South Africa, 1999. Start week of year 35 (25/08/1999). Week of 50% budbreak = 35.6 (combined spur and cane).

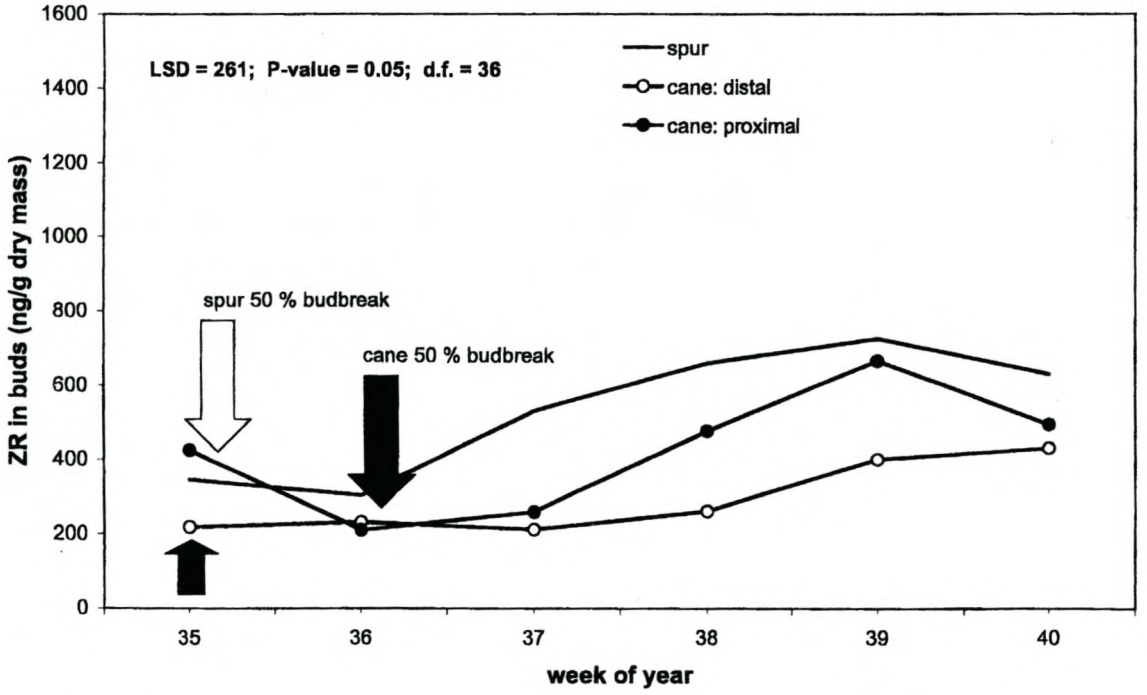

Fig. 5 b. Free zeatin riboside (ZR) levels in buds of spurs and canes of Sultanina vines measured from budswell (solid arrow), Wellington, South Africa, 1999.

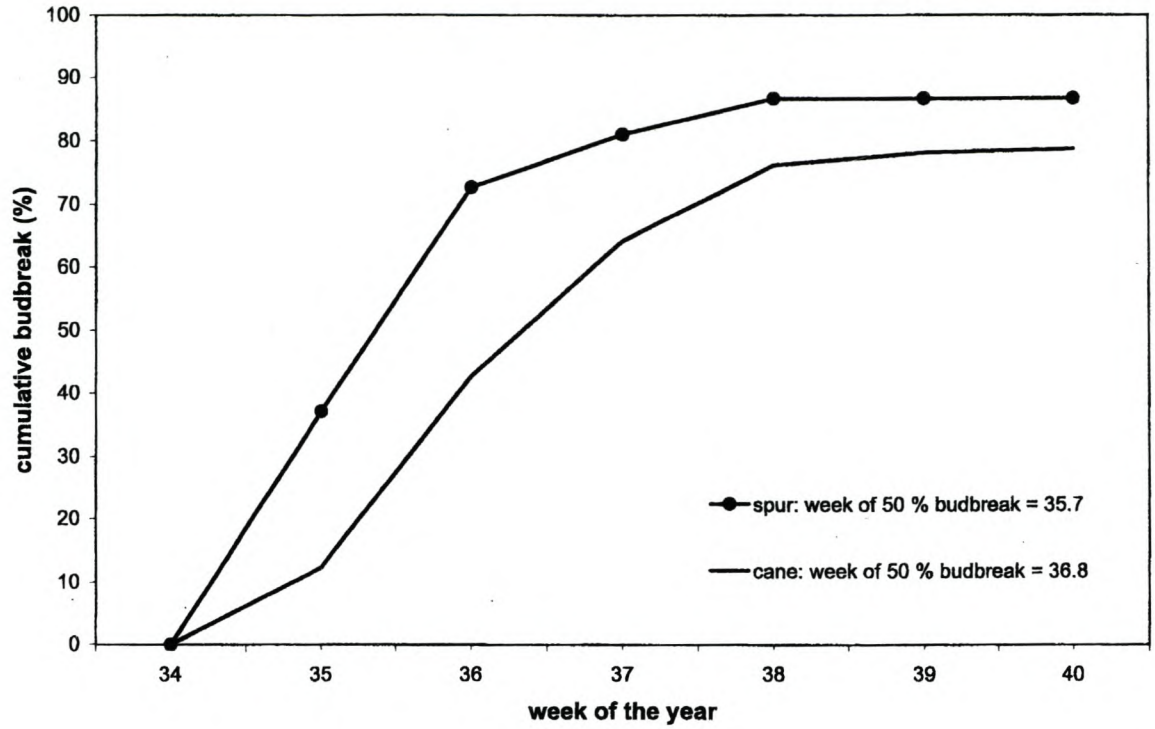

Fig. 6 a. Cumulative budbreak of Sunred Seedless vines, Wellington, South Africa, 1999. Start week of year 35 (22/08/1999). Week of 50% budbreak = 36.3 (combined spur and cane).

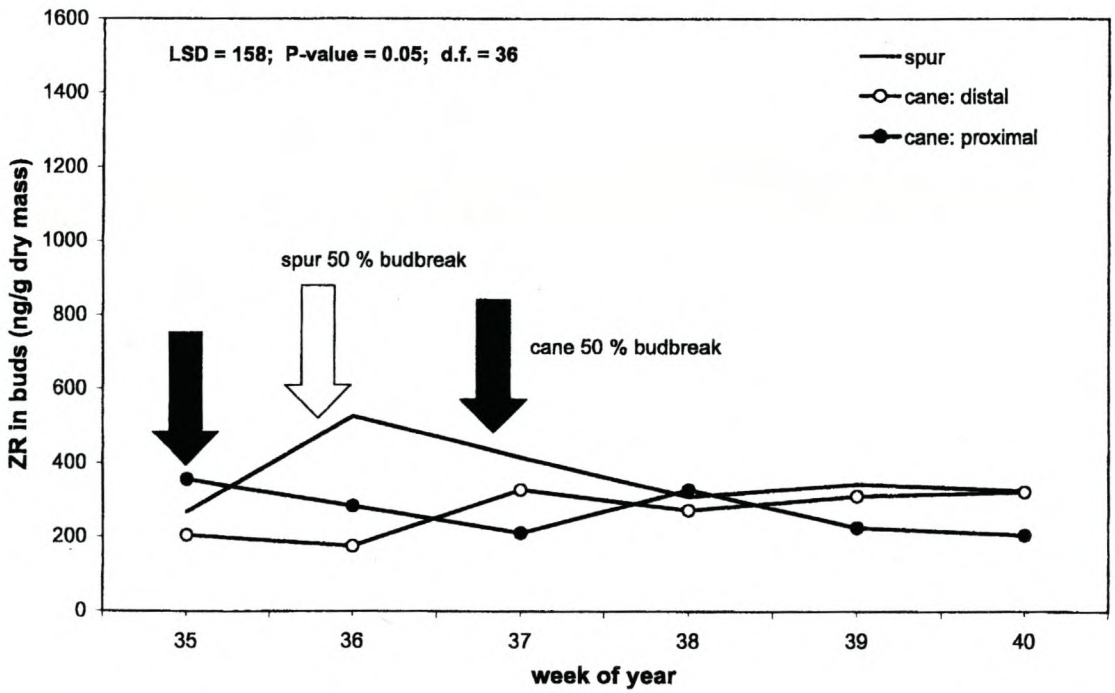

Fig. 6 b. Free zeatin riboside (ZR) levels in buds of spurs and canes of Sunred Seedless vines measured from budswell (solid arrow), Wellington, South Africa, 1999.

