Cytokinins and bud break in rose combination plants

J.A. Dieleman

Promotor: dr. J. Tromp,
emeritus-hoogleraar in de tuinbouwplantenteelt, in het bijzonder de overblijvende gewassen

Co-promotor: dr. D. Kuiper,
senior wetenschappelijk onderzoeker plantenfysiologie AB-DLO

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Proefschrift

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Cover: rose combination plants in a growth room (photo: SC-DLO)

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Stellingen

1. Onderstamgenotypes bij de roos verschillen in de hoeveelheid geproduceerde cytokininen die naar de ent getransporteerd worden.
   Dit proefschrift

2. In bladeren van de roos vindt *de novo* synthese van cytokininen plaats.
   Dit proefschrift

3. Bij de roos wordt knopuitloop voorafgegaan door een verhoogde concentratie cytokininen in het bloedingssap.
   Dit proefschrift

4. Het gebruik van ethanol als oplosmiddel voor cytokininen bij toediening aan planten dient vermeden te worden.
   J.A. Dieleman, R.R.J. Perik and D. Kuiper. J. Plant Physiol. 145: 377-378 (1995)

5. Gezien het aantal onderzoekers dat de laatste jaren aan het rozenonderzoek in Wageningen is onttrokken kan men met betrekking tot de rozenteelt niet spreken van Kenniscentrum Wageningen.

6. Het gezegde 'geen roosje zo mooi of er zit een doorntje aan' is op planten-morfologische gronden onjuist.

7. Erger dan iets niet te weten is er niet over nagedacht te hebben.

8. Een praktisch probleem van chemisch werk is dat zo vaak iets op water lijkt maar dat niet blijkt te zijn.

9. Het voordeel van plantenfysiologisch boven oecologisch onderzoek is dat met de omgevingsfactor 'konijnen' geen rekening gehouden hoeft te worden.

10. De moderne versie van het sabbatsjaar is de braaklegging.
    Leviticus 25: 3-4. Zes jaar zult gij uw akker bezaaien (...), maar in het zevende jaar zal het land een volkomen sabbat hebben (...): uw akker zult gij niet bezaaien (...)
    Verordening (EEG) nr. 1765/92 tot instelling van een steunregeling voor producenten van bepaalde akkerbouwgewassen
    Verordening (EG) nr. 762/94 houdende toepassingsbepalingen inzake het uit produktie nemen van grond als bedoeld in Verordening (EEG) nr. 1765/92

11. Bij het beoefenen van de voetbalsport is de 'sliding' een van de meest spectaculaire technieken. Het verbieden daarvan zou derhalve een verarming van de voetbalsport betekenen.

Stellingen behorende bij het proefschrift 'Cytokinins and bud break in rose combination plants' door J.A. Dieleman. Wageningen, 20 mei 1998

Abstract

Dieleman, J.A., 1998. Cytokinins and bud break in rose combination plants. Thesis Wageningen Agricultural University, Wageningen, The Netherlands, 109 pp; English and Dutch summaries.

In the Netherlands, the rose is the most important glasshouse cut flower. Approximately 50% of the glasshouse roses are combination plants, consisting of a scion cultivar and a rootstock of a different genotype. Rootstocks inducing differences in the vigour of the scion are suggested to differ in cytokinin production and export to the shoot, thereby affecting bud break and outgrowth of the scion. In the present study, the course of endogenous cytokinins in rose combination plants was determined. The high contribution of isopentenyladenine-type cytokinins in young leaves indicated that these leaves, as the roots, were capable of *de novo* synthesis of cytokinins. Export of cytokinins from the roots was estimated based on the assumption that the cytokinin concentration in bleeding sap is representative for the concentration in xylem sap *in situ*, which was experimentally verified. The concentration of zeatin riboside (ZR) in bleeding sap was shown to be correlated with bud break of axillary shoots and bottom breaks, it increased before bud break and decreased thereafter. This suggests a quantitative relationship between cytokinin export from the roots and shoot development. Growth of the scion was also influenced by environmental factors, since bud break was advanced at higher root temperatures in the range of 11 to 26°C. However, this effect could not be correlated reliably with the cytokinin export from roots to shoot. Grafting the scion Madelon on rootstocks varying in vigour revealed that the rootstock that induces earlier bud break of the scion supplies larger quantities of cytokinins to the shoot. As a consequence, the concentrations of cytokinins in bleeding sap may be used as an early selection criterion for rose rootstocks.

An attempt was made to describe correlative inhibition in rose. The apex of the primary shoot is thought tot exert apical dominance over the axillary buds along the shoot, until the terminal flower is visible. As at that time, the auxin export from the apex decreases, the inhibition of the uppermost 2-4 axillary buds is released, resulting in bud break. Later, the young axillary shoots will take over apical dominance and inhibit bud break further down the stem by their auxin production. When the terminal flower buds of the axillary shoots are visible, apical dominance is reduced, leading, following the same reasoning as above, to basal bud break.

The effectivity of ZR in side-shoot formation, as was shown in *in vitro* experiments, combined with the fact that ZR is the major translocation form of cytokinins in the xylem points at a key role for ZR or its immediate metabolite, which might be zeatin, in growth and development of rose plants.

Key words: apical dominance, auxin, axillary shoots, bleeding sap, bottom breaks, bud break, bud growth, combination plants, cytokinins, *in vitro* culture, metabolism, root temperature, rootstock genotype, *Rosa hybrida*, rose, scion, xylem.

Contents

Abbreviations

AMP	adenosine-5'-monophosphate
BA	benzyladenine
BAR	benzyladenosine
C	total cytokinin content in the shoot (pmol)
DDAR	dideoxyadenosine
DHZ	dihydrozeatin
DHZ7G	dihydrozeatin riboside-7-glucoside
DHZ9G	dihydrozeatin riboside-9-glucoside
DHZMP	dihydrozeatin riboside-5'-monophosphate
DHZOG	dihydrozeatin-O-glucoside
DHZR	dihydrozeatin riboside
DHZROG	dihydrozeatin riboside-O-glucoside
EIA	enzyme immuno assay
GA	gibberellic acid
GC-MS	gas chromatography-mass spectrometry
HPLC	high-performance liquid chromatography
I	import of cytokinins into the shoot (pmol)
IAA	indole-3-acetic acid
IAC	immunoaffinity chromatography
IBA	indole-3-butyric acid
iP	N^6-(Δ^2-isopentenyl)adenine
iPAR	N^6-(Δ^2-isopentenyl)adenosine
iPMP	N^6-(Δ^2-isopentenyl)adenosine-5'-monophosphate
Δ^2-iPP	Δ^2-isopentenyl pyrophosphate
KMP	kinetin riboside-5'-monophosphate
LC-MS	liquid chromatography-mass spectrometry
PBS	phosphate buffered saline (pH 7.0)
PBST	phosphate buffered saline + Tween 20 (pH 8.4)

RDR	relative degradation rate (pmol $pmol^{-1}$ day^{-1})
RH	relative humidity
T_{day}	transpiration rate during the photoperiod (ml h^{-1})
T_{half}	half-life of the cytokinin pool in the shoot (days)
T_{night}	transpiration rate during the night (ml h^{-1})
Tris	2-amino-2-hydroxymethyl-1,3-propanediol
UR	unidentified cytokinin-like compound
Z	zeatin
Z7G	zeatin-7-glucoside
Z9G	zeatin-9-glucoside
Z-ala	lupinic acid
ZMP	zeatin riboside-5'-monophosphate
ZOG	zeatin-O-glucoside
ZR	zeatin riboside
ZROG	zeatin riboside-O-glucoside

Chapter 1

General Introduction

1.1 Introduction

In the Netherlands, the rose is the most important cut flower. In 1996, 913 ha cut roses divided over 898 nurseries produced more than 2100 million flowers, with a value of almost Dfl. 856 million (Anonymous, 1997). At present, to improve flower production (De Vries and Dubois, 1983; Kool and Van de Pol, 1992), by estimation 50% of the cut roses in the Netherlands are grown on rootstocks (Van der Salm, 1996). Although a considerable amount of rootstock research has been done, the way rootstocks affect the physiology of the scion is still little understood. A number of authors have suggested that rootstocks inducing differences in the vigour of the scion might differ in cytokinin production and export to the shoot (Zieslin and Khayat, 1983; De Vries, 1993; Kool and Van de Pol, 1996), thereby affecting bud break and outgrowth of the scion. Until now, reports on endogenous cytokinins in xylem sap and plant tissues in relation to the development of roses are not available. In the present study, endogenous cytokinins in rose combination plants are described, which should ultimately lead to a better understanding of the role of cytokinins in the relationship between the rootstock and the scion.

1.2 Roses

1.2.1 Culture

Roses are perennial woody shrubs that are genetically programmed to initiate an apical flower bud on each shoot, when a certain more or less constant number of leaves is formed. Cut roses continuously form new shoots, which are harvested by cutting just above their first or second full-grown leaf from below. The remnant stem part bears several axillary buds, from which, after bud break, new flowering shoots can be formed.

1.2.2 Plant development

Rose cultivars can be grafted onto rootstocks of different genotypes. The axillary bud of a so-called combination plant develops into a primary shoot that flowers approximately 8 weeks after grafting. At the same time, the uppermost 2-4 axillary buds break, forming

lateral shoots. Several weeks later, at the base of the primary shoot, one or more vigorous shoots appear. These so-called bottom breaks form the frame of a rose bush and determine mainly its future yield of flowers (Zieslin *et al.*, 1973; De Vries and Dubois, 1983). Bottom breaks are thicker, longer, have a higher anthocyanin content, bear more thorns than the lateral shoots, and form a greater number of leaves before the flower appears (Zieslin and Moe, 1985). The number of bottom breaks can be stimulated by cutting back the primary shoot and removing axillary shoots, by environmental conditions like high CO_2, high humidity, high light intensity and a period of low temperature, by exposure to ethylene and application of cytokinins (Khayat and Zieslin, 1982).

1.2.3 Rootstocks

Rose rootstocks are primarily selected for high vigour, as basal buds of plants grafted onto vigorous rootstocks break earlier and more profusely, resulting in more bottom-breaks at an earlier stage of plant development, and consequently in higher shoot yield (De Vries, 1993; De Vries and Dubois, 1989). Other important selection criteria are rooting ability (Van der Salm, 1996), resistance to diseases and pests, ability to tolerate continuous harvesting of the scion, compatibility with various rose cultivars, and ability to perform well under different environmental conditions (Leemans and Van de Laar, 1977). Using combination plants enables breeding of the scion cultivars to be aimed at optimizing shoot properties, like colour, shape and longevity of the flower, disregarding its rooting characteristics.

Numerous experiments have demonstrated that rootstocks are able to improve flower production of the scion cultivar (De Vries, 1993). However, due to the large number of cultivars and of rootstock genotypes used, the literature present on rootstock/scion performance is very elusive. Little is known on the precise influence of rootstocks on plant development (Fuchs, 1994). The effect of rootstocks on axillary bud break of the scion, together with the ability of exogenous cytokinins to break bud dormancy (Pillay and Railton, 1983) points at the involvement of root produced cytokinins. De Vries (1993) suggested that vigorous rootstocks, by supplying a relative high amount of cytokinins to the scion might advance bud break and induce outgrowth of more buds. Control of scion growth via cytokinin production was also suggested for *Vitis vinifera* (Skene and Antcliff, 1972) and watermelon (Yamasaki *et al.*, 1994), and led to the idea that the performance of a rootstock might be improved by exogenous application of cytokinins. Indeed, application of BA in lanolin paste to intact rose plants was found to stimulate bud break of axillary buds (Ohkawa, 1984) and bottom breaks (Parups, 1971). Until now, rose breeders usually recommend certain cultivar/rootstock combinations on basis of greenhouse trials. Regarding the variation of cultivars and rootstocks, the selection process of rootstocks takes several years. Therefore, a rootstock characteristic that can be used as an early selection criterion would accelerate this process considerably. Possibly, cytokinin production might be used as such.

1.3 Apical dominance

Outgrowth of axillary buds is under the control of the shoot apex, a process referred to as apical dominance (Cline, 1991). Several theories have been formulated to explain the

phenomenon of apical dominance (Hillman, 1984; Cline, 1991, 1994). In the 'direct auxin action' theory, auxin, primarily produced by the shoot apex and the young, expanding leaves, moves basipetally down the shoot to axillary buds and inhibits their outgrowth. Decapitation of the plant involves the removal of the auxin source and, hence, depletion of auxin in the inhibited lateral bud, and its subsequent outgrowth (Hillman, 1984; Cline, 1991). This theory is supported by the fact that application of auxin to the decapitated shoot stump eliminated the effect of shoot tip removal. One serious criticism of the direct auxin inhibition hypothesis is the finding that endogenous auxin levels in lateral buds do not decrease prior to release from apical dominance as would be expected. Therefore, second messengers, such as ethylene or abscisic acid, have been proposed to play a role in the transfer of the inhibiting message to the lateral buds (Cline, 1991, 1994). This is referred to as the 'indirect auxin hypothesis'.

In the 'nutrient-diversion' theory, auxin creates a flow of nutrients towards the apex, the main site of auxin production, such that the axillary buds become starved, thereby inhibiting their outgrowth.

In the 'vascular-connection' theory, vascular connections between the lateral bud and the main stem are assumed to be necessary for the initiation of bud outgrowth. Auxin is thought to prevent the entry of factors into the lateral buds by an effect on the vascular connections between bud and stem, perhaps by an effect on vascular differentiation. Analysis of bud growth, however, indicated that a correlation does not always exist between vascular development and the loss of the quiescent state (Hillman, 1984).

In the 'hormonal balance' theory, a balance between the inhibitory and stimulatory effects of hormones on bud development has been suggested (Hillman, 1984). Cytokinins promote axillary bud break (Sachs and Thimann, 1967; Pillay and Railton, 1983), and are likely to play a secondary interacting role with auxins (Cline, 1991; Li *et al.*, 1995). Bangerth (1994) showed that decapitating bean plants led to a 40-fold increase in cytokinin concentration in the xylem sap. Application of auxin to the shoot of decapitated plants almost eliminated the effect of shoot tip removal on cytokinin concentration, suggesting that cytokinins in the xylem sap of intact plants are under the control of the polar auxin transport system. Transformed plants expressing the *ipt* (isopentenyl transferase) gene exhibited increased cytokinin concentrations and a bushy phenotype, i.e. a low degree of correlative inhibition (Van Loven *et al.*, 1993; Zhang *et al.*, 1995; Redig *et al.*, 1996).

1.4 Cytokinins

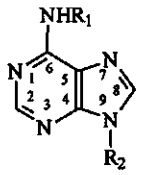
The idea that specific substances are required for cell division occurring in plants dates back to Wiesner who proposed in 1892 that initiation of cell division is evoked by endogenous factors. Haberlandt (1913) discovered that phloem diffusates could induce cell division in parenchyma tissue of cut potato tubers. In the 1950s, Skoog and co-workers (Miller *et al.*, 1955, 1956) investigated the nutritional requirements of tissue cultures derived from tobacco stem pith. On defined media in the presence of auxin, the pith cells enlarged, but failed to divide. Cell division was restored after the addition of substances like coconut milk, autoclaved DNA and yeast extract. The active compound from autoclaved herring sperm DNA was isolated and called 'kinetin' (Miller *et al.*, 1955). This is an artefactual rearrangement product of heated DNA and is not found in

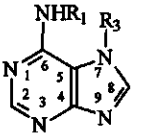
plant tissues. The first naturally occurring plant cytokinin was purified from immature kernels of *Zea mays* by Letham (1963), and identified as zeatin.

Cytokinins have been defined as substances which stimulate cell division in plants and which interact with auxin in determining the pattern of cell differentiation. Figure 1.1 shows the structure of the major naturally occurring cytokinins. Nowadays the term cytokinin is used for all purine compounds with an 5-carbon N^6-substituent, regardless of whether they exhibit cell dividing activity. Structure/activity relationships of cytokinins have been extensively studied in several bioassays (reviewed by Matsubara, 1990). In plants, three groups of cytokinins can be found, namely Z-, DHZ- and iP-type cytokinins. In addition, in some plants the presence of aromatic cytokinins has been reported (Ernst *et al.*, 1983; Strnad *et al.*, 1992). In general, free bases and perhaps also ribosides, nucleotides and O-glucosides are considered to be active cytokinin forms (Letham and Palni, 1983; Van der Krieken *et al.*, 1990). Ribosides are important translocation forms in the xylem (Jameson *et al.*, 1987; Wagner and Beck, 1993). Nucleotides may be associated with cytokinin uptake and transport across membranes (Letham and Palni, 1983). The 9-alanyl conjugates of Z and DHZ and the 7- and 9-glucosides are biologically inactive and extremely stable, and are considered to be important in the deregulation of levels of active cytokinins. In contrast, the O-glucosides are biologically active. They are suggested to be storage forms that can easily be hydrolyzed to their aglycones when required (McGaw, 1987).

Biosynthesis of cytokinins starts with the synthesis of iPMP from AMP and Δ^2-iPP, catalysed by the enzyme isopentenyl transferase (Letham and Palni, 1983). IPMP is stereospecifically hydroxylated to form zeatin derivatives, which has to occur rapidly, since iPMP, iPAR and iP are rarely found as free compounds in most plants (Letham and Palni, 1983; McGaw and Burch, 1995). This hydroxylation step occurs at the level of the nucleotide as was shown by feeding studies (McGaw and Burch, 1995). Ribosides, free bases and glucosides are formed thereafter by dephosphorylation, reduction and glucosylation. The biosynthesis and metabolism of cytokinins is depicted in Figure 1.2 (after Wagner, 1991 and Jameson, 1994). This Figure shows that hydroxylation of iPMP to ZMP is irreversible; dehydroxylation of Z-type into iP-type cytokinins was never reported in plants. Reduction of Z to DHZ-type cytokinins was first reported for *Phaseolus vulgaris* by Sondheimer and Tzou (1971). Characteristics of an enzyme mediating this reduction in *Phaseolus* embryos were described by Martin *et al.* (1989).

Cytokinins are mainly produced in root tips and move via the xylem to the shoot where they participate in the control of growth and development (Van Staden and Davey, 1979). Furthermore, cytokinins are synthesized in actively growing tissues, like young leaves and stems (Chen *et al.*, 1985). Most likely, cytokinins transported into the buds are involved in the induction of bud break and the subsequent outgrowth (Letham, 1994). In addition to promoting bud break by reducing the dominance of the apical bud, cytokinins induce cell division (Skoog *et al.*, 1965), delay senescence of intact plants (Noodén *et al.*, 1990) and excised plant parts (Singh *et al.*, 1992c), affect source/sink relationships (Kuiper, 1988), stimulate chloroplast development and enhance the resistance of plants to various forms of stress (Kamínek, 1992).





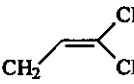
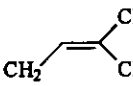
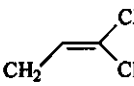
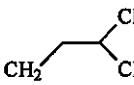
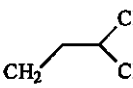
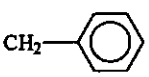
R_1	R_2	R_3	trivial name	abbreviation
	H	-	$N^6(\Delta^2$-isopentenyl) adenine	iP
	ribosyl	-	$N^6(\Delta^2$-isopentenyl) adenosine	iPAR
	ribotide	-	$N^6(\Delta^2$-isopentenyl) adenosine-5'-monophosphate	iPMP
	H	-	zeatin	Z
	ribosyl	-	zeatin riboside	ZR
	ribotide	-	zeatin riboside-5'-monophosphate	ZMP
	glucosyl	-	zeatin-9-glucoside	Z9G
	-	glucosyl	zeatin-7-glucoside	Z7G
	alanyl	-	lupinic acid	Z-ala
	H	-	zeatin-O-glucoside	ZOG
	ribosyl	-	zeatin riboside-O-glucoside	ZROG
	H	-	dihydrozeatin	DHZ
	ribosyl	-	dihydrozeatin riboside	DHZR
	ribotide	-	dihydrozeatin riboside-5'-monophosphate	DHZMP
	glucosyl	-	dihydrozeatin-9-glucoside	DHZ9G
	-	glucosyl	dihydrozeatin-7-glucoside	DHZ7G
	H	-	dihydrozeatin-O-glucoside	DHZOG
	ribosyl	-	dihydrozeatin riboside-O-glucoside	DHZROG
	H	-	benzyladenine	BA
	ribosyl	-	benzyladenosine	BAR

Figure 1.1 *Structure of the major naturally occurring cytokinins in plants.*

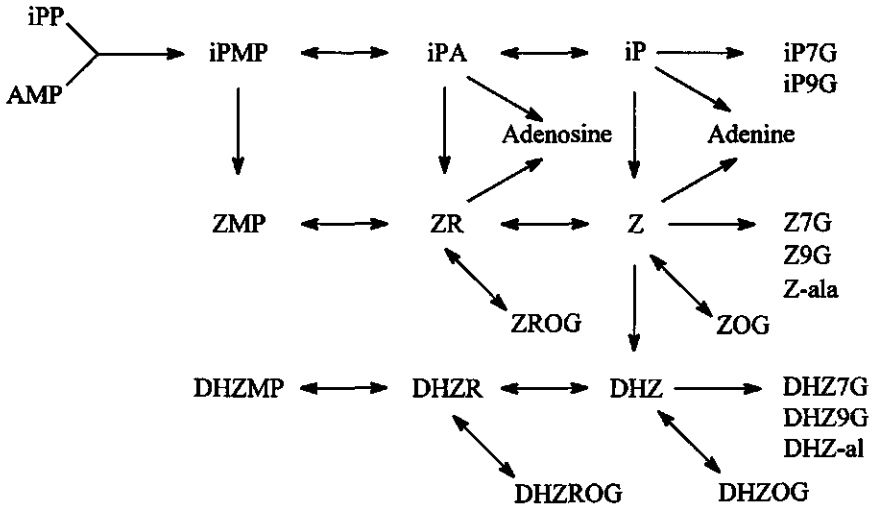


Figure 1.2 *Biosynthesis and metabolism of cytokinins in plants (after Wagner, 1991 and Jameson, 1994).*

1.5 Aim of the thesis

The aim of the present study is to obtain knowledge on the relationship between endogenous cytokinins and shoot development of rose combination plants. This knowledge can be used to determine if cytokinins can be used as an early selection criterion for rose rootstocks. Firstly, we tried to gain insight in the course of endogenous cytokinins of rose plants throughout plant development. Secondly, we tried to establish a quantitative relationship between cytokinin export from the roots and developmental processes occurring in the shoot. Lastly, an attempt was made to relate the cytokinin production of a rootstock genotype to its vigour, expressed in terms as the readiness of bottom breaks to sprout.

1.6 Outline of the thesis

In Chapter 2, based on data obtained at three stages of development, the course of the endogenous cytokinin concentration in bleeding sap and in roots, stems, leaves, axillary shoots and bottom breaks of Madelon/Multic combination plants was described. Export of cytokinins from the roots and their degradation rate in the shoot in relation to plant development were estimated in Chapter 3. These estimations are based on the assumption that the cytokinin concentration in bleeding sap is representative for that in xylem sap *in situ*. This was tested by comparing the cytokinin concentration in bleeding sap and in sap obtained after pressurizing the root system to a level equivalent to the leaf water potential (Chapter 3). The hypothesis that a rootstock that advances bud break of the scion does so by supplying more cytokinins to the shoot was tested by measuring cytokinin levels in

bleeding sap of Madelon grafted on two rootstock genotypes differing in vigour during plant development (Chapter 4). In this Chapter, furthermore an effort was made to describe bud break in rose combination plants as the result of an interaction between auxin and cytokinins. Besides by grafting on a rootstock of a different genotype, growth and development of the scion might be influenced by environmental conditions. This was examined in Chapter 5, in which the effect of root temperature on plant development and on the cytokinin concentration in bleeding sap was examined. In Chapter 6, the attention was focussed on the effectiveness of a series of cytokinin free bases and ribosides on bud break and outgrowth of *in vitro* grown axillary buds. The more effective cytokinins might either have a high rate of uptake, or be more readily metabolized, resulting in a high concentration of 'active' cytokinin. In Chapter 7, we aimed at resolving this point, by using data on uptake of ^{3}H-labelled cytokinins varying in effectiveness. In Chapter 8, in a general discussion an attempt was made to integrate the results of the previous chapters.

Chapter 2

Cytokinins in Relation to Bud Break

2.1 Abstract

To assess the role of endogenous cytokinins in growth and development of *Rosa hybrida*, their concentrations in bleeding sap and in roots, stem, leaves, axillary shoots and bottom breaks in three stages of development were quantified. Cytokinins were purified by means of immunoaffinity chromatography and HPLC, and identified by retention time, UV spectrum and GC-MS. The major translocation form in the xylem was ZR. In all mature tissues, cytokinins of the Z-type were predominant, amounting to 80-90% of the total cytokinin concentration. The stems contained high concentrations of cytokinins, probably caused by lateral movement of ZR from the xylem to adjacent stem tissue and the ability of the stem to metabolize cytokinins. In young leaves the contribution of iP-type cytokinins to the total cytokinin pool was about 50%, which indicates that these leaves might be capable of *de novo* synthesis of cytokinins. In older leaves, the concentration of an unidentified cytokinin-like compound increased to more than 50% of total cytokinins. This compound, which was also found in the roots might be a storage form of cytokinins. In young axillary shoots, about 50% of the cytokinins are iP-compounds, suggesting either import of iP-type cytokinins via the phloem or *de novo* synthesis of cytokinins. In buds forming bottom breaks, ZR and ZMP are the main cytokinins, indicating that these buds receive their cytokinins from the roots.

2.2 Introduction

In commercial cut-rose growing in The Netherlands, cultivars are usually grafted onto rootstocks of a different genotype (De Vries and Dubois, 1983). The primary shoot of these combination plants flowers approximately 7 weeks after grafting (Figure 2.1A). At the same time, the uppermost 2-4 axillary buds break, forming axillary shoots that will grow longer than the primary shoot itself (Figure 2.1B). Several weeks later, at the base of the primary shoot, one or more vigorous shoots appear. These bottom breaks form the frame of a rose bush and determine mainly their future yield of flowers. The genotype of the rootstock affects bud break of the scion (Kool and Van de Pol, 1992), possibly caused by differences in cytokinin production. It is widely accepted that cytokinins are mainly produced in the root tips and are translocated via the xylem to the shoot. Most likely,

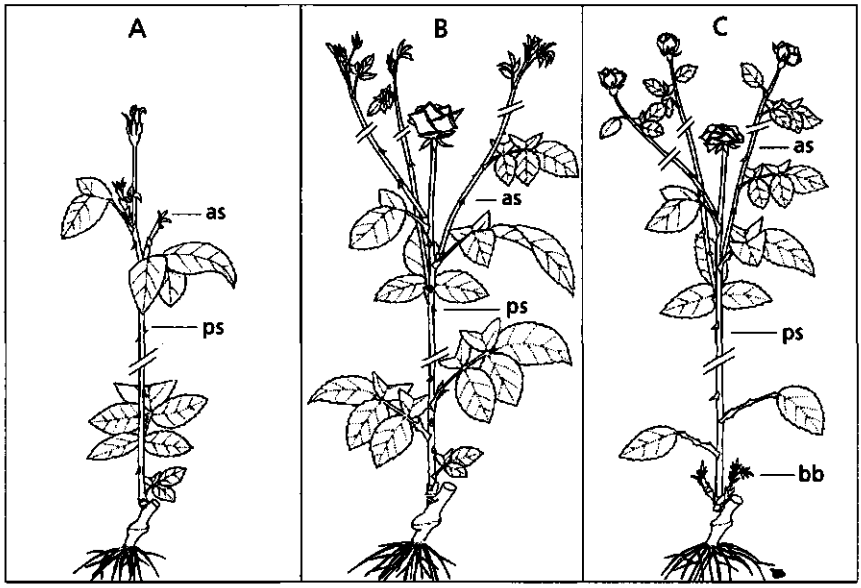


Figure 2.1 *Schematic representation of the developmental stages of Madelon/Multic combination plants; (A) stage 1, primary shoot with 1-2 cm axillary shoots; (B) stage 2, flower buds of the axillary shoots visible, buds forming the bottom breaks swelling; (C) stage 3, flowers of the axillary shoots open and bottom breaks 8-10 cm long. Stages 1, 2 and 3 were reached 7, 9 and 12 weeks after grafting respectively. ps = primary shoot, as = axillary shoot, bb = bottom break.*

root-derived cytokinins transported into the buds are involved in the induction of bud break (Qamaruddin *et al.*, 1990; Letham, 1994). Exogenous application of BA to rose plants broke the quiescence of axillary buds (Ohkawa, 1984). Pillay and Railton (1983) demonstrated bud break of intact seedlings of *Pisum sativum* following a single application of BA or Z. In addition to promoting bud break by reducing the dominance of the apical bud, cytokinins delay senescence, stimulate chloroplast development and enhance the resistance of plants to various forms of stress (Kamínek, 1992). In plants, three groups of cytokinins can be found, namely Z-, DHZ- and iP-type cytokinins. In addition, in some plants aromatic cytokinins are reported (Ernst *et al.*, 1983; Strnad *et al.*, 1992). In general, the free base cytokinins, and perhaps also the ribosides, ribotides and O-glucosides are active, whereas the N-glucosides and alanines are considered inactive (McGaw and Burch, 1995).

To assess the role of endogenous cytokinins in the development of rose plants, it is essential to know how and where they are synthesized within the plant, how they are transported from their site(s) of synthesis, and how they are metabolized in various tissues. Growth and development of a rose plant is primarily determined by bud break of the axillary shoots and bottom breaks. Knowing the cytokinin concentrations in bleeding sap and in various plant parts at developmental stages around bud break is essential to

establish the involvement of cytokinins in these processes of bud break. There are only a few reports describing cytokinin patterns in certain rose plant parts. Lukaszewska *et al.* (1994) were able to detect Z, ZR, iP and iPAR in rose petals. Van Staden *et al.* (1981) reported the presence of Z and ZR in rose axillary buds. To our knowledge no extensive attempts have been made to quantify cytokinins in xylem sap and plant parts of *Rosa hybrida*.

In *Xanthium strumarium*, ZR was the major cytokinin in the apical buds and youngest developing leaves, whereas it did not greatly contribute to the cytokinin content of mature leaves (Henson and Wareing, 1976). In stems and roots, Z exceeded ZR. In mature leaves, the proportion of an unknown cytokinin, probably a zeatin conjugate, increased until it accounted for about 80% of the detectable cytokinins. More recently, Wagner and Beck (1992) determined the cytokinin patterns of young and mature leaves, stems and roots of the herbaceous plant *Urtica dioica*. ZR appeared to be the major cytokinin in roots, stems and young, developing leaves, whereas Z and ZOG predominated in fully developed leaves. In the bleeding sap of several plant species, cytokinin ribosides predominate (Henson and Wareing, 1976; Jameson *et al.*, 1987, Wagner and Beck, 1992). However, in some cases, free bases, O-glucosides and nucleotides could also be detected (Jameson *et al.*, 1987).

The present study describes the cytokinin patterns of *Rosa hybrida* in three stages of development around bud break and outgrowth of axillary shoots and bottom breaks. The endogenous cytokinin levels will be correlated to bud break and outgrowth. These data may form a basis for further studies on the role and mode of action of cytokinins in processes concerning bud break and outgrowth.

2.3 Materials and methods

2.3.1 Plant material

A shoot of the rootstock Multic was cut into segments, each bearing a dormant bud and an adherent leaf. The bases of the segments were dipped in 0.5% IBA and prerooted for 7 days in a mixture of sand and peat (1:1, v/v) at a RH of 90-100% and a temperature of 25°C. The stem of a harvestable shoot (sepals down) of the rose cultivar Madelon was cut into segments bearing one intact five-leaflet leaf and a dormant bud. The cultivar was grafted onto the prerooted rootstock of which the leaf and bud were removed (Van de Pol and Breukelaar, 1982). The combination plants were placed in a mixture of sand and peat (1:1, v/v) at a RH of 90-100% and a temperature of 25°C. After 2.5 weeks, the plants were transferred to nutrient solution (Steiner, 1984) in a growth room at a temperature of 20°C, RH of 75% and a photoperiod of 14 h. The photosynthetically active radiation was 330 $\mu mol\ m^{-2}\ s^{-1}$ (Philips TLD, 50 W) at plant base level at the start of the experiment.

Uniform plants were selected and randomly allocated to 6 groups of 3 x 3 plants. Three developmental stages were defined: (1) primary shoot with 1-2 cm axillary shoots; (2) flower buds of the axillary shoots visible, buds forming the bottom breaks swelling; (3) flowers of axillary shoots open and bottom breaks 8-10 cm long (Figure 2.1). Stages 1, 2 and 3 were reached 7, 9 and 12 weeks after grafting respectively.

2.3.2 *Sample collection*

At each of the three defined developmental stages, one group of plants was used to collect bleeding sap. One hour after the start of the photoperiod, the plants were cut off just below the primary shoot. The first drop of exudate contaminated by rests of damaged cells was discarded. A piece of silicone tubing was mounted onto the stump. A capillary tube was inserted and led the exudate to a vial kept at 2°C, containing 1 µmol Na_2MoO_4 to inhibit phosphatase activity. The exudate was collected for 30 min, and stored at -80°C until purification.

The remaining three groups of plants were used to determine the cytokinin concentration in various plant parts. From each plant, the upper 4 leaves, the lowest 4 leaves, the middle leaves and the stem of the primary shoot, the axillary shoots, the bottom breaks and the roots were weighed and immediately dropped into liquid nitrogen and stored at -80°C until extraction. In stages 1 and 2, the fresh weights of the buds forming the bottom breaks were approximately 20 and 60 mg respectively. In order to determine the cytokinin content in the buds, the sample included 1 cm adherent stem tissue (approximately 0.5 g); later the cytokinin content of a piece of stem with a similar weight was subtracted. For comparison, the sample of stage 3 bottom breaks included 1 cm stem tissue as well.

2.3.3 *Extraction and purification*

The pH of the bleeding sap was adjusted to 3.7 with 10 mM NH_4-formate buffer, and passed by gravity through a Sep-pak C_{18}-cartridge (Waters, Milford, MA, USA), which had been primed with 10 ml 96% ethanol and 10 ml 10 mM NH_4-formate buffer at pH 3.7. The cartridge was washed with 10 ml NH_4-formate buffer and 60 ml deionized water, and the cytokinins eluted with 10 ml 96% ethanol. The eluate was evaporated to dryness.