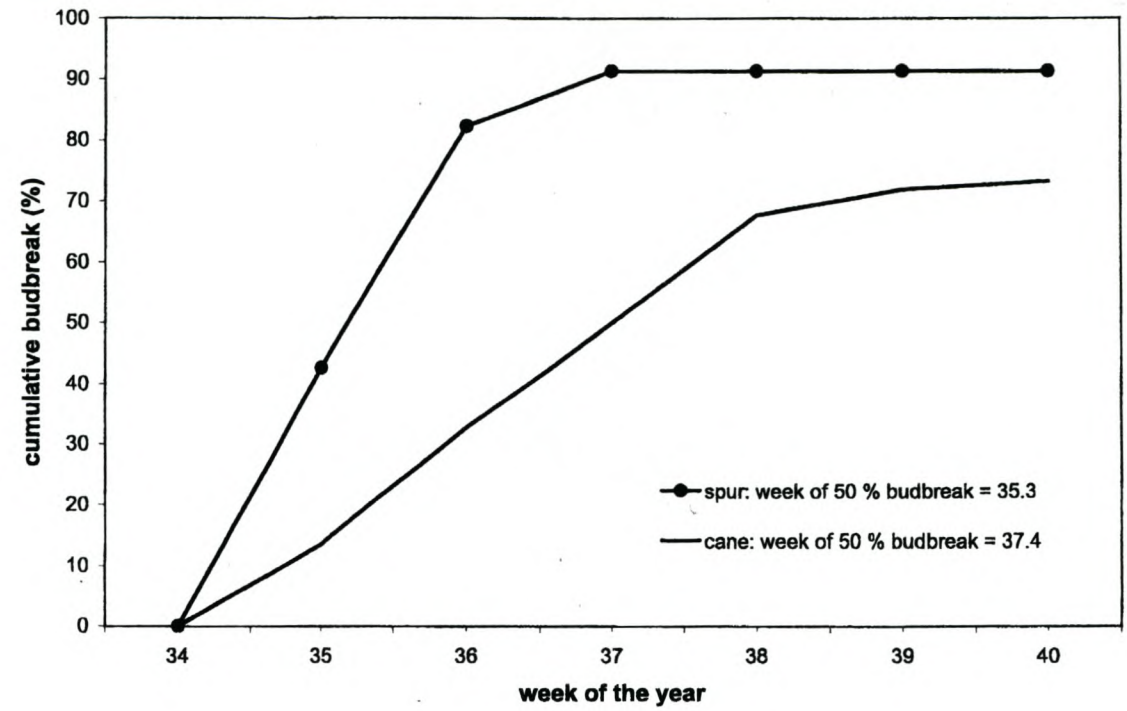

Fig. 7 a. Cumulative budbreak of Alphonse Lavalleé vines, Wellington, South Africa, 1999. Start week of year 36 (31/08/1999). Week of 50% budbreak = 36.4 (combined spur and cane).

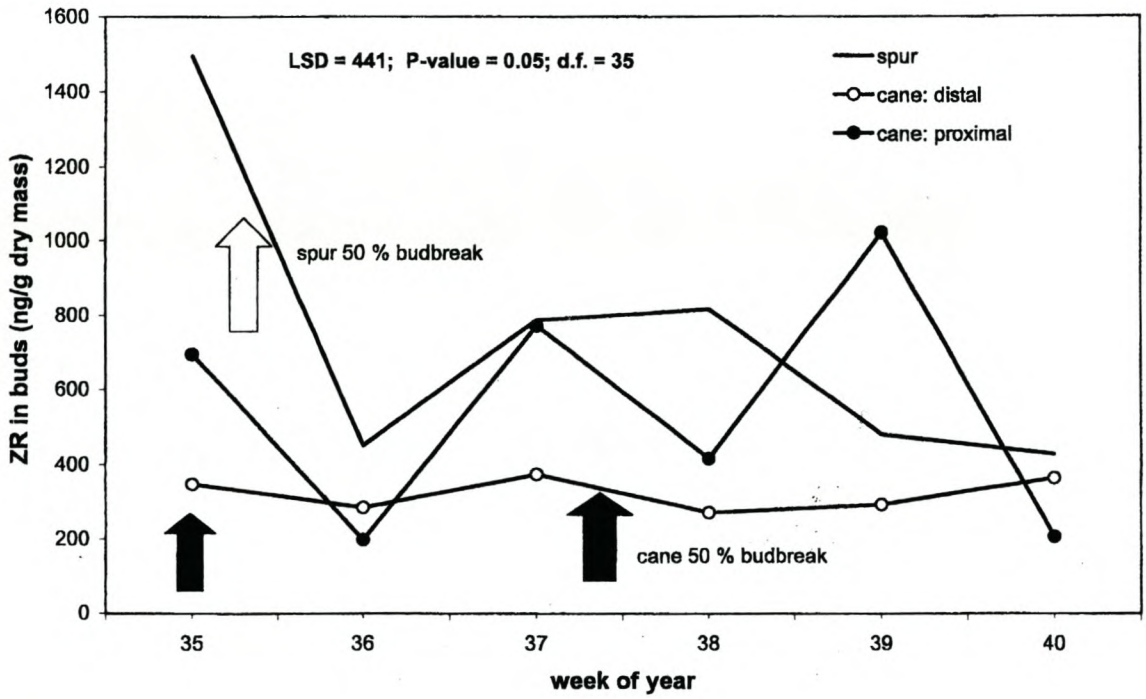

Fig. 7 b. Free zeatin riboside (ZR) levels in buds of spurs and canes of Alphonse Lavalleé vines measured from budswell (solid arrow), Wellington, South Africa, 1999.

similar to those obtained by Cutting *et al.* (1991) on xylem sap ZR levels of apple shoots, where the ZR peak was at one week before BB.

Spur pruning of all three cultivars resulted in earlier and higher final BB than cane pruning. Regardless of cultivar, the proximal portions of shoots, whether spur pruned or the proximal portions of canes had the highest ZR levels. Spurs had the highest ZR levels of both these portions for all three cultivars. Of the three cultivars, Alphonse Lavalleé had the highest spur and proximal cane portion bud ZR levels and Sunred Seedless the lowest ZR levels. Sultanina and especially Alphonse Lavalleé's ZR levels seemed to be declining from a previous higher peak. This could have been a first peak that coincides with the xylem sap peak observed in the free bleeding xylem sap trial. The peaks that were observed after BB may have been due to later active growth of the vines. This fluctuation in ZR levels in bud tissue of different portions of the shoot would suggest a difference in ZR consumption or turnover. Higher ZR levels were observed in the buds of spurs than in the buds of distal portions of canes, while distal portions of canes had the higher xylary ZR levels as found in the free xylem sap trial.

Apical dominance is a phenomenon of long pruned shoots of trees, possibly due to higher growth activity at the distal portions in buds of the shoots. In vines, distal (i.e. apical) buds at the apex of a cane burst prior to and inhibit the growth of the proximal (i.e. basal) buds (Tassie and Freeman 1992). This is most obvious in early spring when apical buds burst early and grow vigourously, while middle and proximal buds grow weakly. Thus, ZR turnover may be high in buds of distal cane portions, resulting in lower ZR levels compared to buds of short spur pruned shoots. The higher ZR levels in proximal buds, whether from the cane or spur, were not observed in the free bleeding xylem sap of spur-pruned canes. The pruning system as used, plays a role to balance vigour with fruit set. Pruning removes the apical inhibition of the cane and the removal thereof can be seen in the generally very good BB of the spurs and high bud ZR levels of spurs found in all three cultivars. This would suggest a proximally dominant system.

The higher free ZR levels observed in the xylem sap of the longer cane pruned canes compared to the short spur pruned canes would suggest a cumulative ZR release in one year old canes from either stored forms in the cane, or from local synthesis in the cane, resulting in a higher final ZR at the end of the cane as cane length increases, compared to the short spurs. These higher ZR levels could lead to stronger growth, consistent with apical dominant growth. Work on apples by Cutting *et al.* (1991) and Cook *et al.* (2001), have suggested that either wood-stored ZR or *de novo* synthesis of ZR in the shoot could

play a role in initial BB. The release of wood-storage forms of ZR could explain the increase in ZR as the cane length increases.

It is likely that the increased ZR levels in the free xylem sap of cane-pruned vines have a profound effect on the development of inflorescence primordia and hence bunch development and ultimately production. It has been shown that the application of CKs to vine buds can increase inflorescence formation and reduce tendril formation (Srinivasan and Mullins 1978, Srinivasan and Mullins 1980). These higher ZR levels could then possibly lead to better fruit set at the distal portions of the cane. Thus, these studies provide a plausible explanation as to why a long cane pruning system is essential to ensure sufficient fruit set in Sultanina vines.

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APPENDIX

In this appendix the SAS input file names and files are given which correspond to the relevant data of each of the trials as used for this study.

Chapter 5

Effect of hydrogen cyanamide on cytokinin levels

1. Statistical file title: 'J.Lombard -StorageZR.dat- WW15/14-MSc-Warmzand:1997:Sultanina:NA DMX';
The data used to investigate the levels of storage form ZR in different cane tissues in 1997.
2. Statistical file title: 'J.Lombard -Wzand.dat- Warmzand Proef: 1997: Sultanina: NA DMX beh ';
The data used to investigate the levels of ZR in various cane tissues over time after HC application in 1997.
3. Statistical file title: 'J.Lombard - Wz98tyd-6tot+8.dat-Warmzand: 1998: Sultanina: NA DMX behandeling';
The data used to investigate the levels of ZR in cane buds over time after HC application in 1998.

Chapter 6

Effect of different pruning systems on cytokinin levels in different cultivars

1. Statistical file title: 'J.Lombard -oegies.dat-Bud-Buds-MSc-Wellington Proef:1999:cane vs spur';
The data used to investigate the levels of ZR in buds from canes and spurs of Sultana ("s"), Sunred Seedless ("ss") and Alphonse Lavalleé ("a") in the pruning system trial in 1999.
2. Statistical file title: 'J.Lombard -xileem.dat- Vry xileemsap:Sultanina';
The data used to investigate ZR levels in free xylem sap of unpruned Sultana canes in 1999.
3. Statistical file title: 'Johann Lombard -Stats2001xileemsap.dat- Wellington :WW15/14: MSc';
The data used to investigate ZR levels in free xylem sap of unpruned, Sultana ("s"), Sunred Seedless ("ss") and Alphonse Lavalleé ("a") canes in 2001.

Chapter 5

1. title 'J.Lombard -StorageZR.dat- WW15/14-MSc-Warmzand:1997:Sultanina:NA DMX';

```
title2 'SLEGS op TIME 0 geneem (drie weke na DMX toediening)';
options ls=78 ps=62;
data a;
input Treat Treatand$ Block Position$ What$ freeZR storageZR @;
output;
cards;
1 control   1       distal     bark      64.07         -11.67
1 control   2       distal     bark      485.38        -116.07
2 control   1       distal     wood      74.58          1.87
2 control   2       distal     wood      178.44         5.12
3 control   1       proximal   bark      122.28        -10.23
3 control   2       proximal   bark      96.81          2.73
4 control   1       proximal   wood      124.81        -7.61
4 control   2       proximal   wood      145.96         9.75
5   HC      1       distal     bark      335.79         10.66
5   HC      2       distal     bark      305.15        -53.71
6   HC      1       distal     wood      330.57         54.18
6   HC      2       distal     wood      385.25        -46.00
7   HC      1       proximal   bark      380.14        -45.90
7   HC      2       proximal   bark      60.86         -2.32
8   HC      1       proximal   wood      253.90        -20.58
8   HC      2       proximal   wood      84.56         -8.50
;
proc print data=a;
run;

proc glm data=a;
 class Treatand Block Position What;
 model freeZR storageZR = Block Treatand Block(Treatand) Position What What*Position
      What*Treatand Treatand*Position What*Treatand*Position / ss1;
 means Treatand|Position|What;
 means Treatand  / lsd lines e=Block(Treatand);
 means What Position / lsd lines;
output out=res r=RFreeZR RStorageZR;
proc univariate data=res plot normal vardef=df;
 var RFreeZR RStorageZR;
run;

proc glm data=a;
 class Treat Block Treatand;
 model freeZR storageZR = Block Treat Block(Treatand) / ss1;
 means Treat;
 means Treat / lsd lines;
run;
```

```
2.      title 'J.Lombard -Wzand.dat- Warmzand Proef: 1997: Sultanina: NA DMX treat
';
options ls=78 ps=62;
data a;
input treat$ time$ block pos$ tipe$ what$ ngZR @;
output;
cards;

control  -3      1       distal      internode     bark     64.81
control  -3      2       distal      internode     bark     183.54
control  -2      1       distal      internode     bark     159.60
control  -2      2       distal      internode     bark     80.61
control  -1      1       distal      internode     bark     81.62
control  -1      2       distal      internode     bark     69.84
control  0       1       distal      internode     bark     64.07
control  0       2       distal      internode     bark     485.38
control  1       1       distal      internode     bark     199.02
control  1       2       distal      internode     bark     87.63
control  2       1       distal      internode     bark     132.58
control  2       2       distal      internode     bark     100.42
control  3       1       distal      internode     bark     98.61
control  3       2       distal      internode     bark     100.01
control  4       1       distal      internode     bark     204.35
control  4       2       distal      internode     bark     258.80
control  5       1       distal      internode     bark     246.22
control  5       2       distal      internode     bark     268.12
control  6       1       distal      internode     bark     121.12
control  6       2       distal      internode     bark     98.10
  HC    -3       1       distal      internode     bark     58.04
  HC    -3       2       distal      internode     bark     57.93
  HC    -2       1       distal      internode     bark     69.41
  HC    -2       2       distal      internode     bark     179.35
  HC    -1       1       distal      internode     bark     313.37
  HC    -1       2       distal      internode     bark     392.04
  HC     0       1       distal      internode     bark     335.79
  HC     0       2       distal      internode     bark     305.15
  HC     1       1       distal      internode     bark     1140.45
  HC     1       2       distal      internode     bark     435.87
  HC     2       1       distal      internode     bark     1747.31
  HC     2       2       distal      internode     bark     247.63
  HC     3       1       distal      internode     bark     1168.07
  HC     3       2       distal      internode     bark     322.17
  HC     4       1       distal      internode     bark     715.78
  HC     4       2       distal      internode     bark     551.53
  HC     5       1       distal      internode     bark     409.59
  HC     5       2       distal      internode     bark     237.91
  HC     6       1       distal      internode     bark     364.36
  HC     6       2       distal      internode     bark     531.64
control  -3      1       distal      internode     wood     259.18
control  -3      2       distal      internode     wood     242.41
control  -2      1       distal      internode     wood     99.89
control  -2      2       distal      internode     wood     144.83
control  -1      1       distal      internode     wood     77.48
control  -1      2       distal      internode     wood     140.29
control  0       1       distal      internode     wood     74.58
control  0       2       distal      internode     wood     178.44
```