Frozen plant material, maximally 20 g per sample, was ground to powder in a mortar under liquid nitrogen. The stems were powdered in a Junke/Kunkel mill (IKA-Werk, Staufen im Breisgrau, Germany). The powder was extracted in boiling 96% ethanol (10 ml g^{-1} fresh weight) for 10 min. After cooling down to room temperature, the extract was centrifuged (10 min, 4500 g) and the pellet re-extracted twice in 80% cold ethanol for 30 min. The combined supernatants were reduced by evaporation until free of ethanol. The extract was kept at -20°C overnight, thawed and centrifuged (10 min, 4000 g, 4°C). The supernatant was reduced to approximately 5 ml. An equal volume of 20 mM NH_4-formate buffer was added (final pH 3.7). The sample was passed through a column of 10-15 ml of polyvinylpolypyrrolidone (Sigma, St. Louis, MO, USA) and lyophilized. To all samples 0.75 pmol ^{3}H-ZR (0.9 TBq $mmol^{-1}$, obtained from Dr M. Strnad, Inst. of Experimental Botany, Czech Academy of Sciences, Olomouc, Czech Republic) was added as an internal standard at the beginning of the extraction procedure.

2.3.4 *Immunoaffinity chromatography (IAC)*

Immunoaffinity gels were prepared as described by Nicander *et al.* (1993). The antibodies were able to recognize modifications at positions 2, 3 and 9 of the purine ring, but not at 7 or at the hydroxyl group of the N^6-side chain. This implies that the cytokinin free bases, ribosides, nucleotides and 9-glucosides did bind to the affinity columns, but that O- and 7-

glucosides did not. The samples were taken up in PBS buffer (35 mM Na_2HPO_4, 17 mM NaH_2PO_4, 0.14 M NaCl), and after adjusting the pH to 7.0, filtered through a Munktell filter paper (Stora, Grycksbo, Sweden) and passed through a pre-immune column containing 2 ml Sepharose 6B (Pharmacia, Uppsala, Sweden). The samples were split in fractions corresponding to maximally 3.5 g fresh weight, each fraction was run separately on an IAC column. Ethanol 30%, PBS and water were degassed; PBS and water were kept at 37°C. After washing the column with 4 ml PBS, the sample was loaded. The column was washed with 14 ml PBS and 400 µl water. Each column was eluted with 1 ml water, 1 ml 30% ethanol and 4 ml 99.5% ethanol. The IAC columns were washed with 4 ml 99.5% ethanol, 4 ml water and 6 ml PBS, and were then ready for another cycle of purification. The eluates were reduced by evaporation and run on IAC columns a second time. It was necessary to do IAC twice, since after one IAC run non-cytokinin like compounds were still present in the eluate (data not shown). DDAR (100 pmol) was added as a standard for HPLC retention times. The eluates were reduced to approximately 300 µl by evaporation (Evapotek; Haake-Büchler Instruments, Saddle Brook, NJ, USA). At regular intervals, the wash of 14 ml PBS and 400 µl water was IAC purified and ran on HPLC to check for the presence of cytokinins. No loss of binding capacity of the IAC columns could be detected during the experiment.

2.3.5 High-performance liquid chromatography (HPLC)

Cytokinins were separated on a 75 x 4 mm Supersphere RP-Select B + 125 x 4 mm Lichrosphere RP-Select B column (Merck, Darmstadt, Germany). The eluents contained 2% HPLC-grade acetic acid (Merck, Darmstadt, Germany), and the column was eluted with a gradient from 1 to 40% acetonitrile (HPLC-grade, Rathburn, Walkerburn, UK). A stepped linear gradient with the following profile was used: 0 min 1% acetonitrile, 8 min 3%, 21 min 16.6%, 25 min 40%, 30 min 40%, 34 min 1%, 42 min 1%.

The nucleotidase treated peaks were separated on a 125 x 4 mm Lichrosphere RP-Select B column (Merck, Darmstadt, Germany). A stepped linear gradient with the following profile was used: 0 min 1% acetonitrile, 3 min 3%, 13 min 16.6%, 16 min 40%, 19 min 40%, 22 min 1%, 30 min 1%.

In both systems, the flow rate was 2 ml min^{-1}, and the column temperature 35°C. The HPLC pump was a Merck-Hitachi L6200A. Spectra were obtained with a scanning spectrophotometric HPLC detector (Spectra-Focus, Spectra-Physics, San José, CA, USA). The detection limit was 10 pmol per sample. Fractions containing peaks of interest were collected.

To quantify the recovery of the method, the radioactivity of the ZR fraction was determined by scintillation counting in Ultima gold (Packard, Downers Grove, IL, USA).

2.3.6 Treatments with Crotalus 5'-nucleotidase

HPLC peaks eluting at the ZMP, DHZMP and iPMP positions were evaporated to dryness and dissolved in 350 µl 100 mM Tris-HCl (pH 8.0). Samples were incubated with 0.1 U of *Crotalus* 5'-nucleotidase (EC 3.1.3.5; Sigma, St. Louis, MO, USA) in the presence of 10 mM $MgCl_2$ and 50 pmol KMP as an internal standard at 37°C for 30 min. The incubation was stopped by addition of 550 µl ice-cold methanol. The samples were left on ice

for 30 min, centrifuged, reduced by evaporation to approximately 150 µl and injected into the HPLC.

2.3.7 Gas chromatography-mass spectrometry (GC-MS)

HPLC peaks of interest were evaporated to dryness and dissolved in methanol. Standards and samples were permethylated according to Kovác (1993) with some modifications as chloroform (p.a., Merck, Darmstadt, Germany) was used instead of dichloromethane, and the organic phase was washed twice with water before it was evaporated to dryness in a stream of nitrogen at 60°C. The samples were dissolved in 3 µl chloroform; 1 µl was injected. GC-MS was performed using a gas chromatograph (model 8000 Carlo Erba) to which a Fisons MD 800 EI 60 mass spectrometer (Fisons Instrument, UK) was coupled. The mass spectrometry conditions were: EI ionizing voltage 70 eV; source temperature 300°C; interface temperature 290°C. GC-MS chromatograms were obtained by selected ion monitoring (SIM) with a dwell time of 50 ms and a mass range span of 0.3 amu. The GC system had an on column injector and a DB-5MS column (J&W Scientific, Folsom, CA, USA; 15 m x 0.25 mm x 0.1 µm) with helium as the carrier gas at 4 psi. The temperature programme was 1 min at 70°C, 10°C min^{-1} to 290°C and finally 10 min at 290°C.

2.3.8 Liquid chromatography-mass spectrometry (LC-MS)

The HPLC peak eluting at the ZMP position after treatment with nucleotidase, referred to as UR, was collected, evaporated to dryness and dissolved in 50 µl methanol/ammonium acetate 0.01 M (70/30, v/v). A 10 µl aliquot was analysed by LC-MS as described by Prinsen *et al.* (1995).

2.3.9 Enzyme immunoassay (EIA)

UR was examined for cross-reactivity with an antibody raised against ZR, by means of the indirect competitive EIA, as described in detail by Vonk *et al.* (1986).

2.3.10 Experimental set-up and calculations

The experiment was arranged in a randomized block design in which each of the 6 groups consisted of 3 repetitions. For each repetition, 3 plants were combined. Cytokinin contents were corrected for recovery, and expressed per ml bleeding sap or per gram fresh weight of the plant parts. To enable calculations, the amount of an unidentified cytokinin-like compound (UR) was expressed in pmol using the specific response of ZMP. The ratio Z-type cytokinins to DHZ-type to iP-type was calculated by dividing the sum of the concentrations of ZMP, UR, Z and ZR by the total cytokinin content, the sum of the concentrations of DHZMP and DHZR by the total cytokinin content and the sum of the concentrations of iPMP and iPAR by the total cytokinin content. The results are presented as Z : DHZ : iP in Tables 2.2-2.8, or, if no DHZ-type cytokinins could be detected, as Z : iP.

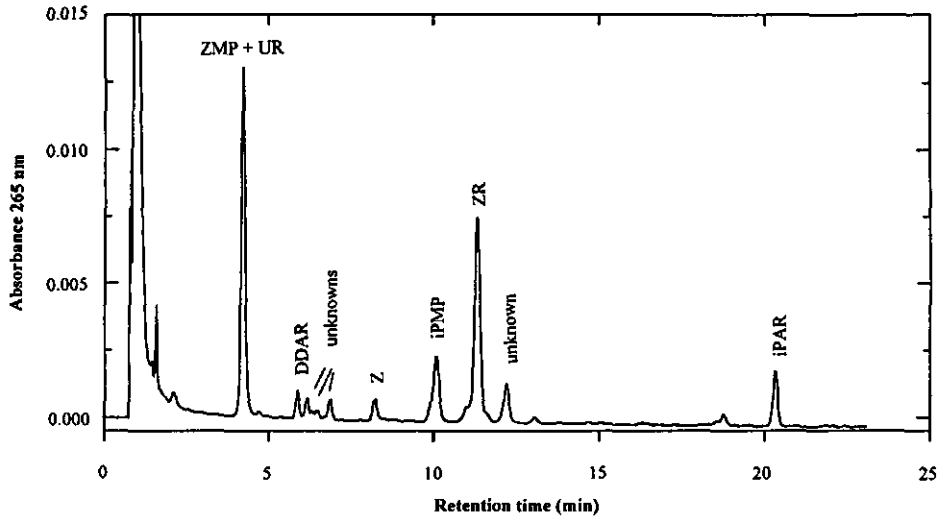


Figure 2.2 *Separation by HPLC of a root extract (stage 1). Column: 75 x 4 mm Supersphere RP-Select B + 125 x 4 mm Lichrosphere RP-Select B (Merck, Darmstadt, Germany). Flow rate: 2 ml min^{-1}. Column temperature: 35°C. Eluent: 2% acetic acid in water, with a gradient from 1-40% acetonitrile.*

2.4 Results

2.4.1 Characterization of cytokinins

The HPLC chromatogram of an IAC-purified root sample (Figure 2.2) showed 4 major peaks at the positions of ZMP, iPMP, ZR and iPAR with UV spectra typical of N^6-modified adenines with a substituent at the 9-position of the purine ring (N^6 + 9) and a peak at the position of Z with a UV spectrum typical of an N^6-modified adenine (see Nicander *et al.*, 1993). At retention times of 6.14, 6.45 and 6.85 min, peaks eluted with N^6 + 9 UV spectra. GC-MS results indicated that none of these peaks was Z9G, against expectation based on retention time. Just after ZR, an unknown compound eluted with a maximal UV absorption at 271 nm. In none of the samples could the free base iP be detected.

In order to check for peak purity, the peak co-eluting with the ZMP standard was treated with 5'-nucleotidase. In the HPLC chromatogram of the nucleotidase-treated sample a peak resulting from the dephosphorylation of ZMP was found at the position of ZR. A second peak with a retention time slightly later than ZMP had a N^6 + 9 UV spectrum and was referred to as UR (unidentified cytokinin-like compound). As can be concluded from the concentrations of ZMP and UR in Tables 2-8, 13% (leaves) to 97% (stems) of the peak at the ZMP position before nucleotidase treatment was ZMP indeed. The remaining part was found to be UR. The EIA showed that UR had cross-reactivity with the ZR-antibody, suggesting it to be a Z-metabolite. Furthermore, comparison of the LC-MS mass spectra of UR and the 9-alanyl conjugate of Z showed that UR was not this

Table 2.1 *Quantifications of cytokinins (pmol) by HPLC and GC-MS. The monitored ion-pairs are between parentheses. ZMP, iPMP and DHZMP were treated with nucleotidase before analysing by GC-MS as the corresponding ribosides.*

Cytokinin	HPLC	GC-MS (ion pairs monitored)
iPAR	86	95 (217/223), 93 (391/397)
Z	54	56 (230/235), 50 (261/266)
ZMP	101	96 (216/221), 98 (390/395)
iPMP	243	255 (217/223), 272 (391/395)
DHZMP	89	127 (250/253), 131 (393/395)

Z-9-alanyl conjugate. Treating the peak eluting at the iPMP position with 5'-nucleotidase revealed that the iPMP peak was about 60% (leaves) to 90% (stems) pure.

Scintillation counting of the ZR peaks showed that the recovery of all samples was about 50%. Comparison of the quantification of a number of cytokinins by HPLC and GC-MS (Table 2.1) shows that the HPLC peaks were sufficiently pure to allow routine quantification by UV absorbance. ZMP, DHZMP and iPMP had to be treated with nucleotidase before analysing by GC-MS as the corresponding ribosides.

2.4.2 Quantification of cytokinins

The only cytokinin that could be detected in bleeding sap was ZR. Its concentration was higher in stage 1 (36.5 ± 11.4 pmol ml^{-1}) than in stages 2 (16.9 ± 1.7 pmol ml^{-1}) and 3 (21.1 ± 2.0 pmol ml^{-1}).

The cytokinin concentrations in the roots decreased during the development of the plant, except for UR. The ratio of Z-type to iP-type cytokinins remained approximately 0.9:0.1 (Table 2.2). No DHZ-type cytokinins could be detected in the roots.

Total cytokinins in the leaves increased strongly as they became older, primarily due to an increase in UR (Tables 2.3-2.5). In full-grown leaves UR accumulated up to more than 50% of total cytokinins. In upper and middle leaves, the Z to iP ratio gradually increased. In stage 3, leaf yellowing started, followed by leaf drop.

In the stems, the total cytokinin concentrations and the Z to iP ratios remained approximately constant during their development (Table 2.6). Small amounts of DHZMP and DHZR could be detected in the stems. The concentration of UR in stems was lower than in the roots and leaves.

In stage 1, the axillary buds had developed into 1-2 cm long shoots (Figure 2.1A). In stage 2 these shoots consisted of a stem, mainly immature leaves and a flower bud that was just visible (Figure 2.1B). In stage 3 the axillary shoots were full-grown with fully opened flowers (Figure 2.1C). Their cytokinin concentration increased almost 3-fold from stage 1 to stage 3 (Table 2.7). The contribution of the iP-compounds to the total cytokinin pool, which was about 50% in stages 1 and 2, decreased as the axillary shoots matured.

The main cytokinins in the buds forming the bottom breaks in stages 1 and 2, ZR and ZMP were found to be present in high concentrations (Table 2.8). The total concentration of cytokinins decreased strongly during bud break and outgrowth. In stage 3, when the

bottom breaks had developed into shoots of 8-10 cm, the contribution of DHZMP and DHZR to the total cytokinin content reached 17%, which was the highest value measured in any of the rose tissues.

2.5 Discussion

Overviews of all cytokinins and their organ distribution are rarely reported. Most studies have been limited to the Z-type cytokinins determined in one or only a few plant parts (Henson and Wareing, 1976; Carmi and Van Staden, 1983; Turnbull and Hanke, 1985). To our knowledge, the most extensive study was performed by Wagner and Beck (1992), who determined the cytokinin pattern in *Urtica dioica* plants. They found the Z-type cytokinins to make up more than 90% of the total cytokinin content, which is in accordance with our results showing that in all mature tissues of *Rosa hybrida*, cytokinins of the Z-type were predominant, amounting to 80-90% of the total cytokinin concentration.

Cytokinin biosynthesis in the roots starts with the synthesis of iPMP from AMP and Δ^2-iPP, catalysed by the enzyme isopentenyltransferase (Letham and Palni, 1983). IPMP is stereospecifically hydroxylated to form zeatin derivatives, which has to occur rapidly, since iPMP, iPAR and iP are rarely found as free compounds in most plants (Letham and Palni, 1983; McGaw and Burch, 1995). Feeding studies have indicated that the hydroxylation step occurs at the level of the nucleotide (McGaw and Burch, 1995). In rose roots, ZR, ZMP and UR were the main cytokinins (Table 2.2). ZMP is most likely the immediate precursor of ZR, which is transported to the shoot via the xylem. The concentration of UR is constant during the development of the roots, in contrast to the other cytokinins of which the concentration decreased. This leads to a relative accumulation of UR, suggesting that UR might function as a storage form of cytokinins, which can be metabolized to an active form when required.

The only cytokinin that we could detect in the bleeding sap was ZR, suggesting it to be the major translocation form of cytokinins in rose, as was found for several other plant species (Jameson *et al.*, 1987; Wagner and Beck, 1992). The rise in xylem cytokinins occurring before and during bud break in apple (Tromp and Ovaa, 1990) and bean (Bangerth, 1994), coupled with the ability of exogenous cytokinins to break bud dormancy (Pillay and Railton, 1983) have lead to the idea that xylem cytokinins transported into the buds are involved in the induction of bud break and/or outgrowth (Letham, 1994). The high ZR concentration in the bleeding sap appeared to coincide with the outgrowth of axillary buds and to precede bud break of bottom breaks. This suggests that ZR in the xylem might be involved in bud break and/or outgrowth.