control	1	1	distal	internode	wood	162.86
control	1	2	distal	internode	wood	77.20
control	2	1	distal	internode	wood	57.02
control	2	2	distal	internode	wood	82.09
control	3	1	distal	internode	wood	60.02
control	3	2	distal	internode	wood	201.33
control	4	1	distal	internode	wood	93.93
control	4	2	distal	internode	wood	159.60
control	5	1	distal	internode	wood	203.23
control	5	2	distal	internode	wood	158.94
control	6	1	distal	internode	wood	132.76
control	6	2	distal	internode	wood	87.00
HC	-3	1	distal	internode	wood	163.25
HC	-3	2	distal	internode	wood	165.88
HC	-2	1	distal	internode	wood	144.63
HC	-2	2	distal	internode	wood	121.87
HC	-1	1	distal	internode	wood	320.59
HC	-1	2	distal	internode	wood	297.93
HC	0	1	distal	internode	wood	330.57
HC	0	2	distal	internode	wood	385.25
HC	1	1	distal	internode	wood	583.75
HC	1	2	distal	internode	wood	281.60
HC	2	1	distal	internode	wood	962.60
HC	2	2	distal	internode	wood	145.32
HC	3	1	distal	internode	wood	858.50
HC	3	2	distal	internode	wood	139.41
HC	4	1	distal	internode	wood	370.39
HC	4	2	distal	internode	wood	240.78
HC	5	1	distal	internode	wood	123.45
HC	5	2	distal	internode	wood	121.52
HC	6	1	distal	internode	wood	235.41
HC	6	2	distal	internode	wood	120.30
control	-3	1	distal	bud	bud	421.60
control	-3	2	distal	bud	bud	269.43
control	-2	1	distal	bud	bud	271.20
control	-2	2	distal	bud	bud	319.42
control	-1	1	distal	bud	bud	206.01
control	-1	2	distal	bud	bud	298.36
control	0	1	distal	bud	bud	247.01
control	0	2	distal	bud	bud	333.34
control	1	1	distal	bud	bud	273.76
control	1	2	distal	bud	bud	274.41
control	2	1	distal	bud	bud	162.63
control	2	2	distal	bud	bud	177.31
control	3	1	distal	bud	bud	185.27
control	3	2	distal	bud	bud	213.63
control	4	1	distal	bud	bud	269.80
control	4	2	distal	bud	bud	386.89
control	5	1	distal	bud	bud	242.78
control	5	2	distal	bud	bud	219.45
control	6	1	distal	bud	bud	93.06
control	6	2	distal	bud	bud	145.31
HC	-3	1	distal	bud	bud	297.44
HC	-3	2	distal	bud	bud	230.67
HC	-2	1	distal	bud	bud	84.57
HC	-2	2	distal	bud	bud	222.75

HC	-1	1	distal	bud	bud	450.33
HC	-1	2	distal	bud	bud	462.64
HC	0	1	distal	bud	bud	464.47
HC	0	2	distal	bud	bud	950.16
HC	1	1	distal	bud	bud	1236.86
HC	1	2	distal	bud	bud	1780.77
HC	2	1	distal	bud	bud	804.61
HC	2	2	distal	bud	bud	639.19
HC	3	1	distal	bud	bud	802.31
HC	3	2	distal	bud	bud	628.09
HC	4	1	distal	bud	bud	348.06
HC	4	2	distal	bud	bud	617.79
HC	5	1	distal	bud	bud	441.97
HC	5	2	distal	bud	bud	290.32
HC	6	1	distal	bud	bud	366.01
HC	6	2	distal	bud	bud	509.72
control	-3	1	proximal	internode	bark	37.72
control	-3	2	proximal	internode	bark	24.49
control	-2	1	proximal	internode	bark	19.79
control	-2	2	proximal	internode	bark	34.68
control	-1	1	proximal	internode	bark	74.19
control	-1	2	proximal	internode	bark	75.42
control	0	1	proximal	internode	bark	122.28
control	0	2	proximal	internode	bark	96.81
control	1	1	proximal	internode	bark	66.04
control	1	2	proximal	internode	bark	98.94
control	2	1	proximal	internode	bark	169.63
control	2	2	proximal	internode	bark	237.30
control	3	1	proximal	internode	bark	184.15
control	3	2	proximal	internode	bark	69.04
control	4	1	proximal	internode	bark	239.84
control	4	2	proximal	internode	bark	142.35
control	5	1	proximal	internode	bark	137.40
control	5	2	proximal	internode	bark	157.19
control	6	1	proximal	internode	bark	152.86
control	6	2	proximal	internode	bark	149.17
HC	-3	1	proximal	internode	bark	156.85
HC	-3	2	proximal	internode	bark	31.22
HC	-2	1	proximal	internode	bark	39.49
HC	-2	2	proximal	internode	bark	28.53
HC	-1	1	proximal	internode	bark	74.50
HC	-1	2	proximal	internode	bark	243.45
HC	0	1	proximal	internode	bark	380.14
HC	0	2	proximal	internode	bark	60.86
HC	1	1	proximal	internode	bark	60.20
HC	1	2	proximal	internode	bark	164.89
HC	2	1	proximal	internode	bark	107.78
HC	2	2	proximal	internode	bark	154.12
HC	3	1	proximal	internode	bark	205.18
HC	3	2	proximal	internode	bark	255.64
HC	4	1	proximal	internode	bark	307.51
HC	4	2	proximal	internode	bark	189.04
HC	5	1	proximal	internode	bark	195.30
HC	5	2	proximal	internode	bark	221.79
HC	6	1	proximal	internode	bark	349.99
HC	6	2	proximal	internode	bark	164.11

control	-3	1	proximal	internode	wood	72.90
control	-3	2	proximal	internode	wood	120.74
control	-2	1	proximal	internode	wood	100.86
control	-2	2	proximal	internode	wood	106.14
control	-1	1	proximal	internode	wood	137.07
control	-1	2	proximal	internode	wood	114.61
control	0	1	proximal	internode	wood	124.81
control	0	2	proximal	internode	wood	145.96
control	1	1	proximal	internode	wood	88.54
control	1	2	proximal	internode	wood	129.97
control	2	1	proximal	internode	wood	122.01
control	2	2	proximal	internode	wood	98.45
control	3	1	proximal	internode	wood	59.92
control	3	2	proximal	internode	wood	70.74
control	4	1	proximal	internode	wood	137.66
control	4	2	proximal	internode	wood	136.91
control	5	1	proximal	internode	wood	121.80
control	5	2	proximal	internode	wood	138.42
control	6	1	proximal	internode	wood	118.96
control	6	2	proximal	internode	wood	121.45
HC	-3	1	proximal	internode	wood	115.98
HC	-3	2	proximal	internode	wood	56.02
HC	-2	1	proximal	internode	wood	196.95
HC	-2	2	proximal	internode	wood	69.95
HC	-1	1	proximal	internode	wood	257.78
HC	-1	2	proximal	internode	wood	209.57
HC	0	1	proximal	internode	wood	253.90
HC	0	2	proximal	internode	wood	84.56
HC	1	1	proximal	internode	wood	74.54
HC	1	2	proximal	internode	wood	90.83
HC	2	1	proximal	internode	wood	170.42
HC	2	2	proximal	internode	wood	158.02
HC	3	1	proximal	internode	wood	86.65
HC	3	2	proximal	internode	wood	111.14
HC	4	1	proximal	internode	wood	157.56
HC	4	2	proximal	internode	wood	84.12
HC	5	1	proximal	internode	wood	88.57
HC	5	2	proximal	internode	wood	119.27
HC	6	1	proximal	internode	wood	99.87
HC	6	2	proximal	internode	wood	89.90
control	-3	1	proximal	bud	bud	156.86
control	-3	2	proximal	bud	bud	170.30
control	-2	1	proximal	bud	bud	257.66
control	-2	2	proximal	bud	bud	179.50
control	-1	1	proximal	bud	bud	268.53
control	-1	2	proximal	bud	bud	199.88
control	0	1	proximal	bud	bud	344.75
control	0	2	proximal	bud	bud	387.73
control	1	1	proximal	bud	bud	203.07
control	1	2	proximal	bud	bud	200.04
control	2	1	proximal	bud	bud	469.44
control	2	2	proximal	bud	bud	403.38
control	3	1	proximal	bud	bud	219.91
control	3	2	proximal	bud	bud	269.97
control	4	1	proximal	bud	bud	290.98
control	4	2	proximal	bud	bud	355.25

```
control  5    1    proximal  bud    bud    164.10
control  5    2    proximal  bud    bud    214.03
control  6    1    proximal  bud    bud    370.52
control  6    2    proximal  bud    bud    246.95
  HC    -3    1    proximal  bud    bud    643.87
  HC    -3    2    proximal  bud    bud    369.07
  HC    -2    1    proximal  bud    bud    209.22
  HC    -2    2    proximal  bud    bud    879.62
  HC    -1    1    proximal  bud    bud    509.95
  HC    -1    2    proximal  bud    bud    545.28
  HC    0     1    proximal  bud    bud    835.04
  HC    0     2    proximal  bud    bud    531.81
  HC    1     1    proximal  bud    bud    798.74
  HC    1     2    proximal  bud    bud    229.67
  HC    2     1    proximal  bud    bud    423.15
  HC    2     2    proximal  bud    bud    367.63
  HC    3     1    proximal  bud    bud    326.67
  HC    3     2    proximal  bud    bud    527.14
  HC    4     1    proximal  bud    bud    318.72
  HC    4     2    proximal  bud    bud    288.79
  HC    5     1    proximal  bud    bud    219.88
  HC    5     2    proximal  bud    bud    314.96
  HC    6     1    proximal  bud    bud    620.17
  HC    6     2    proximal  bud    bud    289.07
;

proc print data=a;
run;

proc glm data=a;
 class Block Treat Time Pos What;
 model ngZR = Block Treat|Time|Pos|What / ss1;
 means Treat|Time|Pos|What;
 means Treat Time Pos What / lsd lines;
output out=res r=rngZR;
proc univariate data=res plot normal vardef=df noprint;
 var rngZR;
run;
```