Our results show that rose stems contained high concentrations of cytokinins (Table 2.6), especially ZMP and ZR. Similar data were obtained for *Phaseolus vulgaris* by Palmer *et al.* (1981) and Carmi and Van Staden (1983). Jameson *et al.* (1987) found that if ^{3}H-ZR was supplied exogenously into the transpiration stream of *Lupinus angustifolius*, a considerable portion moved directly from the xylem into the bark. In the bark, the ^{3}H detected was primarily due to DHZ-O-glucosides and adenosine, in the xylem and pith to AMP and adenosine (Jameson *et al.*, 1987). Incubation of *Pisum sativum* stem segments with ^{3}H-iP yielded 10 radioactive peaks, the main ones being DHZ and adenosine (King and Van Staden, 1990). Both the lateral movement of ZR from the xylem to adjacent stem tissue and the ability of the stem to metabolize cytokinins may explain the presence of

Table 2.2 *Endogenous cytokinin concentrations (pmol g^{-1}, means ± SE, n = 3) in roots and the ratio Z-type to iP-type cytokinins. UR is expressed in pmol g^{-1} using the specific response of ZMP. nd = not detectable.*

	stage 1	stage 2	stage 3
ZMP	51.7 ± 4.9	32.0 ± 2.4	25.6 ± 4.9
UR	26.2 ± 1.0	24.3 ± 4.7	21.2 ± 2.1
Z	6.1 ± 0.8	4.8 ± 0.4	2.9 ± 0.3
ZR	79.4 ± 3.9	54.5 ± 10.4	23.3 ± 3.0
DHZMP	nd	nd	nd
DHZR	nd	nd	nd
iPMP	11.1 ± 3.7	8.6 ± 0.8	2.9 ± 0.2
iPAR	10.3 ± 3.0	6.1 ± 2.0	1.6 ± 0.2
Total	184.8 ± 8.0	130.3 ± 11.9	77.5 ± 6.1
Z : iP	0.88 : 0.12	0.89 : 0.11	0.94 : 0.06

Table 2.3 *Endogenous cytokinin concentrations (pmol g^{-1}, means ± SE, n = 3) in upper leaves and the ratio Z-type to iP-type cytokinins. UR is expressed in pmol g^{-1} using the specific response of ZMP. nd= not detectable.*

	stage 1	stage 2	stage 3
ZMP	8.8 ± 2.1	4.5 ± 0.4	16.1 ± 1.9
UR	8.4 ± 0.8	28.6 ± 1.7	85.6 ± 3.5
Z	nd	nd	nd
ZR	10.7 ± 2.9	11.0 ± 7.3	38.4 ± 1.6
DHZMP	nd	nd	nd
DHZR	nd	nd	nd
iPMP	14.3 ± 0.9	9.5 ± 0.5	12.6 ± 2.8
iPAR	11.5 ± 0.4	6.2 ± 1.0	3.7 ± 0.5
Total	53.7 ± 3.8	59.8 ± 7.6	156.4 ± 5.1
Z : iP	0.52 : 0.48	0.74 : 0.26	0.90 : 0.10

Table 2.4 *Endogenous cytokinin concentrations (pmol g^{-1}, means ± SE, n = 3) in middle leaves and the ratio Z-type iP-type cytokinins. UR is expressed in pmol g^{-1} using the specific response of ZMP. nd = not detectable.*

	stage 1	stage 2	stage 3
ZMP	12.3 ± 1.8	13.2 ± 0.4	15.6 ± 3.5
UR	21.8 ± 3.8	50.9 ± 0.4	108.1 ± 14.0
Z	nd	2.4 ± 1.0	nd
ZR	9.8 ± 1.9	18.2 ± 6.0	21.9 ± 14.7
DHZMP	nd	nd	nd
DHZR	nd	nd	nd
iPMP	18.2 ± 2.9	13.9 ± 0.4	16.4 ± 1.8
iPAR	9.4 ± 1.2	4.4 ± 0.2	10.1 ± 3.6
Total	71.5 ± 5.6	103.0 ± 6.1	172.1 ± 21.0
Z : iP	0.61 : 0.39	0.82 : 0.18	0.85 : 0.15

Table 2.5 *Endogenous cytokinin concentrations (pmol g^{-1}, means ± SE, n = 3) in lowest leaves and the ratio Z-type to iP-type cytokinins. UR is expressed in pmol g^{-1} using the specific response of ZMP. nd = not detectable.*

	stage 1	stage 2	stage 3
ZMP	16.5 ± 1.3	18.2 ± 1.5	16.8 ± 2.6
UR	32.6 ± 2.1	87.8 ± 9.0	109.7 ± 11.6
Z	nd	nd	nd
ZR	17.5 ± 6.1	24.6 ± 8.3	8.8 ± 0.9
DHZMP	nd	nd	nd
DHZR	nd	nd	nd
iPMP	14.4 ± 0.4	7.6 ± 1.5	17.5 ± 4.0
iPAR	12.1 ± 0.3	7.1 ± 0.1	6.4 ± 0.8
Total	93.1 ± 6.6	145.3 ± 12.4	159.2 ± 12.6
Z : iP	0.72 : 0.28	0.90 : 0.10	0.85 : 0.15

Table 2.6 *Endogenous cytokinin concentrations (pmol g^{-1}, means ± SE, n = 3) in stems and the ratio Z-type to DHZ-type to iP-type cytokinins. UR is expressed in pmol g^{-1} using the specific response of ZMP. nd = not detectable.*

	stage 1	stage 2	stage 3
ZMP	74.6 ± 4.4	89.0 ± 16.1	69.6 ± 15.9
UR	2.0 ± 0.5	4.8 ± 0.1	8.2 ± 1.5
Z	7.9 ± 1.3	7.1 ± 0.6	12.1 ± 1.5
ZR	56.2 ± 1.1	50.0 ± 0.7	50.7 ± 10.9
DHZMP	3.2 ± 0.4	5.5 ± 0.9	6.2 ± 1.5
DHZR	nd	2.4	3.8 ± 0.9
iPMP	27.4 ± 1.4	20.8 ± 0.4	40.2 ± 3.3
iPAR	5.1 ± 0.5	3.4 ± 1.4	4.5 ± 0.6
Total	176.4 ± 5.0	183.0 ± 16.2	195.3 ± 19.8
Z : DHZ : iP	0.80 : 0.02 : 0.18	0.83 : 0.04 : 0.13	0.72 : 0.05 : 0.23

Table 2.7 *Endogenous cytokinin concentrations (pmol g^{-1}, means ± SE, n = 3) in axillary shoots and the ratio Z-type to DHZ-type to iP-type cytokinins. UR is expressed in pmol g^{-1} using the specific response of ZMP. nd = not detectable.*

	stage 1	stage 2	stage 3
ZMP	7.8 ± 2.0	11.7 ± 1.8	21.4 ± 4.1
UR	0.9 ± 0.2	4.1 ± 0.8	32.3 ± 3.3
Z	nd	nd	2.0 ± 0.2
ZR	11.5 ± 1.8	6.9 ± 1.4	13.7 ± 6.3
DHZMP	nd	nd	2.1 ± 0.3
DHZR	nd	nd	nd
iPMP	9.1 ± 1.4	18.6 ± 0.8	24.1 ± 4.5
iPAR	4.5 ± 0.4	2.6 ± 0.5	2.6 ± 0.3
Total	33.8 ± 3.1	43.9 ± 2.6	98.2 ± 9.4
Z : DHZ : iP	0.60 : 0 : 0.40	0.52 : 0 : 0.48	0.71 : 0.02 : 0.27

Table 2.8 *Endogenous cytokinin concentrations (pmol g^{-1}, means ± SE, n = 3) in buds forming the bottom breaks (stages 1 and 2) and in bottom breaks (stage 3), and the ratio Z-type to DHZ-type to iP-type cytokinins. UR is expressed in pmol g^{-1} using the specific response of ZMP. nd = not detectable.*

	stage 1	stage 2	stage 3
ZMP	6080 ± 1140	1580 ± 156	67.6 ± 16.1
UR	nd	nd	6.5 ± 0.9
Z	68 ± 52	95 ± 14	7.5 ± 0.7
ZR	3850 ± 1210	738 ± 98	22.9 ± 4.2
DHZMP	106 ± 40	63 ± 28	14.2 ± 2.0
DHZR	nd	nd	10.2 ± 1.1
iPMP	nd	nd	17.8 ± 1.8
iPAR	311 ± 32	nd	nd
Total	10420 ± 1660	2476 ± 187	146.7 ± 16.9
Z : DHZ : iP	0.96 : 0.01 : 0.03	0.97 : 0.03 : 0.00	0.71 : 0.17 : 0.12

high concentrations of various cytokinins in the stem (Letham, 1994). The stem and the bottom breaks in stage 3, which primarily consist of stem tissue, are the only *Rosa hybrida* plant parts in which DHZR and DHZMP could be detected.

In young leaves of *Rosa hybrida*, the contribution of iP-type cytokinins to the total cytokinin pool was about 50%. Most likely, these leaves are capable of *de novo* synthesis of cytokinins, because iP-compounds were not detectable in bleeding sap, and to our current knowledge, dehydroxylation of Z-type into iP-type cytokinins has never been reported in plants. As the concentration of iP-type cytokinins in the leaves remained approximately constant, but their contribution decreased due to an increase in Z-type cytokinins, the relative importance of *de novo* synthesis of cytokinins decreased during the development of the plant. *De novo* synthesis of cytokinins in leaves was reported earlier for sunflower (Salama and Wareing, 1979), pea (Chen *et al.*, 1985) and *Perilla* (Grayling and Hanke, 1992). As the leaves matured, the contribution of UR to the total cytokinins increased to more than 50% in stage 3. In the roots, the contribution of UR increased to 27% in stage 3, whereas the contribution in the stems was found to be maximally 4%. These data suggest that UR might be a storage form of cytokinins, which accumulates at sites of cytokinin production. UR eluted early in the HPLC chromatogram, had an N^6 + 9 UV spectrum and showed cross-reactivity with the ZR antibody in the EIA, thereby suggesting it to be a Z-type cytokinin. UR was not affected by nucleotidase, indicating that it is not zeatin riboside mono-, di- or triphosphate. Its HPLC retention time suggested it to be the 9-alanyl conjugate of zeatin, a biologically inactive and extremely stable compound, but comparison with the standard on LC-MS excluded this possibility. Thus, the structure of UR remains a matter of conjecture.

In the axillary shoots in stages 1 and 2, about 50% of the cytokinins are iP-compounds. As these young shoots have a limited transpiration capacity, they depend on the phloem as their source of cytokinins (Komor *et al.*, 1993). Cytokinins were found in phloem sap of a number of plant species (Hoad, 1995), originating either from the xylem (Jameson *et al.*, 1987) or from mature leaves (Vonk, 1979; Van Staden, 1982). In *Ricinus communis* (Komor *et al.*, 1993), *Perilla* (Grayling and Hanke, 1992) and *Sinapis alba* (Lejeune *et al.*, 1994), iP-compounds were the main phloem cytokinins. If this is also true for rose, phloem import might cause the high contribution of iP-type cytokinins in axillary shoots in stages 1 and 2. In addition, *de novo* synthesis of cytokinins in (the leaves of) the axillary shoots cannot be ruled out. As we do not have data on cytokinins in phloem of rose, it is not possible to attribute the high percentage of iP-type cytokinins in axillary shoots fully or partially either to *de novo* synthesis or to phloem import.

The total cytokinin concentration of the buds forming the bottom breaks was very high in stage 1. Apparently the cytokinins are necessary for bud break and outgrowth. ZR and ZMP were the main cytokinins in stages 1 and 2 (Table 2.8), indicating that the buds receive their cytokinins directly from the roots. When the bottom breaks had developed into shoots (stage 3), the cytokinin pattern changed considerably. The amounts of iP and DHZ-type cytokinins increased, resulting in contributions of 15% each. The increase in iPMP and iPAR might be the result of either cytokinin import via the phloem or *de novo* synthesis of cytokinins in the very young stem and leaf tissue.

In this Chapter we have established the cytokinin patterns of *Rosa hybrida* at three stages of development and correlated these with bud break and outgrowth of axillary shoots and bottom breaks. Most likely, the buds forming the bottom breaks derive their cytokinins from the roots, whereas the axillary shoots either import their cytokinins from the phloem or are capable of *de novo* synthesis of cytokinins. A high ZR concentration in the bleeding sap appeared to coincide with the outgrowth of axillary shoots and to precede bud break of bottom breaks. This study provides the framework for succeeding studies in which a causal relationship between endogenous cytokinins and bud break and outgrowth will be investigated.

Chapter 3

Quantification of the Export of Cytokinins from Roots to Shoots and their Degradation Rate in the Shoot

3.1 Abstract

Cytokinins from the roots may be involved in regulating rose shoot growth and development. The objective of this study was to estimate the export of cytokinins from the roots and their degradation rate in the shoot, which were expected to be correlated to plant development. Hence, the total cytokinin content of the shoot, the concentration of ZR in bleeding sap, and the transpiration rates in three stages of development were determined. The estimations performed are based on the assumption that the cytokinin concentration in bleeding sap is representative for the cytokinin concentration in xylem sap in situ. This was verified by comparing the ZR concentration in bleeding sap and in sap obtained after pressurizing the root system to a level equivalent to the leaf water potential; no significant differences could be found. The import of cytokinins could not be correlated to plant development, as it increased linearly in time. The estimated relative degradation rate of cytokinins in the shoot decreased as the plants matured. The half-life of cytokinins in the shoot was found to be approximately 1 day, indicating that cytokinins are rapidly metabolized in the shoot.

3.2 Introduction

In rose combination plants, the genotype of the rootstock influences bud break of the scion, ultimately reflected in yield of cut flowers (De Vries and Dubois, 1989). Rootstock effects on shoot growth may be correlated with levels of cytokinins in xylem sap, as was shown for *Vitis vinifera* (Skene and Antcliff, 1972) and *Prunus avium* (Stevens and Westwood, 1984). It is generally accepted that cytokinins are mainly produced in root tips and move via the xylem to the shoot where they participate in the control of growth and development. They are known to induce cell division (Skoog *et al.*, 1965), promote growth of lateral buds by counteracting the dominance of the apex (Pillay and Railton, 1983), delay senescence of intact plants (Noodén *et al.*, 1990) and excised plant parts (Singh *et al.*, 1992c), and affect source/sink relationships (Kuiper, 1988).

In Chapter 2, endogenous cytokinins in bleeding sap and in various plant parts of *Rosa hybrida* in three stages of development were described. In all mature tissues, cytokinins of the Z-type predominated, amounting to 80-90% of the total cytokinin concentration. ZR was found to be the major translocation form in the xylem sap.

According to the hormone concept of Jackson (1993), measurements of merely the concentration of cytokinins are insufficient to correlate cytokinins and physiological processes in the plants. For this, the amount of cytokinins leaving the roots should be known. To calculate the cytokinin export from the roots, both the volume flow rate of the xylem sap and its cytokinin concentration must be quantified. Unfortunately, it is not possible to sample the transpiration stream directly (Jackson, 1993). Thus, estimates are usually made indirectly, using osmotically driven root pressure exudate, i.e. bleeding sap (Heindle *et al.*, 1982) or root exudate obtained after pressurizing the root system (Beck and Wagner, 1994). These methods generate different sap flows and may result in confounding estimates of cytokinin export from roots to shoot (Jackson, 1993). This may be dealt with by measuring cytokinins in sap samples obtained after pressurizing the detached root system to generate sap flows close to those of intact transpiring plants (Else *et al.*, 1993). The disadvantage of this method is that the relationship between the applied pressure and the resulting sap flow rate has to be established first (see Else *et al.*, 1995). An alternative approach is to pressurize the roots to a level equivalent to the leaf water potential of intact plants (Jackson, 1993), which can be determined more rapidly.

Cytokinins from the roots may be involved in the regulation of shoot growth and development of *Rosa hybrida*. The objective of our study was to estimate the export of cytokinins from the roots and their degradation rate in the shoot, which we expected to be correlated to plant development. Hence, the cytokinin content of the shoot, the ZR concentration in xylem sap (Experiment 1) and the transpiration rate (Experiment 2) were measured. For practical reasons, these were determined at three stages of development that are crucial for rose growth. The estimations performed were based on the assumption that the cytokinin concentration in bleeding sap is representative for the cytokinin concentration in xylem sap in situ. This was verified by comparing the ZR concentrations in bleeding sap and in sap obtained after pressurizing the root system to a level equivalent to the leaf water potential (Experiment 3). Furthermore, the pattern of cytokinin concentration in bleeding sap during the day was determined by measuring the ZR concentration in the sap at 2-hourly intervals during 24 h (Experiment 4). These data may form the basis for further studies on the involvement of cytokinins from the roots in the effect of rose rootstocks on bud break and outgrowth of the scion.

3.3 Materials and methods

3.3.1 Plant material

Rose combination plants were obtained by grafting a piece of stem of the cultivar Madelon bearing one intact five-leaflet leaf and an adherent bud onto a single internode of the rootstock Multic (Van de Pol and Breukelaar, 1982). After dipping the basal part of the rootstock in 0.5% indole butyric acid (w/v), the plants were placed in a mixture of sand and peat (1:1, v/v) at a RH of 95-100% and a temperature of 25°C. After 3 weeks, the rooted plants were transferred to nutrient solution (Steiner, 1984) in a growth chamber

at 20°C, at a RH of 75%, at a photosynthetically active radiation of 300 μmol m^{-2} s^{-1} at plant base level (Philips TLD, 50 W) and at a photoperiod of 14 h (8:00 to 22:00). These conditions were used in all experiments described.