3. 'J.Lombard - Wz98tyd-6tot+8.dat-Warmzand: 1998: Sultanina: NA DMX behandeling';

```
options ls=90 ps=62;
data a;
input Treat$ Time Block Position$ ZR @;
output;
cards;

control    1       1       distal     129.6973
control    1       2       distal     200.4586
control    1       1    proximal      262.9065
control    1       2    proximal      193.3159
control    2       1       distal     194.3522
control    2       2       distal     286.5633
control    2       1    proximal      144.6989
control    2       2    proximal      209.2454
control    3       1       distal     237.7104
control    3       2       distal     223.0179
control    3       1    proximal      227.6540
control    3       2    proximal      213.8689
control    4       1       distal     270.5314
control    4       2       distal     365.2010
control    4       1    proximal      137.2337
control    4       2    proximal      209.8359
control    5       1       distal     363.5456
control    5       2       distal     240.1993
control    5       1    proximal      352.8302
control    5       2    proximal      340.8348
control    6       1       distal     453.6983
control    6       2       distal     455.2698
control    6       1    proximal      301.0753
control    6       2    proximal      169.5057
control    7       1       distal     486.2165
control    7       2       distal     386.7210
control    7       1    proximal      701.8150
control    7       2    proximal      552.3206
control    8       1       distal     195.5186
control    8       2       distal     294.0826
control    8       1    proximal      219.2930
control    8       2    proximal      242.5268
control    9       1       distal     347.9825
control    9       2       distal     200.4918
control    9       1    proximal      278.1739
control    9       2    proximal      158.2852
control    10      1       distal     361.4692
control    10      2       distal     440.7394
control    10      1    proximal         .
control    10      2    proximal      236.6637
control    11      1       distal     195.8457
control    11      2       distal     248.0930
control    11      1    proximal      174.6468
control    11      2    proximal      119.6900
control    12      1       distal     276.4354
control    12      2       distal     281.8067
control    12      1    proximal      278.0463
control    12      2    proximal      359.5833
```

control	13	1	distal	197.0913
control	13	2	distal	113.5887
control	13	1	proximal	214.3131
control	13	2	proximal	140.3048
control	14	1	distal	107.9423
control	14	2	distal	113.6029
control	14	1	proximal	100.7064
control	14	2	proximal	122.8199
control	15	1	distal	186.5382
control	15	2	distal	85.7600
control	15	1	proximal	119.4984
control	15	2	proximal	77.1352
hydcyan	1	1	distal	234.1749
hydcyan	1	2	distal	99.4856
hydcyan	1	1	proximal	163.4336
hydcyan	1	2	proximal	79.4666
hydcyan	2	1	distal	143.7261
hydcyan	2	2	distal	162.5223
hydcyan	2	1	proximal	148.2979
hydcyan	2	2	proximal	239.4668
hydcyan	3	1	distal	387.1927
hydcyan	3	2	distal	612.6040
hydcyan	3	1	proximal	577.0025
hydcyan	3	2	proximal	331.4840
hydcyan	4	1	distal	679.7427
hydcyan	4	2	distal	569.7136
hydcyan	4	1	proximal	449.1352
hydcyan	4	2	proximal	779.3295
hydcyan	5	1	distal	485.6205
hydcyan	5	2	distal	407.6859
hydcyan	5	1	proximal	302.1018
hydcyan	5	2	proximal	454.1997
hydcyan	6	1	distal	535.6216
hydcyan	6	2	distal	593.0482
hydcyan	6	1	proximal	341.5994
hydcyan	6	2	proximal	475.8569
hydcyan	7	1	distal	273.9351
hydcyan	7	2	distal	349.5362
hydcyan	7	1	proximal	371.7320
hydcyan	7	2	proximal	224.4913
hydcyan	8	1	distal	487.2798
hydcyan	8	2	distal	393.3798
hydcyan	8	1	proximal	318.5921
hydcyan	8	2	proximal	232.7768
hydcyan	9	1	distal	707.0141
hydcyan	9	2	distal	478.7478
hydcyan	9	1	proximal	209.8324
hydcyan	9	2	proximal	327.5500
hydcyan	10	1	distal	360.3569
hydcyan	10	2	distal	230.6282
hydcyan	10	1	proximal	316.0482
hydcyan	10	2	proximal	277.4725
hydcyan	11	1	distal	267.0860
hydcyan	11	2	distal	407.9019
hydcyan	11	1	proximal	240.7259
hydcyan	11	2	proximal	207.4695

```
hydcyan    12      1        distal     189.1135
hydcyan    12      2        distal     186.3858
hydcyan    12      1     proximal      269.3210
hydcyan    12      2     proximal      244.3712
hydcyan    13      1        distal     263.3403
hydcyan    13      2        distal     159.8289
hydcyan    13      1     proximal      229.2234
hydcyan    13      2     proximal      128.4131
hydcyan    14      1        distal     211.9513
hydcyan    14      2        distal     193.6463
hydcyan    14      1     proximal      139.3635
hydcyan    14      2     proximal      138.1766
hydcyan    15      1        distal     235.3508
hydcyan    15      2        distal     154.7475
hydcyan    15      1     proximal      162.3711
hydcyan    15      2     proximal      144.5963
;

proc print data=a;
run;

proc glm data=a;
 class Block Treat Position Time;
 model ZR = Block Treat|Position|Time / ss1;
 means Treat|Time|Position|Time;
 means Treat Time  Position / lsd lines;
output out=res r=rZR;
proc univariate data=res plot normal vardef=df;
 var rZR;
run;
PROC SORT DAta=A;
  BY Treat;

PROC PLOT vpercent=60;
  PLOT  ZR*Time=Position ;
  BY Treat;
Run;
```

Chapter 6

1. title 'J.Lombard -oegies.dat-Bud-Buds-MSc-Wellington Proef:1999:cane vs spur';

```
options ls=78 ps=62;
data a;
input Treatment Cult$ Treat Time Block Shoot$28-46 What$  ZR @;
output;
cards;
```

1	a	1	1	1	spurspur	bud	2191.329
1	a	1	1	2	spurspur	bud	586.4777
1	a	1	1	3	spurspur	bud	953.4373
1	a	1	1	4	spurspur	bud	2252.788
2	s	1	1	1	spurspur	bud	330.13
2	s	1	1	2	spurspur	bud	547.7855
2	s	1	1	3	spurspur	bud	311.398
2	s	1	1	4	spurspur	bud	190.2455
3	ss	1	1	1	spurspur	bud	345.0362
3	ss	1	1	2	spurspur	bud	471.1538
3	ss	1	1	3	spurspur	bud	156.317
3	ss	1	1	4	spurspur	bud	98.95363
4	a	2	1	1	canedistal	bud	610.2278
4	a	2	1	2	canedistal	bud	166.7276
4	a	2	1	3	canedistal	bud	243.2127
4	a	2	1	4	canedistal	bud	367.8825
5	s	2	1	1	canedistal	bud	249.9525
5	s	2	1	2	canedistal	bud	175.3854
5	s	2	1	3	canedistal	bud	234.0389
5	s	2	1	4	canedistal	bud	211.4724
6	ss	2	1	1	canedistal	bud	144.9531
6	ss	2	1	2	canedistal	bud	369.759
6	ss	2	1	3	canedistal	bud	133.827
6	ss	2	1	4	canedistal	bud	168.0884
7	a	3	1	1	caneproximal	bud	1500.869
7	a	3	1	2	caneproximal	bud	198.2447
7	a	3	1	3	caneproximal	bud	229.403
7	a	3	1	4	caneproximal	bud	847.8632
8	s	3	1	1	caneproximal	bud	183.3088
8	s	3	1	2	caneproximal	bud	575.2508
8	s	3	1	3	caneproximal	bud	558.7302
8	s	3	1	4	caneproximal	bud	376.4386
9	ss	3	1	1	caneproximal	bud	411.7181
9	ss	3	1	2	caneproximal	bud	365.3504
9	ss	3	1	3	caneproximal	bud	380.1327
9	ss	3	1	4	caneproximal	bud	267.7733
10	a	4	2	1	spurspur	bud	790.7436
10	a	4	2	2	spurspur	bud	322.0763
10	a	4	2	3	spurspur	bud	336.5753
10	a	4	2	4	spurspur	bud	356.9411
11	s	4	2	1	spurspur	bud	461.7815
11	s	4	2	2	spurspur	bud	264.7083
11	s	4	2	3	spurspur	bud	108.3572
11	s	4	2	4	spurspur	bud	372.5148
12	ss	4	2	1	spurspur	bud	381.6813
12	ss	4	2	2	spurspur	bud	1010.777

12	ss	4	2	3	spurspur	bud	400.2502
12	ss	4	2	4	spurspur	bud	313.0904
13	a	5	2	1	canedistal	bud	235.7795
13	a	5	2	2	canedistal	bud	489.8213
13	a	5	2	3	canedistal	bud	155.8974
13	a	5	2	4	canedistal	bud	253.9683
14	s	5	2	1	canedistal	bud	295.3333
14	s	5	2	2	canedistal	bud	158.7531
14	s	5	2	3	canedistal	bud	262.126
14	s	5	2	4	canedistal	bud	206.5715
15	ss	5	2	1	canedistal	bud	147.125
15	ss	5	2	2	canedistal	bud	204.2858
15	ss	5	2	3	canedistal	bud	149.1927
15	ss	5	2	4	canedistal	bud	198.7856
16	a	6	2	1	caneproximal	bud	120.3913
16	a	6	2	2	caneproximal	bud	358.1009
16	a	6	2	3	caneproximal	bud	118.8291
16	a	6	2	4	caneproximal	bud	195.0218
17	s	6	2	1	caneproximal	bud	244.5863
17	s	6	2	2	caneproximal	bud	266.3337
17	s	6	2	3	caneproximal	bud	123.1381
17	s	6	2	4	caneproximal	bud	203.7202
18	ss	6	2	1	caneproximal	bud	368.4576
18	ss	6	2	2	caneproximal	bud	292.8945
18	ss	6	2	3	caneproximal	bud	275.5571
18	ss	6	2	4	caneproximal	bud	197.4327
19	a	7	3	1	spurspur	bud	1060.681
19	a	7	3	2	spurspur	bud	971.8621
19	a	7	3	3	spurspur	bud	653.7907
19	a	7	3	4	spurspur	bud	460.6898
20	s	7	3	1	spurspur	bud	406.8254
20	s	7	3	2	spurspur	bud	386.3248
20	s	7	3	3	spurspur	bud	396.6568
20	s	7	3	4	spurspur	bud	933.1828
21	ss	7	3	1	spurspur	bud	628.5692
21	ss	7	3	2	spurspur	bud	483.2444
21	ss	7	3	3	spurspur	bud	291.7379
21	ss	7	3	4	spurspur	bud	256.9411
22	a	8	3	1	canedistal	bud	252.4806
22	a	8	3	2	canedistal	bud	327.1173
22	a	8	3	3	canedistal	bud	252.1587
22	a	8	3	4	canedistal	bud	663.451
23	s	8	3	1	canedistal	bud	175.7754
23	s	8	3	2	canedistal	bud	297.5625
23	s	8	3	3	canedistal	bud	200.6614
23	s	8	3	4	canedistal	bud	171.1648
24	ss	8	3	1	canedistal	bud	414.7932
24	ss	8	3	2	canedistal	bud	481.3139
24	ss	8	3	3	canedistal	bud	203.7546
24	ss	8	3	4	canedistal	bud	212.1837
25	a	9	3	1	caneproximal	bud	1322.483
25	a	9	3	2	caneproximal	bud	438.7727
25	a	9	3	3	caneproximal	bud	722.9073
25	a	9	3	4	caneproximal	bud	599.3122
26	s	9	3	1	caneproximal	bud	205.314
26	s	9	3	2	caneproximal	bud	220.7173

26	s	9	3	3	caneproximal	bud	316.6994
26	s	9	3	4	caneproximal	bud	289.8893
27	ss	9	3	1	caneproximal	bud	232.0794
27	ss	9	3	2	caneproximal	bud	199.3251
27	ss	9	3	3	caneproximal	bud	214.6815
27	ss	9	3	4	caneproximal	bud	194.182
28	a	10	4	1	spurspur	bud	519.3325
28	a	10	4	2	spurspur	bud	914.5299
28	a	10	4	3	spurspur	bud	.
28	a	10	4	4	spurspur	bud	1250.971
28	a	10	4	5	spurspur	bud	584.0456
29	s	10	4	1	spurspur	bud	711.7743
29	s	10	4	2	spurspur	bud	556.5268
29	s	10	4	3	spurspur	bud	876.9534
29	s	10	4	4	spurspur	bud	493.1806
30	ss	10	4	1	spurspur	bud	256.8441
30	ss	10	4	2	spurspur	bud	394.417
30	ss	10	4	3	spurspur	bud	367.4198
30	ss	10	4	4	spurspur	bud	218.463
31	a	11	4	1	canedistal	bud	205.3833
31	a	11	4	2	canedistal	bud	378.1898
31	a	11	4	3	canedistal	bud	221.9572
31	a	11	4	4	canedistal	bud	274.2769
32	s	11	4	1	canedistal	bud	226.1029
32	s	11	4	2	canedistal	bud	184.2726
32	s	11	4	3	canedistal	bud	353.3046
32	s	11	4	4	canedistal	bud	275.4641
33	ss	11	4	1	canedistal	bud	298.0312
33	ss	11	4	2	canedistal	bud	279.1838
33	ss	11	4	3	canedistal	bud	343.5829
33	ss	11	4	4	canedistal	bud	168.9731
34	a	12	4	1	caneproximal	bud	439.1124
34	a	12	4	2	caneproximal	bud	424.8045
34	a	12	4	3	caneproximal	bud	529.5429
34	a	12	4	4	caneproximal	bud	270.8294
35	s	12	4	1	caneproximal	bud	422.4122
35	s	12	4	2	caneproximal	bud	457.8486
35	s	12	4	3	caneproximal	bud	725.7631
35	s	12	4	4	caneproximal	bud	297.4278
36	ss	12	4	1	caneproximal	bud	205.3
36	ss	12	4	2	caneproximal	bud	334.5922
36	ss	12	4	3	caneproximal	bud	456.4371
36	ss	12	4	4	caneproximal	bud	314.6138
37	a	13	5	1	spurspur	bud	622.2584
37	a	13	5	2	spurspur	bud	475.5144
37	a	13	5	3	spurspur	bud	458.8082
37	a	13	5	4	spurspur	bud	367.7804
38	s	13	5	1	spurspur	bud	678.9934
38	s	13	5	2	spurspur	bud	698.4127
38	s	13	5	3	spurspur	bud	1003.167
38	s	13	5	4	spurspur	bud	524.4755
39	ss	13	5	1	spurspur	bud	361.153
39	ss	13	5	2	spurspur	bud	401.5737
39	ss	13	5	3	spurspur	bud	247.2178
39	ss	13	5	4	spurspur	bud	364.2463
40	a	14	5	1	canedistal	bud	347.0955