3.3.2 Experiment 1

This experiment was started in June 1994, and was aimed at determining the cytokinin content in shoot and roots and the cytokinin concentration in bleeding sap. Uniform plants were selected and randomly allocated to 6 groups of 3 repetitions each. For each repetition, 3 plants were combined. Three stages of development were defined: (1) primary shoot with 1-2 cm axillary shoots; (2) flower buds of the axillary shoots are visible, buds that will form the bottom breaks are swelling; (3) flowers of the axillary shoots are open, bottom breaks are 8-10 cm long. Stages 1, 2 and 3 were reached 7, 9 and 12 weeks after grafting, respectively. At each stage, one group of plants was used to collect bleeding sap. One hour after the start of the photoperiod, the plants were cut just below the primary shoot. The first drop of bleeding sap which may have been contaminated by rests of damaged cells was discarded. A piece of silicone tubing was mounted onto the stump. A capillary tube was inserted and led the exudate into a vial kept at 2°C, containing 10 μl 100 mM Na_2MoO_4 to inhibit phosphatase activity. Bleeding sap was collected during 30 min, and stored at -80°C until analysis. Furthermore, at each stage, from one group of plants, roots and shoots were separated, weighed, and immediately dropped into liquid N_2 and stored at -80°C until extraction, to determine total cytokinins in roots and shoots. Cytokinins were purified by means of immunoaffinity chromatography and HPLC, and identified by retention time, UV spectrum and gas chromatography-mass spectrometry (GC-MS) as described in Chapter 2. Total cytokinins in roots and shoot were determined by summing up the amounts of the separate cytokinins.

3.3.3 Experiment 2

In August 1995, a separate experiment was started to determine transpiration rates during plant development. To that end, weight of a plant in a beaker with aerated nutrient solution was recorded every 10 min during 5 weeks, from shortly before stage 1 until stage 3. The transpiration rate (expressed in ml h^{-1}) was estimated by measuring the weight loss of the plant and beaker, corrected for evaporation of the nutrient solution in the beaker. The measurements were performed in duplicate.

3.3.4 Experiment 3

In this experiment, done in February 1995, the daily course of the ZR concentration in bleeding sap of plants at stage 1 (1-2 cm long axillary shoots) was followed. At each two-hourly interval, starting at 9.00 a.m., one hour after the start of the photoperiod until 7.00 next morning, six plants were cut just below the primary shoot. Bleeding sap was collected as described for experiment 1.

After adjusting the pH of bleeding sap to 3.7 with 10 mM NH_4-formate buffer, it was passed through a Sep-pak C_{18}-cartridge (Waters, Milford, MA, USA) by gravity, which had been primed with 10 ml 96% ethanol and 10 ml 10 mM NH_4-formate buffer at pH

3.7. After washing the cartridge with 10 ml NH_4-formate buffer and 60 ml deionized water, the cytokinins were eluted with 10 ml 96% ethanol. The eluate was evaporated to dryness and redissolved in HPLC-eluens. For HPLC purification of ZR a μBondapak C_{18}-column (Waters, 30 x 0.39 cm) was used which was eluted with 22% methanol/1 mM NH_4-formate pH 3.7 at a flow rate of 2 ml min^{-1} and a column temperature of 25°C. The ZR fraction was collected, evaporated to dryness and taken up in PBST (10 mM Na_2HPO_4, 0.15 mM KH_2PO_4, 2.7 mM KCl, 0.14 M NaCl, 0.05% Tween 20, pH 8.4). ZR was quantified by means of the indirect competitive enzyme immunoassay as described by Vonk *et al.* (1986).

3.3.5 Experiment 4

Plants were grafted in December 1994 and used for comparison of ZR concentrations in bleeding sap and in root pressure exudate. Root pressure exudate was obtained by pressurizing the detopped root system to a level equivalent to the tension in the xylem. This tension is regarded equal to the water potential of the plant. To determine the water potential, a single leaf was wrapped in aluminium foil for 2 h, before cutting it from the plant. Immediately after cutting, the leaf was mounted in a pressure chamber and increasing pressure was applied to the leaf from a cylinder of compressed gas, until sap appeared at the cut surface (Scholander *et al.*, 1965). This pressure is regarded equal to the water potential of the plant and was determined to be 0.4 MPa. The detopped root system was placed in a dry pressure chamber, with the stump protruding. On the stump a piece of silicon tubing was mounted. The pressure in the chamber was gradually increased to 0.4 MPa over 30 s and maintained at this level for 3 min. Then the sap yield was equal to that obtained during a 30-min collection period of bleeding sap. Root pressure exudate was collected from the cut surface with a microlitre syringe and transferred to a vial kept at 2°C, containing 100 mM Na_2MoO_4. Of uniform groups of plants in stages 1 and 3, 4 plants were used to collect bleeding sap, and 4 to collect root pressure exudate. ZR concentrations were determined as described in experiment 3.

3.3.6 Statistics

Where relevant, data were submitted to analysis of variance (ANOVA) by means of the statistical package GENSTAT 5. The significance of differences was determined by Student's t-test.

3.4 Results

3.4.1 Experiment 1

The cytokinin content in shoot and roots increased severalfold with stage of development ($P<0.001$; Table 3.1). Of the total cytokinin content, in stage 1 27% was ZR, and in stages 2 and 3 22 and 17% respectively. As the ZR concentrations in bleeding sap did not differ significantly ($P=0.19$) between developmental stages 1, 2 and 3, the average value (24.8 pmol ml^{-1}) was used for calculations.

Table 3.1 *Endogenous cytokinin contents in shoot and roots (pmol plant^{-1}, means ± SE) and ZR concentration in bleeding sap (pmol ml^{-1}, means ± SE) of Madelon/ Multic combination plants in three stages of development (n=3).*

	stage 1	stage 2	stage 3
shoot	3523 ± 241	6556 ± 322	18327 ± 1532
roots	923 ± 17	1642 ± 194	3827 ± 236
bleeding sap	36.5 ± 11.4	16.9 ± 1.7	21.1 ± 3.0

3.4.2 Experiment 2

The transpiration rates of the plants during photoperiod and night were recorded for 5 weeks. When these values were plotted against time (data not shown), the transpiration rates were found to increase linearly in time according to the following equations:

$$T_{day} = 0.54 \cdot t + 7.7 \qquad (R^2 = 0.95) \qquad (1)$$

$$T_{night} = 0.12 \cdot t + 2.1 \qquad (R^2 = 0.93) \qquad (2)$$

where T_{day} is transpiration rate during the photoperiod (ml h^{-1}), T_{night} is transpiration rate during night (ml h^{-1}) and t is time (days).

3.4.3 Experiment 3

The ZR concentration in bleeding sap of stage 1 plants fluctuated during the day, but did not show a distinct diurnal pattern (Figure 3.1). This implies that for calculations the concentration of ZR in bleeding sap collected at 9.00 a.m. can be used as representative for the ZR concentration during 24 h. Analysis of variance showed that the volume of bleeding sap collected was significantly higher ($P < 0.001$) during the light (0.249 ml [30 min]$^{-1}$) than during the dark (0.183 ml [30 min]$^{-1}$) period (no further data shown).

3.4.4 Experiment 4

Analysis of variance revealed that the concentrations of ZR in bleeding sap and root pressure exudate did not differ significantly in the two stages of development tested (Table 3.2). The ZR concentrations differed between stages 1 and 3. This is in contrast with the results of experiment 1, where the ZR concentrations in bleeding sap did not differ significantly between stages 1 and 3. Furthermore, the ZR concentrations differed greatly between experiments 1 and 4. These differences cannot be attributed to differences in experimental conditions between the experiments. They might be at least partially due to seasonal effects, as the plants in experiment 1 were obtained from shoots grown in summer, and in experiment 4 from shoots that developed during winter.

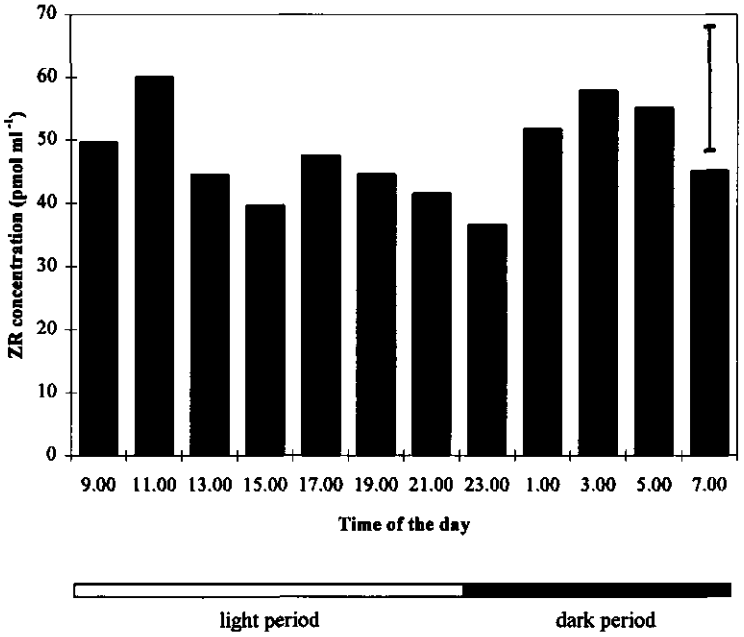


Figure 3.1 *Daily course of the ZR concentration in bleeding sap (pmol ml^{-1}, n=6). Vertical bar indicates LSD (P=0.05).*

Table 3.2 *Concentration of ZR (pmol ml^{-1}) in about the same volume (ml) of bleeding sap (collected in 30 minutes) and root pressure exudate (collected at 0.4 MPa in 3 minutes) of Madelon/Multic combination plants in developmental stages 1 and 3 (n=4).*

	stage 1		stage 3	
	sap volume	ZR	sap volume	ZR
bleeding sap	0.188	100.8	0.582	12.6
root pressure exudate	0.191	123.4	0.522	8.8
LSD (P=0.05)	0.062	42.5	0.160	6.5

3.5 Discussion

3.5.1 Daily course of ZR concentration in bleeding sap

No distinct diurnal pattern in ZR concentration in bleeding sap during 24 h could be detected (Figure 3.1). Although the transpiration rate of the plants increased approximately 4-fold from night to day (Equation 2, T_{night} = 2.1 ml h^{-1} at stage 1;

Equation 1, T_{day} = 7.7 ml h^{-1}), the ZR concentration in the bleeding sap did not change significantly. This implies that the ZR export from the roots increased approximately 4-fold as well, possibly resulting from an increase in cytokinin biosynthesis. In contrast, Beck and Wagner (1994) found that the cytokinin concentration in bleeding sap of *Urtica dioica* was higher in the morning than during the rest of the light period. They interpreted this in terms of a more or less steady state of cytokinin synthesis in the roots and loading into the xylem. The cytokinin concentration in the xylem is then modulated by the daily dynamics of the transpiration stream. Apparently, in *Rosa hybrida*, cytokinin production or its loading in the xylem are more strongly regulated by the shoot than in *Urtica dioica*.

3.5.2 Calculation of ZR export from the roots and degradation of cytokinins in the shoot

In intact plants, water transport in the xylem is primarily determined by the negative hydrostatic pressure of the transpiring leaves and to a much smaller extent by root pressure. Our results show that when the shoot is removed, the sap flow is reduced to about 5% of the daytime transpiration rate (Equation 1, transpiration rate during photoperiod in stage 1 is 7.7 ml h^{-1}; Table 3.2, rate of bleeding in stage 1 is 0.188 ml in 30 min, i.e. 0.367 ml h^{-1}). In general, calculation of the cytokinin export from the roots by multiplying the ZR concentration in this slow-flowing bleeding sap by the amount of water transpired may lead to a serious overestimation of the export (Jackson, 1993), unless the volume of the collected sap is small and may be considered to be captured transpiration sap in transit to the shoot. This situation probably holds for the present experiments with rose. Otherwise it is difficult to explain why no significant differences could be found when the ZR concentrations in bleeding sap and root pressure sap were compared, whereas the volume flow rates differed 10-fold (Table 3.2).

From equations 1 and 2, given a 14-h photoperiod, the rate of xylem sap import is calculated to be $8.76 \cdot t + 128.8$ (ml day^{-1}). As the concentration of ZR in bleeding sap did not differ significantly between developmental stages 1, 2 and 3 (Experiment 1), the average value of 24.8 pmol ml^{-1} is used to calculate the import of cytokinins into the shoot (*dI*; pmol) during the interval *dt* (days), which is:

$$\frac{dI}{dt} = 217.2 \cdot t + 3194.2 \qquad (3)$$

At stages 1(t = 0) and 2 (t = 14 days), the amount of cytokinins imported into the shoot in 1 day is approximately the amount of cytokinins present in the shoot at that day (3500 and 6500 pmol respectively). However, at day 35 (stage 3), the estimated cytokinin import is about 10800 pmol, whereas the amount present in the shoot at that time is 18300 pmol. These data suggest that the metabolic degradation rate is higher in the interval between stages 1 and 2 than between stages 2 and 3. The relative degradation rate (*RDR*; pmol $pmol^{-1}$ day^{-1}) can be expressed as:

$$RDR = \frac{dC}{dt} \cdot \frac{1}{C} \qquad (4)$$

where C is the total cytokinin content of the shoot and dC is the increment in cytokinin content (pmol) during the interval dt (days). Estimation of the *RDR* is based on the assumption that all cytokinins present in the shoot or imported via the xylem are subjected to the same degradation rate during the time they are present in the shoot. Furthermore, it is supposed that this degradation rate is constant during the time interval studied. For the interval between stages 1 and 2 (14 days) holds that the cytokinin content of the shoot at day 14 (C_{day14}) is the cytokinin content at day 0 (C_{day0}) subjected to *RDR* for 14 days, plus the integral of the import of cytokinins into the shoot (dI) during time (dt) subjected to *RDR* for the time these cytokinins are present in the shoot, expressed as:

$$C_{day14} = C_{day0} \cdot e^{RDR \cdot 14} + \int_0^{14} \frac{dI}{dt} \cdot e^{RDR \cdot (14-t)} \cdot dt \qquad (5)$$

The half-life (T_{half}; days) of the cytokinin pool C is calculated by:

$$\tfrac{1}{2}C = C \cdot e^{RDR \cdot T_{half}} \qquad (6)$$

resulting in:

$$T_{half} = \frac{\ln 2}{-RDR} \qquad (7)$$

By resolving equation 7, the *RDR* was estimated to be -0.91 pmol pmol^{-1} day^{-1} for the interval between developmental stages 1 and 2, resulting in a half-life of 0.76 days. These results indicate that root-derived cytokinins are rapidly metabolized in the shoot. Similar results were obtained for *Urtica dioica* by Wagner and Beck (1992) who estimated a metabolic turnover of the entire pool of endogenous cytokinins of one or at most a few days. Studies in which ^{3}H-ZR was applied through the xylem to *Lupinus angustifolius* (Jameson *et al.*, 1987) or soybean explants (Noodén and Letham, 1986) also revealed that ZR was rapidly metabolized.

A similar calculation for the interval between stages 2 and 3 (day 14 to 35) resulted in a *RDR* of -0.57 pmol pmol^{-1} day^{-1} and a half-life of 1.22 days. In the interval between stage 1 and stage 2, the plants grow vigorously. Between stages 2 and 3, the leaves start yellowing, and fresh weight increase is primarily caused by thickening of stems and leaves. In a number of plant species, O-glucoside cytokinins accumulate as the leaves mature (Letham and Palni, 1983). These glucosides can remain unmetabolized over long periods (Letham and Palni, 1983), but they remain part of the cytokinin pool of the plant (C). Therefore, the fact that as plants mature, part of the cytokinins are more stable, might cause the differences in *RDR* and T_{half} between the first and second interval. The supposition that the *RDR* is constant in time, is most likely incorrect. The observation that the *RDR* varied considerably between the first and the second interval suggests a gradual decrease.

The calculations presented are based on the assumption that the concentration of ZR in the transpiration stream is constant both during the day (Figure 3.1) and during plant development (Table 3.1). If this assumption would not be valid, the concentration of ZR should be described as a function of time in stead of the fixed value of 24.8 pmol ml^{-1}. If, on average, this function would yield a value for ZR higher than 24.8, the import of ZR into the shoot would be higher, and, given that the cytokinin content of the shoot would not be altered, the relative degradation rate would be more negative, i.e. the metabolic degradation would be higher. To indicate the order of magnitude, the effect of a 4-fold increase in concentration of ZR (to 100 pmol ml^{-1} as in Table 3.2) in the interval between stages 1 and 2 on the *RDR* and T_{half} was estimated. This would result in a 4-fold increase of the *RDR* to approximately 3.7 pmol $pmol^{-1}$ day^{-1}, and an almost 7-fold decrease of T_{half} to 0.19 day.

In this study, we have estimated the export of cytokinins from the roots and their degradation rate in the shoot, which we expected to be correlated to plant development. Although between stages 1 and 3 important developmental processes occur, like outgrowth of axillary shoots, and the appearance and outgrowth of bottom breaks, the transpiration rate, and therefore the import of cytokinins into the shoot (Equation 3) were found to increase linearly in time and did not show fluctuations that could be correlated to these processes. Therefore, based on these data, no relationship between cytokinin import and plant development could be established, which falsifies our expectations. The degradation of cytokinins in the shoot was found to be higher in the interval between stages 1 and 2 than between stages 2 and 3, suggesting that it varies during plant development. However, these data are not sufficient to correlate *RDR* to developmental processes. This study provides the framework to determine whether the effect of rootstocks on bud break and outgrowth of the scion in rose combination plants is mediated via cytokinins. This will be the object of the next Chapter.

Chapter 4

Bud Break and Cytokinin Concentration in Bleeding Sap as affected by the Genotype of the Rootstock

4.1 Abstract

Grafting *Rosa hybrida* Madelon on six rootstock genotypes revealed that growth of the scion was affected by the genotype of the rootstock. If rootstocks would mediate scion growth via their cytokinin production, it might be possible to improve the performance of a rootstock by exogenous application of cytokinins. Indeed, BA application was found to stimulate the number of bottom breaks, mainly on the low-yielding rootstocks Vivaldi and Madelon. To test the hypothesis that a rootstock that induces earlier bud break of the scion supplies more cyokinins to the shoot, cytokinin levels in bleeding sap of Vivaldi and the vigorous rootstock Multic were followed during plant development. Axillary bud break was not affected by the genotype of the rootstock, but bottom breaks appeared earlier when Madelon was grafted on Multic than on Vivaldi. The concentration of ZR in bleeding sap of Madelon/Multic combination plants was high at axillary bud break, decreased when the axillary buds grew out, increased thereafter until the bottom breaks appeared, and decreased again. The ZR concentration in bleeding sap of Madelon/Vivaldi plants was considerably lower than that of Madelon/Multic plants, but showed more or less the same pattern. Apical dominance in rose combination plants during their development is discussed in terms of auxin and cytokinins

4.2 Introduction

In commercial cut-rose growing, cultivars are usually grafted onto rootstocks of a different genotype to improve flower production (De Vries and Dubois, 1983; Kool and Van de Pol, 1992). In contrast to fruit tree growing, where a range of rootstocks is used, varying from dwarfing to vigorous (Carlson, 1970), rose rootstocks are primarily selected for high vigour. Other important selection criteria are resistance to diseases and pests, ability to allow continuous harvesting of the scion, compatibility with various rose cultivars, and ability to perform well under various environmental conditions (Leemans and Van de Laar, 1977). From fruit tree growing it is known that rootstocks may affect shoot growth via uptake and translocation of nutrients (Lombard and Westwood, 1987)

and/or the production of cytokinins (Stevens and Westwood, 1984). In grape (Skene and Antcliff, 1972) and watermelon (Yamasaki *et al.*, 1994) rootstocks were selected for their resistance to nematodes and *Fusarium*, respectively. Of these rootstocks, some were also found to improve yield compared to growing on own roots. These higher yields were positively correlated with cytokinin levels in the xylem sap. If rose rootstocks would mediate scion growth via their cytokinin production, it might be possible to improve the performance of a rootstock by exogenous application of cytokinins. Application of BA in lanolin paste to intact rose plants was found to stimulate bud break of axillary buds (Ohkawa, 1984) and bottom breaks (Parups, 1971). Bottom breaks, vigorous shoots at the base of the plant, form the frame of a rose bush and determine mainly the future yield of flowers. Besides by cytokinin application, the number of bottom breaks can be stimulated by cutting back and deshooting, by environmental conditions like high CO_2, low temperatures, high humidity and high light intensity, and by application of ethylene (Khayat and Zieslin, 1982).

Outgrowth of axillary buds is under the control of the shoot apex, a process referred to as apical dominance (Cline, 1991). Apical dominance is supposed to be mediated by auxin, primarily produced by the young expanding leaves of the apex. Auxin moves basipetally down the shoot to axillary buds and inhibits their outgrowth. Cytokinins are thought to play a secondary interacting role with auxins, as was shown by Bangerth (1994). In general, axillary bud growth is found to be well correlated with the level of naturally occurring cytokinins in the buds (Tamas, 1995).

In the present Chapter, six rootstocks were compared for their effects on growth of the scion Madelon. Further, to investigate whether the effect of rootstocks varying in vigour on shoot growth is mediated by cytokinins, the effect of exogenous application of cytokinins to combination plants was studied. Finally, to test the hypothesis that a rootstock that induces earlier bud break of the scion supplies more cytokinins to the shoot, cytokinin levels in bleeding sap of two rootstocks of different vigour were followed during two months. These data were correlated with bud break of axillary buds and bottom breaks. Apical dominance in rose combination plants during their development is discussed in terms of auxin and cytokinins.

4.3 Materials and methods

4.3.1 Growth of Madelon on six rootstock genotypes

In March 1991, *Rosa hybrida* Madelon was grafted (Van de Pol and Breukelaar, 1982) on the rootstocks *R. hybrida* Marleen and Moneyway, *R. indica* Major and on Multic, a selection from *R. multiflora* Cathayensis. The scion cultivars *R. hybrida* Madelon and Vivaldi were included in this experiment as putatively low yielding rootstocks. The base of the rootstock was dipped in 0.5% IBA in talcum powder (w/v), and the combination plants were placed in a mixture of sand and peat at a ratio of 1:1 (v/v) at a RH of 95-100% and a temperature of 25°C. After three weeks, the rooted plants were transferred to nutrient solution in a growth room at 20°C, RH of 75%, photosynthetically active radiation of 135 μmol m^{-2} s^{-1} at plant base level (Philips HPI-T, 400W) and a photoperiod of 18 hours. Fourteen plants per combination were harvested 15 weeks after grafting. The

number of bottom breaks and dry weights of roots, bottom breaks, and primary shoot plus axillary shoots were recorded.

4.3.2 Cytokinin application

Madelon was grafted on the rootstocks Multic, Madelon and Vivaldi in October 1990 (Van de Pol and Breukelaar, 1982). The basal parts of these combination plants were dipped in 0.5% IBA in talcum powder (w/v), and the plants were placed in a mixture of perlite, vermiculite and sand (1:3:1, v/v/v) at 25-30°C, RH of 90-100% with additional lighting of 90 μmol m^{-2} s^{-1} (Philips HPI-T, 400W) for 18 hours. After 3 weeks, the rooted plants were transferred to nutrient solution in a growth chamber (conditions as in the previous experiment). When the flower bud of the primary shoot of an individual plant had reached a diameter of 12 mm (approximately 7 weeks after grafting), the stem was pruned 2 cm above the fourth 5-leaflet leaf from the base of the stem. Flower tubes (Van de Pol and Marcelis, 1988) containing 6 ml 0, 4.4 10^{-5} M or 4.4 10^{-4} M BA dissolved in KOH, pH adjusted to 5.0 with acetic acid, were placed upside down on the freshly pruned stems. The flower tubes were removed after 4 days. At that time more than 90% of the plants had taken up the solution completely. Of each scion/rootstock combination 30 plants were used, 10 per treatment. The moment of bud break of the bottom breaks (length is 1 cm) was recorded during the experiment. Four weeks after application of BA, the number of bottom breaks per plant was counted.

4.3.3 Growth and ZR concentrations in bleeding sap of Madelon on two rootstocks

The scion Madelon was grafted onto the rootstocks Multic and Vivaldi (Van de Pol and Breukelaar, 1982) in August 1995. After 3 weeks, the rooted plants were transferred to nutrient solution (Steiner, 1984) in a growth room at 20°C, RH of 75%, photosynthetically active radiation of 250 μmol m^{-2} s^{-1} at plant base level (Philips TLD, 50 W) and a photoperiod of 14 hours (8:00 to 22:00). Per plant, the day of axillary bud break (first axillary shoot was 1.5 cm long), approximately 8 weeks after grafting, was denoted as day 0. From that day plants were harvested at intervals of two days, from day 10 at intervals of three days until bud break of the bottom breaks, and, in addition, when bottom break length was 0.5, 2, 5 or 20-25 cm. Of both scion/rootstock combinations, four plants per harvest were used. At harvest, dry weights of the primary shoot, axillary shoots and bottom breaks, and of the roots after collection of bleeding sap were recorded.

Bleeding sap was collected by cutting off a plant just below the primary shoot one hour after the start of the photoperiod. The first drop of sap which might be contaminated by rests of damaged cells was discarded. A piece of silicone tubing was mounted onto the stump. A capillary tube was inserted which led the exudate into a vial kept at 2°C, containing 1 μmol Na_2MoO_4 to inhibit phosphatase activity. Bleeding sap was collected during 30 minutes, and stored at -20°C until further analysis. Cytokinins were purified by adjusting the pH to 3.7 with 10 mM NH_4-formate buffer and passing through a Sep-pak C_{18}-cartridge (Waters, Milford, MA, USA) by gravity, which had been primed by passing through 10 ml 96% ethanol and 10 ml NH_4-formate buffer pH 3.7. The cartridge was washed with 10 ml NH_4-formate buffer and 60 ml deionized water, and the cytokinins were eluted with 10 ml 96% ethanol. The eluate was evaporated to dryness and redissolved in HPLC-eluens. ZR is the only cytokinin that can be detected in bleeding sap

of rose, as was previously shown by identification by means of HPLC retention time, UV spectrum and GC-MS (Chapter 2). For HPLC purification of ZR a μBondapak C_{18}-column (Waters, 30 x 0.39 cm) was used which was eluted with 22% methanol/1 mM NH_4-formate pH 3.7, flow rate 2 ml min^{-1} and column temperature 25°C. The ZR fraction was collected, evaporated to dryness and redissolved in PBST (pH 8.4). ZR was quantified by means of the indirect competitive enzyme immunoassay as described accurately by Vonk *et al.* (1986).

4.3.4 Statistics

All experiments were arranged in randomized block designs. Data were submitted to statistical analysis of variance (ANOVA) and the significance of differences was determined by Student's t-test.

4.4 Results

4.4.1 Growth of Madelon on six rootstock genotypes

The rootstocks used can be divided into two distinct groups (Table 4.1). Multic and Moneyway induced the highest number of bottom breaks and the highest shoot dry weight. Of these two, Multic had the highest shoot-to-root ratio. Indica major, Madelon and Vivaldi induced low numbers of bottom breaks and low shoot dry weights. Of these rootstocks, the cultivar Vivaldi induced the weakest growth. With regard to the number of bottom breaks, Marleen was intermediate with 0.8 bottom breaks. Unfortunately, we do not have data on the time of bud break of bottom breaks. However, the high dry weight of the bottom breaks induced by Multic suggests that these shoots broke early. For succeeding experiments, the vigorous rootstock Multic and the weak cultivar Vivaldi were selected.

Table 4.1 *Growth characteristics of Madelon grafted on six rootstock genotypes, 15 weeks after grafting (n=14). # bb = number of bottom breaks; DW = dry weight (g); shoot = primary shoot + axillary shoots + bottom breaks; s/r = shoot to root ratio*

rootstock	# bb	DW bb	DW shoot	DW roots	DW plant	s/r
Multic	1.4	15.7	34.0	2.9	36.9	11.7
Moneyway	1.1	9.3	33.8	4.0	37.8	8.5
Marleen	0.8	2.3	20.9	3.0	23.9	7.0
Indica major	0.5	5.4	24.3	3.0	27.3	8.1
Madelon	0.4	3.2	19.7	2.2	21.9	9.0
Vivaldi	0.5	3.2	14.7	1.6	16.3	9.2
LSD (P=0.05)	0.4	4.7	6.9	0.8	7.4	1.8

Table 4.2 *Effect of the concentration of BA (6 ml) applied via flower tubes to pruned shoots on the number of bottom breaks of Madelon grafted on three rootstocks, 4 weeks after BA application (n=10). Values within one row followed by different letters differ significantly (P=0.05).*

	BA (10^{-4} M)		
rootstock	0	0.44	4.4
Multic	1.5 ab	1.2 a	2.0 b
Vivaldi	0.1 a	0.9 b	1.5 c
Madelon	0.0 a	0.9 b	2.0 c

Table 4.3 *Effect of the rootstock and the concentration of BA (6 ml) applied via flower tubes to pruned shoots on the number of days between BA application and bud break of bottom breaks (n=10). Values followed by different letters differ significantly (P=0.05)*

	BA (10^{-4} M)		
rootstock	0	0.44	4.4
Multic	6.9 a	4.8 a	6.7 a
Vivaldi	-	9.1 b	10.4 b
Madelon	-	12.6 c	13.8 c

4.4.2 Cytokinin application

Within four weeks after pruning the primary shoot of Madelon grafted onto Multic, bottom breaks appeared (Table 4.2), while Vivaldi and Madelon hardly induced bottom breaks. Application of BA favoured bottom break emergence, especially for Vivaldi and Madelon. The highest concentration clearly was most effective. The genotype of the rootstock affected the number of days between BA application and bud break of bottom breaks (Table 4.3), Multic being early and Madelon late. However, BA application did not accelerate bud break of the bottom breaks.

4.4.3 Growth and ZR concentrations in bleeding sap of Madelon on two rootstocks

The axillary buds of Madelon/Multic broke 59.8 ± 0.8 and of Madelon/Vivaldi 59.5 ± 1.0 days after grafting. In Figure 4.1, shoot growth (total dry weight of primary shoot, axillary shoots and bottom breaks) of Madelon/Multic and Madelon/Vivaldi combination plants is presented. Growth curves were fitted for both combinations. Since curve parameters did not differ significantly (P=0.05), shoot growth of both combinations was described by one exponential growth curve.

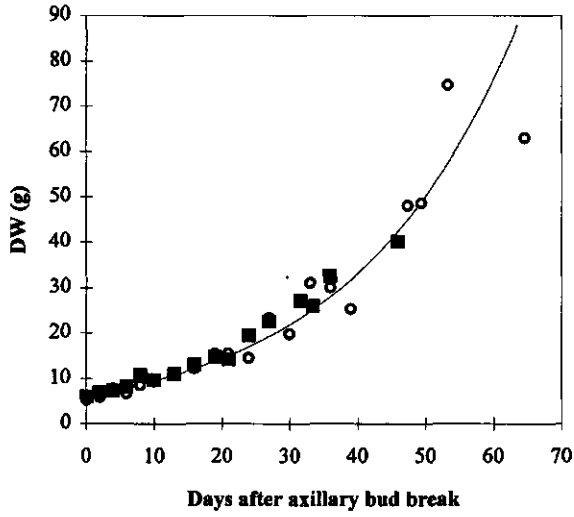


Figure 4.1 *Shoot growth of Madelon grafted on Multic (■) or Vivaldi (○). Each point represents the average value of 4 plants. One exponential curve is fitted ($Y = 6.27e^{0.0416X}$, $R^2 = 0.86$).*

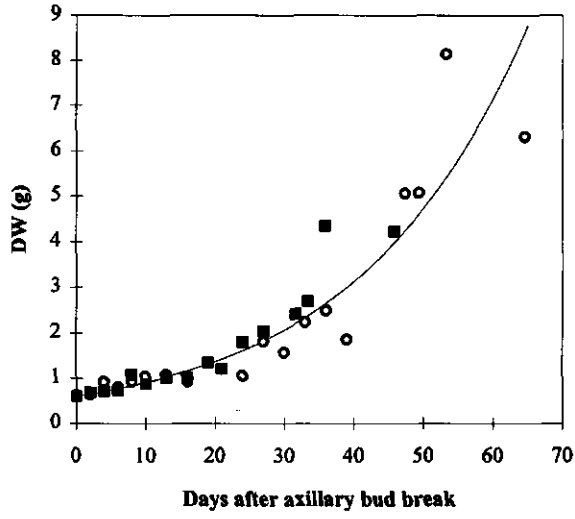


Figure 4.2 *Growth of the roots of Madelon/Multic (■) or Madelon/Vivaldi (○) combination plants. Each point represents the average value of 4 plants. One exponential curve is fitted ($Y = 0.599e^{0.0413X}$, $R^2 = 0.80$).*

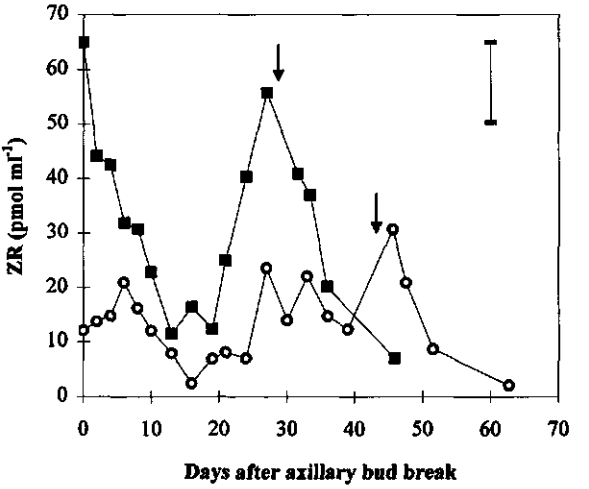


Figure 4.3 *ZR concentrations in bleeding sap (pmol ml^{-1}) of Madelon/Vivaldi (○) or Madelon/Multic (■) combination plants. Axillary shoots of Madelon/Multic appeared 59.8 ± 0.8 and of Madelon/Vivaldi 59.5 ± 1.0 days after grafting (day 0). Arrows indicate the moment that bottom breaks appeared, for Multic at 29.6 ± 1.6 days after axillary bud break, for Vivaldi at 45.6 ± 2.2 days after axillary bud break. Vertical bar indicates LSD (P = 0.05).*

The root systems showed distinct morphological differences; Multic roots were longer, thinner, darker and more branched as compared to Vivaldi. In spite of these morphological differences, the parameters of the fitted root growth curves did not differ significantly (P=0.05). Therefore, root growth of Multic and Vivaldi was described by one exponential growth curve (Figure 4.2).