40	a	14 5	2	canedistal	bud	289.1069
40	a	14 5	3	canedistal	bud	180.7282
40	a	14 5	4	canedistal	bud	348.9659
41	s	14 5	1	canedistal	bud	300.134
41	s	14 5	2	canedistal	bud	639.9611
41	s	14 5	3	canedistal	bud	291.0788
41	s	14 5	4	canedistal	bud	363.9847
42	ss	14 5	1	canedistal	bud	319.5655
42	ss	14 5	2	canedistal	bud	489.6276
42	ss	14 5	3	canedistal	bud	237.1542
42	ss	14 5	4	canedistal	bud	198.5923
43	a	15 5	1	caneproximal	bud	313.2346
43	a	15 5	2	caneproximal	bud	1142.935
43	a	15 5	3	caneproximal	bud	2055.257
43	a	15 5	4	caneproximal	bud	573.9338
44	s	15 5	1	caneproximal	bud	886.8169
44	s	15 5	2	caneproximal	bud	946.9543
44	s	15 5	3	caneproximal	bud	342.5507
44	s	15 5	4	caneproximal	bud	487.6823
45	ss	15 5	1	caneproximal	bud	241.7201
45	ss	15 5	2	caneproximal	bud	232.875
45	ss	15 5	3	caneproximal	bud	272.6616
45	ss	15 5	4	caneproximal	bud	152.5312
46	a	16 6	1	spurspur	bud	600.2331
46	a	16 6	2	spurspur	bud	330.8214
46	a	16 6	3	spurspur	bud	392.9514
46	a	16 6	4	spurspur	bud	392.1569
47	s	16 6	1	spurspur	bud	424.359
47	s	16 6	2	spurspur	bud	529.718
47	s	16 6	3	spurspur	bud	779.5699
47	s	16 6	4	spurspur	bud	787.0018
48	ss	16 6	1	spurspur	bud	193.6267
48	ss	16 6	2	spurspur	bud	285.841
48	ss	16 6	3	spurspur	bud	482.6078
48	ss	16 6	4	spurspur	bud	337.7778
49	a	17 6	1	canedistal	bud	275.3082
49	a	17 6	2	canedistal	bud	402.2122
49	a	17 6	3	canedistal	bud	283.6053
49	a	17 6	4	canedistal	bud	497.2188
50	s	17 6	1	canedistal	bud	812.6264
50	s	17 6	2	canedistal	bud	309.4607
50	s	17 6	3	canedistal	bud	353.4357
50	s	17 6	4	canedistal	bud	244.793
51	ss	17 6	1	canedistal	bud	253.6314
51	ss	17 6	2	canedistal	bud	440.6747
51	ss	17 6	3	canedistal	bud	286.9699
51	ss	17 6	4	canedistal	bud	306.9313
52	a	18 6	1	caneproximal	bud	200.6916
52	a	18 6	2	caneproximal	bud	98.68055
52	a	18 6	3	caneproximal	bud	145.7324
52	a	18 6	4	caneproximal	bud	381.1579
53	s	18 6	1	caneproximal	bud	203.5295
53	s	18 6	2	caneproximal	bud	725.6686
53	s	18 6	3	caneproximal	bud	791.5662
53	s	18 6	4	caneproximal	bud	251.0008
54	ss	18 6	1	caneproximal	bud	254.4797

```
54      ss    18 6      2          caneproximal    bud    295.2863
54      ss    18 6      3          caneproximal    bud    117.5395
54      ss    18 6      4          caneproximal    bud    150.6843
;
proc print data=a;
run;

proc sort data=a;
 by Cult;
run;

proc glm data=a;
 class Block Treat Time Shoot;
 model ZR = Block Treat Block(Time) Block(Shoot*Time) / ss1;
 test h=Block e=Block(Time);
 test h=Treat  e=Block(Shoot*Time);
 means Treat;
 means Treat / lsd lines e=Block(Shoot*Time);
by Cult;
run;

proc glm data=a noprint;
 class Block Time Shoot;
 model ZR = Block Time Block(Time) Shoot Time*Shoot Block(Shoot*Time) / ss1;
 test h=Block Time e=Block(Time);
 test h=Shoot Shoot*Time e=Block(Shoot*Time);
 means Time|Shoot;
 means Time   / lsd lines e=Block(Time);
 means Shoot / lsd lines e=Block(Shoot*Time);
 by Cult;
run;

proc glm data=a noprint;
 class Cult Block Time Shoot;
 model ZR = Block Cult Block(Cult) Time Cult*Time Block(Cult*Time) Shoot
      Shoot*Cult Shoot*Time Shoot*Time*Cult Block(Shoot*Time*Cult) / ss1;
 test h=Block Cult e=Block(Cult);
 test h=Time Cult*Time e=Block(Cult*Time);
 test h=Shoot Shoot*Cult Shoot*Time Shoot*Time*Cult e=Block(Shoot*Time*Cult);
 means Cult|Time|Shoot;
 means Cult  / lsd lines e=Block(Cult);
 means Time   / lsd lines e=Block(Cult*Time);
 means Shoot / lsd lines e=Block(Shoot*Time*Cult);
output out=res r=rZR;
proc univariate data=res plot normal vardef=df noprint;
 var rZR;
run;

means Beh;

proc glm data=a noprint;
 class Block Cult Time Shoot Treat;
 model ZR = Block Treat Block(Cult) Block(Cult*Time) Block(Shoot*Time*Cult) / ss1;
 test h= Block Cult e=Block(Shoot*Time*Cult);
 means Treat;
 means Treat / lsd lines e=Block(Shoot*Time*Cult);
run;
```

```
2.      title 'J.Lombard -xileem.dat- Vry xileemsap:Sultanina';
title2 'Wellington:1999/2000 : WW 15/14';
options ls=78 ps=62;
data a;
input Treat Time Block Position$ ZR ul12ZR @;
output;
cards;
1       1       1       spur    0.61    0.61
1       1       2       spur    0.62    0.62
1       1       3       spur    0.826   0.826
1       1       4       spur    0.514   0.514
2       1       1       cane    6.15    6.82227
2       1       2       cane    2.517   3.4153
2       1       3       cane    2.49    3.49027
2       1       4       cane    5.339   6.09756
3       2       1       spur     .       .
3       2       2       spur    2.576   3.00713
3       2       3       spur    2.765   2.7489
3       2       4       spur    7.811   8.38831
4       2       1       cane    6.02    6.53072
4       2       2       cane    8.203   8.77982
4       2       3       cane    13.122  16.72664
4       2       4       cane    10.409  12.23677
5       3       1       spur    2.409   2.80721
5       3       2       spur    3.483   4.17333
5       3       3       spur    5.097   6.54738
5       3       4       spur    3.057   3.96508
6       3       1       cane    13.872  20.86665
6       3       2       cane    6.714   8.09676
6       3       3       cane    14.818  18.05111
6       3       4       cane    10.666  13.92776
7       4       1       spur    2.713   3.53192
7       4       2       spur    9.79    11.98687
7       4       3       spur    7.61    10.12928
7       4       4       spur    5.643   6.62235
8       4       1       cane    4.839   6.50573
8       4       2       cane    6.704   7.02219
8       4       3       cane    5.844   6.47241
8       4       4       cane    6.445   7.21378
9       5       1       spur    3.094   3.95675
9       5       2       spur     .       .
9       5       3       spur    1.608   2.51566
9       5       4       spur    4.549   6.19752
10      5       1       cane    1.938   2.52399
10      5       2       cane    6.119   6.17253
10      5       3       cane    3.948   4.51486
10      5       4       cane    2.391   2.92383
;
proc print data=a;
run;

proc glm data=a;
 class Block Time Position;
 model ZR ul12ZR = Block Time Position Position*Time / ss1;
 means Time|Position;
 means Position Time / lsd lines;
output out=res r= rZR rul12ZR;
```

```
proc univariate data=res plot normal vadrdef=df noprint;
 var rZR rul12ZR;
run;

proc glm data=a;
 class Block Treat;
 model ZR ul12ZR = Block Treat / ss1;
 means Treat;
 means Treat / lsd lines;
run;
```

```
3.      title 'Johann Lombard -Stats2001xileemsap.dat- Wellington :WW15/14: MSc';
OPTIONS  ls=90 ps=64;
DATA A;
INPUT  Cult$ Day Block Position$ ZR @;
*     Day2 = Day*Day;
     OUTPUT;
CARDS;
alphonse  211      1      spur     0.405
alphonse  211      2      spur     0.319
alphonse  211      3      spur     0.294
alphonse  211      1      cane     0.539
alphonse  211      2      cane     0.583
alphonse  211      3      cane     0.2495
alphonse  220      1      spur     0.385
alphonse  220      2      spur     1.201
alphonse  220      3      spur     0.726
alphonse  220      1      cane     0.869
alphonse  220      2      cane     1.324
alphonse  220      3      cane     0.414
alphonse  232      1      spur     1.391
alphonse  232      2      spur     1.478
alphonse  232      3      spur     1.579
alphonse  232      1      cane     3.878
alphonse  232      2      cane     3.846
alphonse  232      3      cane     3.172
alphonse  236      1      spur     2.079
alphonse  236      2      spur     1.913
alphonse  236      3      spur     2.573
alphonse  236      1      cane     5.864
alphonse  236      2      cane     5.3
alphonse  236      3      cane     2.948
alphonse  240      1      spur     1.645
alphonse  240      2      spur     1.765
alphonse  240      3      spur     2.678
alphonse  240      1      cane     4.037
alphonse  240      2      cane     6.413
alphonse  240      3      cane     5.563
alphonse  247      1      spur     1.962
alphonse  247      2      spur     4.268
alphonse  247      3      spur     4.052
alphonse  247      1      cane     5.16
alphonse  247      2      cane     6.788
alphonse  247      3      cane     7.952
alphonse  255      1      spur     4.026
alphonse  255      2      spur     4.06
alphonse  255      3      spur     1.926
alphonse  255      1      cane     8.624
alphonse  255      2      cane     .
alphonse  255      3      cane     .
alphonse  262      1      spur     4.344
alphonse  262      2      spur     6.008
alphonse  262      3      spur     4.368
alphonse  262      1      cane     11.9
alphonse  262      2      cane     8.12
alphonse  262      3      cane     6.08
sultana   211      1      spur     1.874
```