The ZR concentration in Madelon/Multic bleeding sap decreased strongly from the moment of axillary bud break (Figure 4.3), until day 13, when the axillary shoots were about 20 cm long. The ZR concentration increased thereafter and reached its peak when the bottom breaks emerged (29.6 ± 1.6 days after axillary bud break). From then on, the ZR concentration decreased. When the bottom breaks were 20-25 cm, the ZR concentration had dropped to less than 10 pmol ml^{-1}. At day 0, the ZR concentration in bleeding sap of Madelon/Vivaldi plants was considerably lower than that of Madelon/Multic plants. This concentration decreased until about 18 days after axillary bud break, and increased slowly and irregularly until the bottom breaks emerged (45.6 ± 2.2 days after axillary bud break). Thereafter ZR decreased rapidly to 2 pmol ml^{-1}.

4.5 Discussion

Our results show that growth of the rose cultivar Madelon was affected by the genotype of the rootstock (Table 4.1). Both the dry weights of roots and shoot, and the number of bottom breaks were higher for Multic or Moneyway than for Indica major, Madelon or Vivaldi. These results are in agreement with previous observations that Multic and Moneyway induce high flower yields and Marleen moderately high (De Vries and Dubois, 1994). The way in which rose rootstocks affect shoot growth is not known yet. In grape (Skene and Antcliff, 1972), sweet cherry (Stevens and Westwood, 1984) and watermelon (Yamasaki *et al.*, 1994), positive correlations were found between scion growth and cytokinin export from the rootstock. If the same would hold true for rose, it might be possible to improve the performance of a rootstock by exogenous application of cytokinins. Indeed, BA application to the pruned stem was found to stimulate the number of bottom breaks, mainly in the case of Madelon and Vivaldi (Table 4.2). This suggests that the endogenous cytokinin production of these weaker rootstocks was insufficient to induce bottom breaks, which can be overcome by additional exogenous cytokinin application.

In the bleeding sap experiment, the dry weight increase in time of both the shoot (Figure 4.1) and roots (Figure 4.2) of Madelon/Multic and Madelon/Vivaldi did not differ significantly, which may be partly due to the high level of variability. These results are in contrast with those of the first experiment (Table 4.1). The primary difference between the two experiments is the light intensity, being 135 μmol m^{-2} s^{-1} in the first experiment against 250 μmol m^{-2} s^{-1} in the bleeding sap experiment. Light intensity is known to enhance both shoot and root dry weight accumulation markedly. Apparently the weaker growing combination Madelon/Vivaldi was able to profit more of the increase in light intensity than Madelon/Multic. This might cause the observed differences between these experiments, indicating the difficulty of comparing rootstock experiments performed under different environmental conditions.

Our results show a distinct decrease in cytokinin concentration in bleeding sap after bud break of both axillary shoots and bottom breaks (Figure 4.3). Bud break is considered to be regulated by auxin originating from the shoot apex and cytokinins exported from the roots (Cline, 1991). In a rose plant, the apex of the primary shoot exerts apical dominance over the axillary buds along the shoot, until the terminal flower bud is visible. At that time, auxin export from the apex might decrease, causing the uppermost 2-4 axillary buds to break. In bean, a decrease in basipetal auxin flow was found to increase the cytokinin concentration in xylem sap (Bangerth, 1994). An increase in xylem cytokinin concentration is reported to coincide with or precede the beginning of bud outgrowth in bean (Bangerth, 1994) and apple (Tromp and Ovaa, 1990). This is in agreement with our results, in which a high ZR concentration in bleeding sap coincided with axillary bud break. The young axillary shoots might take over the apical dominance and inhibit axillary bud break further down the stem by their auxin production. When the terminal flower buds of the axillary shoots are visible, their auxin export might decreases, causing the cytokinin export from the roots to increase. The increased ZR concentration in bleeding sap might be the signal for the basal buds to break. In this proposed model, axillary bud break is under the direct influence of the apex, whereas basal bud break is primarily affected by cytokinins produced in the roots.

This theory on the regulation of bud break in rose by apical dominance is supported by data of Beever and Woolhouse (1975), who showed that florally induced *Perilla frutescens* showed precocious development of axillary buds, which might reflect a decrease in auxin status of the shoot. In these plants, the concentration of cytokinins in xylem sap was 5 times higher than in sap from vegetative plants (Beever and Woolhouse, 1974). The suggested link between auxin status and cytokinin export from the roots was experimentally confirmed by Bangerth (1994), who found that application of auxin to a decapitated plant abolished the positive effect of decapitation on the cytokinin concentration in xylem sap, indicating that the effect of the shoot apex is mediated by auxins. Following this line of thought, removal of the axillary shoots should lead to an increased cytokinin export from the roots, which was indeed shown by other experiments with rose performed in our department (data not published). Also Beever and Woolhouse (1974) found that the removal of apical and lateral buds from vegetative plants resulted in similar changes of cytokinin flux as occurred in flowering plants.

Our results show that the number of days from grafting to axillary bud break was not affected by the genotype of the rootstock (Figure 4.3). This is a further indication that axillary bud break in our system is determined by the genotype of the scion, more precisely by the auxin production in the apex. However, basal bud break is affected by the genotype of the rootstock, most likely via its cytokinin production, which corresponds with findings of Skene and Antcliff (1972), Stevens and Westwood (1984) and Yamasaki *et al.* (1994).

Chapter 5

Root Temperature Effects on Growth and Bud Break in Relation to Cytokinin Concentrations in Xylem Sap

5.1 Abstract

The effects of three divergent root temperatures (11, 20 and 26°C) on growth and bud break of *Rosa hybrida* were studied. Root morphology changed considerably with root temperature. Roots at 11°C were white, succulent, short and sparsely branched, whereas at 26°C roots were long, brown thin and branched. However, root dry weight was not affected by root temperature. Axillary bud break was earlier at higher root temperatures, resulting in a higher shoot dry weight. At higher temperatures, bottom breaks tended to appear earlier. Our hypothesis was that the effect of root temperature on growth and bud break of the shoot is mediated by cytokinin production in the roots and/or cytokinin translocation to the shoot. The concentration of ZR in bleeding sap was determined during plant development. ZR was found to decrease slightly after bud break, but was not affected by the root temperature treatments, which falsified our hypothesis. Other processes may be involved in the effect of root temperature on shoot growth and bud break.

5.2 Introduction

Root temperature has been shown to have pronounced effects on shoot growth of a number of plant species (Cooper, 1973; Bowen, 1991), including roses. As early as 1949, Shanks and Laurie (1949a,b) found 18°C soil temperature to be optimal for shoot growth of 'Better Times' roses. Zeroni and Gale (1982) confirmed these results for 'Sonia' on the rootstock *Rosa indica*. Increasing the root temperature above 18°C reduced stem length and shoot dry weight of 'Sonora' on *Rosa manetti* stocks (Brown and Ormrod, 1980). On the contrary, Moss and Dalgleish (1984) reported longer stems and higher flower production when the root temperature of roses grown using nutrient film technique (NFT) increased from 18 to 25°C. In contrast, no effects of a similar increase in root temperature were reported for stem length (Kohl *et al.*, 1949) and flower production (Kohl *et al.*, 1949; Tsujita and Dutton, 1983). When the root temperature was lowered from 18 to 10-

12°C, shoot growth was reduced (Shanks and Laurie, 1949a; Moss and Dalgleish, 1984; Mortensen and Gislerød, 1996).

Main functions of roots are uptake of water, absorption of nutrients and synthesis of plant hormones. Effects of root temperature on shoot growth might be mediated via one or several of these functions. Brown and Ormrod (1980) suggested that increasing the root temperature might alter cytokinin supply to the shoot by enhancing cytokinin synthesis and/or by affecting transport of cytokinins. This hypothesis was examined in a number of plant species. Shoot and root dry weights of *Vitis vinifera* were found to be higher at 30°C than at 20°C. This corresponded to a change in the qualitative pattern of cytokinins detected (Skene and Kerridge, 1967). Atkin *et al.* (1973) found that the concentration of cytokinins in xylem sap of *Zea mays* was highest at a root temperature of 28°C, whereas there was a positive effect of temperature on the rates of leaf extension and production between 8 and 33°C. In cucumber, a root temperature of 11°C resulted in a lower root dry weight and cytokinin concentration in the xylem than at 17 or 23°C (Tachibana, 1988). In the latter experiment, also total cytokinin content of the roots was low at 11°C, which indicates that besides translocation, cytokinin production in the roots is also inhibited at low root temperatures. In contrast to these reports, in which cytokinins and growth are positively correlated, no correlation between cytokinins and shoot weight of apple (Tromp and Ovaa, 1994) and tomato (Ali *et al.*, 1996) or bud break of apple (Belding and Young, 1989) were found.

The aim of the present investigation was to study the effects of three divergent root temperatures (11, 20 and 26°C) on growth, bud break and cytokinin concentrations in xylem sap of *Rosa hybrida*. Our hypothesis was that the effect of root temperature on growth and bud break of the shoot was mediated by cytokinin production in the roots and/or cytokinin translocation to the shoot.

5.3 Materials and methods

5.3.1 Plant material and temperature treatments

Rosa hybrida Madelon stems were cut into segments, bearing one intact five-leaflet leaf and a dormant bud, that were grafted onto internodes of the rootstock *R. multiflora* Cathayensis sel. Multic (Van de Pol and Breukelaar, 1982). After dipping the basal parts of these combination plants in 0.5% (w/v) indole butyric acid, the plants were placed in a mixture of perlite, vermiculite and sand (1:3:1, v/v/v) at 25-30°C, RH of 90-100% with additional lighting of 90 μmol m^{-2} s^{-1} (Philips HPI-T, 400W) for 18 hours. After 3 weeks, the rooted plants were transferred to nutrient solution (Steiner, 1984) in a growth chamber at 20°C root and shoot temperature, RH of 75%, photosynthetically active radiation of 350 μmol m^{-2} s^{-1} at plant base level (Philips TLD, 50 W) and at a photoperiod of 14 hours. One week later, uniform plants were selected and randomly allocated to tanks in which the nutrient solution was gradually brought to the desired temperatures of 11, 20 or 26°C. Root and air temperatures were continuously registered by means of thermocouples. From 3 weeks after the beginning of the temperature treatments (i.e. 7 weeks after grafting), 6 plants per root temperature were harvested weekly. Length of the primary shoot, fresh and dry weights of the primary shoot, axillary shoots and bottom breaks, and of the roots after

collecting bleeding sap were recorded. Dates of emergence of axillary shoots and bottom breaks were recorded throughout the experiment.

5.3.2 Quantification of cytokinins in bleeding sap

Bleeding sap was collected by cutting each individual plant just below the primary shoot, one hour after the start of the photoperiod. The first drop of exudate contaminated by rests of damaged cells was discarded. A piece of silicone tubing was mounted onto the stump. A capillary tube was inserted through the silicon tubing just above the cut end and led the exudate to a vial kept at 2°C, containing 1 µmol Na_2MoO_4 to inhibit phosphatase activity. While exuding, the roots were kept at the same temperature as during the temperature treatment (i.e. 11, 20 or 26°C). Bleeding sap was collected for 30 minutes and stored at -20°C until purification. After adjusting the pH to 3.7 with 10 mM NH_4-formate buffer, the sap was purified by passing by gravity through a Sep-pak C_{18}-cartridge (Waters, Milford, MA, USA), which had been primed with 10 ml 96% ethanol and 10 ml 10 mM NH_4-formate buffer at pH 3.7. The cartridge was washed with 10 ml NH_4-formate buffer and 60 ml deionized water, and the cytokinins were eluted off with 10 ml 96% ethanol. The eluate was split in two parts, for determination of Z and ZR, and of iP and iPAR. The eluates were evaporated to dryness and redissolved in the corresponding HPLC-eluens. For HPLC separation of Z and ZR a µBondapak C_{18}-column (Waters, 30 x 0.39 cm) was used that was eluted with 22% methanol/10 mM NH_4-formate pH 3.7 at a flow rate of 2 ml min^{-1} and a column temperature of 25°C. IP and iPAR were separated on a Lichrosorb 10RP8 column (Chrompack, 25 x 0.46 cm), eluted with 48% methanol/10 mM NH_4-formate pH 3.7 at a flow rate of 2.5 ml min^{-1} and a column temperature of 25°C. The Z, ZR, iP and iPAR fractions were collected, evaporated to dryness and taken up in PBST buffer (0.14 M NaCl, 10 mM Na_2HPO_4, 0.15 mM KH_2PO_4, 2.7 mM KCl, pH 8.4, and 0.05% (v/v) Tween 20). Cytokinins were quantified by means of the indirect competitive enzyme immunoassay as described by Vonk *et al.* (1986).

5.3.3 Statistics

Data were submitted to statistical analysis of variance (ANOVA). If relevant, curves were fitted using the statistical package GENSTAT 5. The significance of differences was determined by Student's t-tests.

5.4 Results

5.4.1 Effect of root temperature on plant growth

Plants at a root temperature of 11°C had white, succulent roots that were short and sparsely branched. At 26°C, the roots were long, brown, thin and well branched. At 20°C, the roots were slightly shorter and less branched than at 26°C. However, root dry weight was not significantly affected by root temperature ($P = 0.25$; Figure 5.1). At the time the root temperature treatments started, i.e. 4 weeks after grafting, the primary shoot was 5-10 cm long. Both stem length and dry weight of the primary shoot, as well as the process of flower bud opening were not affected by root temperature (data not shown). The first

visible effect of root temperature on shoot growth was on the outgrowth of axillary buds. The higher the root temperature, the earlier the axillary shoots appeared (P = 0.05; Figure 5.2). On average, all plants formed 3 axillary shoots. Dry weight of the axillary shoots was significantly higher at 26 than at 20 and 11°C (data not shown). At higher root temperatures, bottom breaks appeared earlier, although not significantly so (P = 0.21; Figure 5.3). Dry weight of the bottom breaks formed at 11°C tended to be lower than at 20 and 26°C, but these differences were not significant (data not shown). Dry weight of the above-ground plant parts, i.e. primary shoot, axillary shoots and bottom breaks taken together, was significantly higher at 26 than at 20 and 11°C (P = 0.02; Figure 5.4). This implies that the shoot/root ratio at 26°C is higher than at 20 and 11°C. Percentages dry matter of the total shoot (about 23%) was not affected by root temperature (data not shown).

5.4.2 Effect of root temperature on cytokinin concentration in bleeding sap

In none of the bleeding sap samples iP or iPAR was detectable (detection limit 3.0 and 1.5 pmol sample^{-1} respectively). As in 60% of the samples Z was found to be below the detection limit (1.5 pmol sample^{-1}), these data are not presented. Although root temperature did not significantly affect the ZR concentration in bleeding sap, ZR at 26°C had its peak earlier than at 11°C, which corresponded to earlier bud break (Figure 5.5).