sultana	211	2	spur	1.446
sultana	211	3	spur	0.798
sultana	211	1	cane	0.914
sultana	211	2	cane	0.797
sultana	211	3	cane	2.893
sultana	220	1	spur	9.16
sultana	220	2	spur	1.884
sultana	220	3	spur	8.166
sultana	220	1	cane	9.82
sultana	220	2	cane	2.54
sultana	220	3	cane	11.588
sultana	232	1	spur	8.904
sultana	232	2	spur	3.532
sultana	232	3	spur	5.44
sultana	232	1	cane	12.7
sultana	232	2	cane	5.088
sultana	232	3	cane	10.676
sultana	236	1	spur	3.21
sultana	236	2	spur	2.795
sultana	236	3	spur	9.772
sultana	236	1	cane	6.328
sultana	236	2	cane	11.956
sultana	236	3	cane	7.556
sultana	240	1	spur	6.863
sultana	240	2	spur	4.178
sultana	240	3	spur	5.668
sultana	240	1	cane	6.52
sultana	240	2	cane	14.52
sultana	240	3	cane	14.18
sultana	247	1	spur	6.858
sultana	247	2	spur	.
sultana	247	3	spur	.
sultana	247	1	cane	8.532
sultana	247	2	cane	2.104
sultana	247	3	cane	9.772
sultana	255	1	spur	3.68
sultana	255	2	spur	5.473
sultana	255	3	spur	2.295
sultana	255	1	cane	3.914
sultana	255	2	cane	6.236
sultana	255	3	cane	3.746
sultana	262	1	spur	6.54
sultana	262	2	spur	1.452
sultana	262	3	spur	3.656
sultana	262	1	cane	5.54
sultana	262	2	cane	4.932
sultana	262	3	cane	4.584
sunred	211	1	spur	0.236
sunred	211	2	spur	1.337
sunred	211	3	spur	0.455
sunred	211	1	cane	0.214
sunred	211	2	cane	0.2895
sunred	211	3	cane	0.2225
sunred	220	1	spur	1.186
sunred	220	2	spur	0.552
sunred	220	3	spur	0.409

```
sunred    220    1    cane    4.444
sunred    220    2    cane    3.508
sunred    220    3    cane    0.974
sunred    232    1    spur    1.942
sunred    232    2    spur    1.925
sunred    232    3    spur    0.842
sunred    232    1    cane    6.16
sunred    232    2    cane    8.376
sunred    232    3    cane    3.652
sunred    236    1    spur    2.088
sunred    236    2    spur    3.258
sunred    236    3    spur    1.715
sunred    236    1    cane    10.884
sunred    236    2    cane    10.524
sunred    236    3    cane    3.728
sunred    240    1    spur    2.589
sunred    240    2    spur    4.221
sunred    240    3    spur    1.755
sunred    240    1    cane    8.472
sunred    240    2    cane    7.952
sunred    240    3    cane    5.856
sunred    247    1    spur    4.367
sunred    247    2    spur    0.868
sunred    247    3    spur    0.867
sunred    247    1    cane    6.4
sunred    247    2    cane    2.488
sunred    247    3    cane    11.284
sunred    255    1    spur    4.296
sunred    255    2    spur    2.787
sunred    255    3    spur    3.986
sunred    255    1    cane    6.024
sunred    255    2    cane    7.936
sunred    255    3    cane    2.844
sunred    262    1    spur    3.258
sunred    262    2    spur    4.741
sunred    262    3    spur    1.766
sunred    262    1    cane    2.827
sunred    262    2    cane    2.039
sunred    262    3    cane    4.175
;
PROC PRINT;
* Cult Day Block Position ZR  ;
PROC GLM  ;
    CLASS Cult Day Block Position;
    MODEL ZR  =Block Cult|Day|Position /SS1;
    MEANS  Cult|Day|Position ;
    MEANS Cult Day Position  / LSD LINES;
    OUTPUT OUT=RESID1
     R= rZR  ;
     PROC UNIVARIATE DATA=RESID1 PLOT NORMAL VARDEF=DF ;
    VAR rZR ;
 RUN;

PROC SORT Data=A;
   BY  Cult ;
PROC GLM  ;
    CLASS Block Position Day  ;
```

```
    MODEL ZR  =Block Position|Day /SS1;
    MEANS  Position|Day ;
    MEANS Day Position  / LSD LINES;
    OUTPUT OUT=RESID1 R= rZR  ;
  BY  Cult ;
PROC UNIVARIATE DATA=RESID1 PLOT NORMAL VARDEF=DF ;
    VAR rZR ;
    BY  Cult ;
RUN;
```