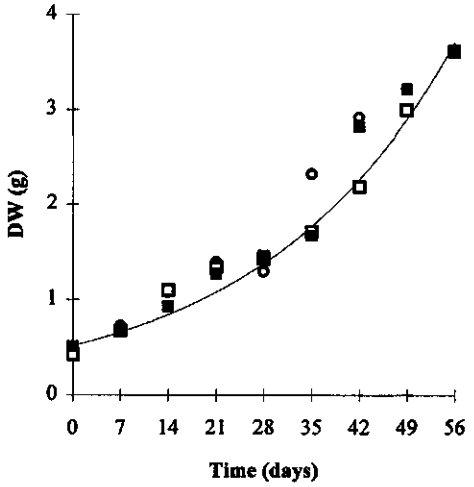


Figure 5.1 *Growth of the roots of Madelon/Multic combination plants at root temperatures of 11 (○), 20 (□) and 26°C (■). Each point represents the average value of 6 plants. One logistical curve was fitted* $Y = 219 / (1 + e^{-0.0360 (X-169)})$. *Day 0 is 3 weeks after the start of the temperature treatment.*

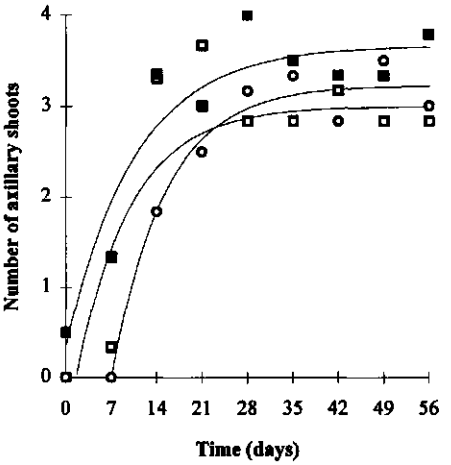


Figure 5.2 *Number of axillary shoots per plant as affected by root temperatures of 11 (○), 20 (□) and 26°C (■). Each point represents the average value of 6 plants. For each root temperature, an exponential curve was fitted.*
11°C, $Y = 3.2 - 7.6\ 0.89^X$; 20°C, $Y = 3.0 - 3.6 \cdot 0.89^X$; 26°C, $Y = 3.6 - 3.3\ 0.91^X$

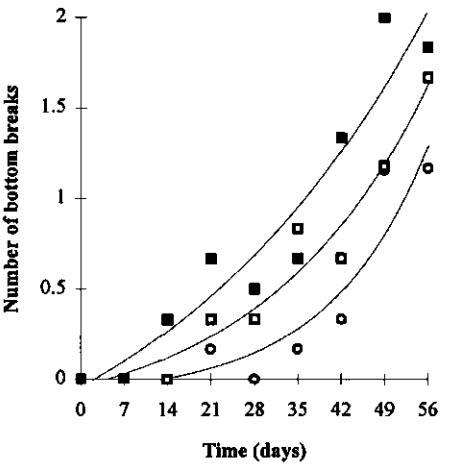


Figure 5.3 *Number of bottom breaks per plant as affected by root temperature of 11 (○), 20 (□) and 26°C (■). Each point represents the average value of 6 plants. For each root temperature, a logistical curve was fitted.*
11°C, $Y = -0.09479 + 1319 / (1 + e^{-0.06239 (X - 165.9)})$
20°C, $Y = -0.2698 + 18173 / (1 + e^{-0.0377 (X - 299.1)})$
26°C, $Y = -0.9012 + 11971 / (1 + e^{-0.02196 (X - 434.5)})$

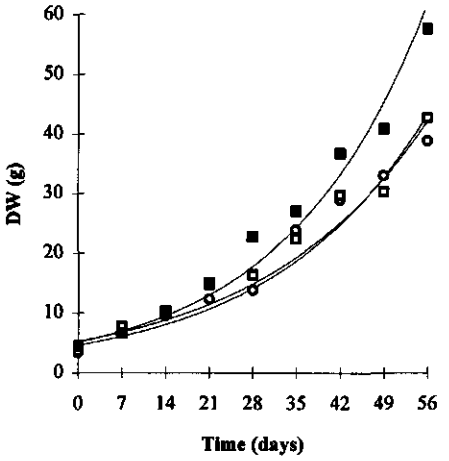


Figure 5.4 *Growth of the shoot (primary shoot + axillary shoots + bottom breaks) at root temperatures of 11 (○), 20 (□) and 26°C (■). Each point represents the average value of 6 plants. For each root temperature, a logistical curve was fitted.*
11°C, $Y = 79122 / (1 + e^{-0.03999 (X - 243.8)})$
20°C, $Y = 16497 / (1 + e^{-0.03729 (X - 216.0)})$
26°C, $Y = 4624445 / (1 + e^{-0.04456 (X - 307.8)})$

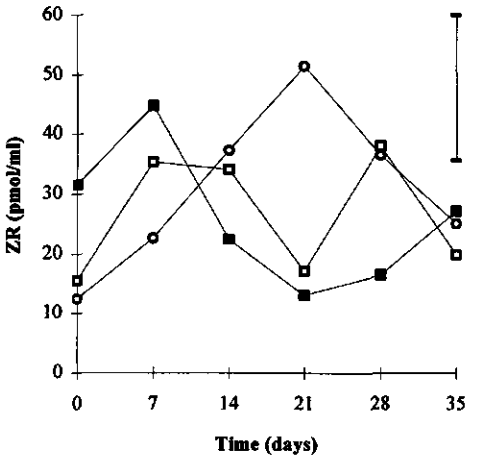


Figure 5.5 *Concentration of ZR (pmol ml^{-1}) in bleeding sap collected in 30 min as affected by root temperatures of 11 (○), 20 (□) and 26°C (■). Vertical bar indicates LSD (P = 0.05).*

5.5 Discussion

The increase of root temperature in the range of 11 to 26°C was reflected in distinct changes in root morphology, which agrees well with other results obtained for rose (Shanks and Laurie, 1949a; Mortensen and Gislerød, 1996). In spite of these changes, dry weight of the roots was not affected by root temperature, which contrasts with the results of Shanks and Laurie (1949b) who found a higher root dry weight at 13°C than at 20°C.

Although the root temperature treatments already started when the primary shoot was 5-10 cm long, neither flower development nor dry weight or stem length of the primary shoot was affected by these treatments. The effect of increasing root temperatures in the range of 10 to 25°C has previously been shown to vary considerably for roses, from negative (Brown and Ormrod, 1980), to none (Kohl *et al.*, 1949), to positive (Shanks and Laurie, 1949a; Moss and Dalgleish, 1984; Mortensen and Gislerød, 1996).

Although growth of the primary shoot was not affected by root temperature, our results show that axillary bud break was advanced at higher root temperatures. Bud break of the bottom breaks also tended to be earlier at higher root temperatures, although due to the high level of variability this effect was not significant. Brown and Ormrod (1980) found that root temperature positively affected the number of bottom breaks formed. They suggested that an increase of root temperature may alter the cytokinin supply to the shoot, by enhancing cytokinin synthesis and/or by alterations in the transport of cytokinins. Exogenous application of benzyladenine in lanolin paste to intact rose plants stimulated bud break of axillary buds (Ohkawa, 1984) and bottom breaks (Parups, 1971). Our results show that the ZR concentration in bleeding sap was not affected by the root temperature treatments ($P = 0.54$; Figure 5.5). However, at 11 and 26°C the ZR concentration decreased when the plants had formed 3 axillary shoots. These data correspond well with the results of Belding and Young (1989), who reported for young apple trees a higher percentage bud break at root temperatures of 20°C than at 10°C. Cytokinin concentration in xylem sap decreased as bud break occurred, but the concentration was not affected by root temperature (Belding and Young, 1989). Tromp and Ovaa (1994) did not find an effect of root temperature on the concentration of cytokinins (primarily ZR) in xylem sap of apple, although shoot growth was markedly reduced at the lower root temperature. On the other hand, in maize (Atkin *et al.*, 1973) and cucumber (Tachibana, 1988), a positive correlation between shoot growth and xylem cytokinins was established.

The effects of root temperature on shoot growth might be mediated via synthesis of plant hormones, uptake of water and/or absorption of nutrients. As our results show that bud break is affected by root temperature, whereas the ZR concentration in bleeding sap is not, other plant hormones than cytokinins might be involved. Atkin *et al.* (1973) found an increase in gibberellic acid (GA) and cytokinin activity and a decrease in abscisic acid activity in maize xylem sap with increasing root temperatures from 8 to 28°C, which correlated positively with shoot growth. However, the mode of action of root temperature cannot be completely explained in terms of hormones, since maize shoot growth was further stimulated when the root temperature increased to 33°C, whereas cytokinin concentration was about 25% of that at 28°C and GA about 40% (Atkin *et al.*, 1973). Furthermore, root temperature may affect shoot growth via water uptake. In our system, in which plants grew in nutrient solution, this is not very likely, since the dry matter percentage of the shoot was not affected by root temperature. Lastly, the uptake of nutrients might be involved. In our experiment, the availability of nutrients was high, growth of the primary shoot was not affected by root temperature, and there were no

visible signs of shortage of nutrients. Hence, it is improbable that the effect of root temperature is mediated via uptake of nutrients.

In previous experimental work, confounding effects of root temperature on shoot growth of roses were reported. Our main results indicate earlier bud break at higher root temperatures, and enhancement of shoot growth, primarily of the axillary shoots. In contrast to the optimal root temperature of 18°C, as was found by Shanks and Laurie (1949b), our results indicate that for roses on nutrient solution the optimal root temperature might be higher. Our hypothesis concerning the mediation of the root temperature effect via an increase in cytokinin translocation to the shoot was not confirmed by our results. The processes involved are so far unclear.

Chapter 6

The Structure of Cytokinins affects Axillary Bud Growth

6.1 Abstract

The effect of the cytokinin free bases BA, Z, iP, (RS)DHZ, R(+)DHZ, S(-)DHZ and their ribosides on axillary bud break and growth of Madelon roses *in vitro* was studied. Addition of cytokinins was necessary for the bud to grow out to a fully developed shoot. Bud break, expressed as the number of days until the first compound leaf was visible, was not affected by the presence of cytokinins. When the cytokinin concentration in the medium was raised from 0 to 32 μM, the dry weight of the total plant increased. Depending on the type of cytokinin added, the optimum was reached at 3.2 or at 32 μM. The number and dry weight of secondary and tertiary shoots increased with increasing exogenous cytokinin concentrations. Cytokinin activity was in the decreasing order Z ≥ BA >> iP >> S(-)DHZ > (RS)DHZ > R(+)DHZ. Cytokinin ribosides were equally active as their cognate free bases. Structure-activity relationships of cytokinins with respect to axillary bud break and outgrowth were discussed.

6.2 Introduction

As is generally accepted, cytokinins are mainly produced in root tips and move via the xylem to the shoot where they participate in the control of growth and development (Van Staden and Davey, 1979). In a number of plant species, ZR is found to be the principal cytokinin in the xylem (Wagner and Beck, 1992; Letham, 1994). Most likely, cytokinins transported into the buds are involved in the induction of bud break and the subsequent outgrowth (Letham, 1994). Exogenous application of BA to rose plants broke the quiescence of axillary buds (Ohkawa, 1984). Pillay and Railton (1983) demonstrated bud break and elongation in intact seedlings of *Pisum sativum* following a single application of BA or Z. Kinetin or iP application appeared to be less effective.

The structure-activity relationships of cytokinins were reviewed by Matsubara (1990) who compared their biological activities in several bioassays. Cytokinin activity depends in the first place on the structure of the substituents on the purine ring. In general, cytokinin activity is in the decreasing order of bases >> ribosides ≥ ribotides (Matsubara, 1990). Furthermore, cytokinin activity decreases when the planarity of the side chain is

disturbed by adding substituents to the double bond (Hecht *et al.*, 1970). Thus, in many bioassays, Z is more active than DHZ. Lastly, cytokinin activity is determined by the stereochemical configuration of the asymmetric carbon atom in the side chain. In several bioassays, the synthetic R(+)DHZ was found to be more active than the naturally occurring S(-)DHZ (Matsubara *et al.*, 1977).

In the present investigation, the response of axillary buds of Madelon roses to the cytokinin free bases BA, Z, iP, (RS)DHZ, R(+)DHZ, S(-)DHZ and their ribosides is studied, using an *in vitro* model system (Marcelis-van Acker and Scholten, 1995). Bud break and subsequent outgrowth were characterized by (1) the number of days until bud break, i.e. first compound leaf visible; (2) dry weight of the plant developing from the bud after 28 days of growth; (3) branching of the plant, reflected in the number and dry weights of secondary and tertiary shoots originating from the primary shoot.

6.3 Materials and methods

One cm long stem segments of *Rosa hybrida* Madelon bearing one quiescent axillary bud and a piece of petiole were cut. Segments belonging to the second, third and fourth five-leaflet leaf counted from the apex were sterilized by dipping in 70% ethanol and immersing in 1% NaOCl for 20 minutes. After rinsing three times with sterile water, the buds were excised and placed on MS medium (Murashige and Skoog, 1962), containing glycine (2 mg l^{-1}), thiamine, nicotinic acid, pyridoxine (each 0.5 mg l^{-1}), *myo*-inositol (100 mg l^{-1}) and glucose (45 g l^{-1}). The pH of the medium was adjusted to 5.7 with 1M NaOH before 0.7% (w/v) BBL agar (Becton Dickinson, USA) was added. The medium was sterilized during 15 minutes at 115°C. Cytokinins, dissolved in 0.07 M KOH (Dieleman *et al.*, 1995) were added filtersterile (Millex-FG, 0.2 µm, Millipore) as well as an equimolar amount of 1 M HNO_3 (Acrodisc CR, 0.2 µm, Gelman Sciences). Thus, per litre medium 0.9 mmol KOH and 0.9 mmol HNO_3 were added, which is 4.6% more K^+ and 2.3% more NO_3^- than was already present in the MS medium. To enable the addition of 320 µM DHZ and DHZR, per litre medium 9 mmol KOH and 9 mmol HNO_3 were added. Testing 32 µM ZR with additional 9 mM KOH and HNO_3 showed that this 46% extra K^+ and 23% extra NO_3^- did not affect bud growth (data not shown). The cytokinins (except for BA and BAR) were purchased from Apex Organics, BA from Sigma and BAR from Aldrich. Explants were incubated at 20°C, photosynthetically active radiation of 60 µmol m^{-2} s^{-1} and a photoperiod of 16 hours for 28 days. The number of days until the first compound leaf was visible was registered. At harvest, fresh and dry weights and number of secondary and tertiary shoots were recorded.

In the first of two experiments, BA, BAR, Z, ZR, iP, iPAR, (RS)DHZ and (RS)DHZR were tested at 0.32, 3.2 and 32 µM. (RS)DHZ is a racemic mixture of R(+)DHZ and S(-)DHZ, which are enantiomers. (RS)DHZR is a mixture of equimolar amounts of R(-)DHZR and S(-)DHZR, which are diastereoisomers. In the second experiment the concentration range of (RS)DHZ and (RS)DHZR was extended to 320 µM due to their low activity. To certify which enantiomers primarily determined the activity of (RS)DHZ and (RS)DHZR, R(+)DHZ, S(-)DHZ, R(-)DHZR and S(-)DHZR were included in the second experiment. Z and ZR were used to compare experiments 1 and 2. Both experiments were arranged in a randomized block design, in which each treatment was represented by 4 replicates of 5 explants. Means and least significant differences (LSD) were calculated using GENSTAT 5.

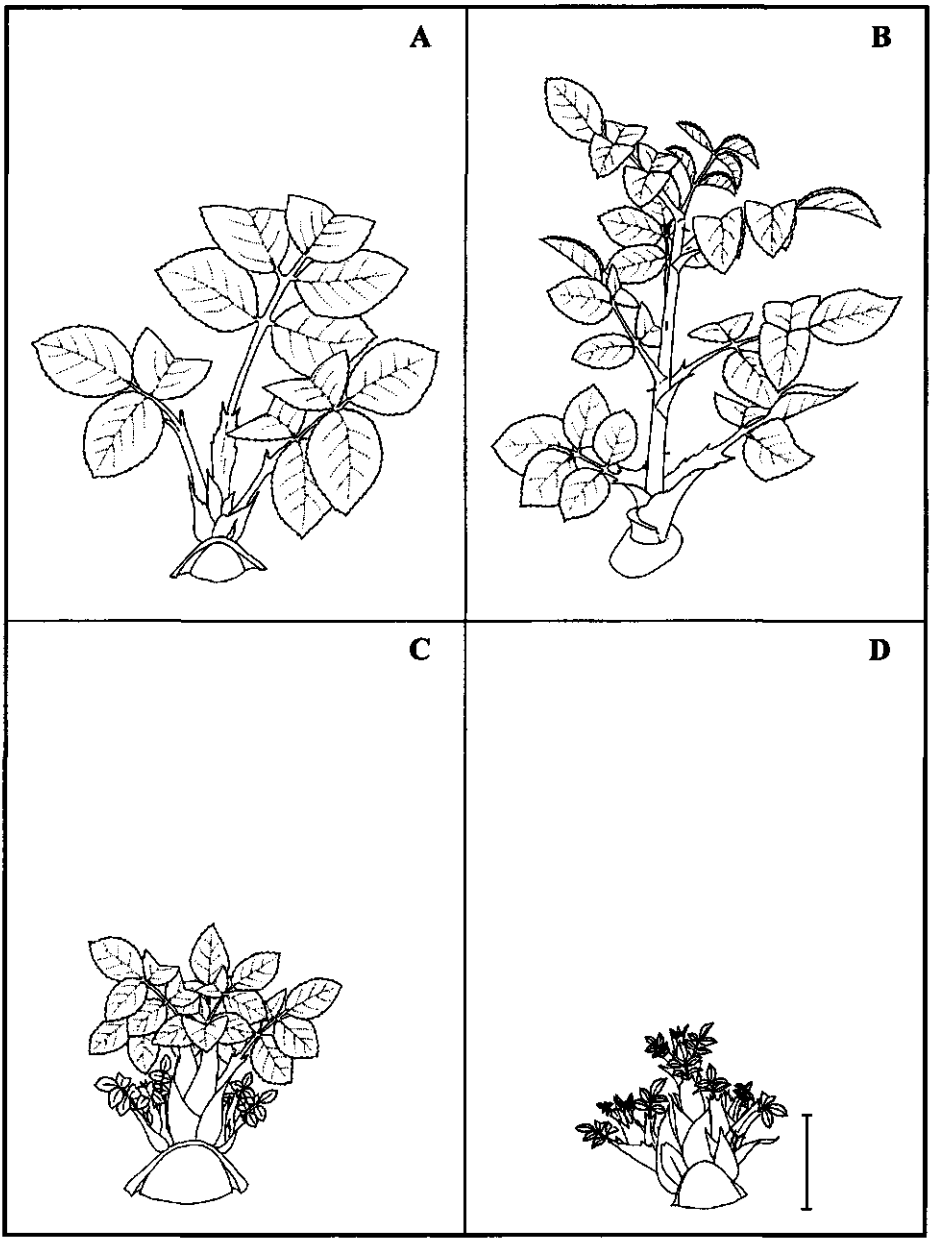


Figure 6.1 *Development of axillary rose buds after 28 days of growth on medium without cytokinins (A), with 0.32 (B), 3.2 (C) or 32 (D) µM ZR. Vertical bar indicates 1 cm.*

6.4 Results

A quiescent axillary bud from the middle region of a harvestable shoot already contained eleven leaves of which the lowermost 7 are scale-like and the upper ones compound leaves (Marcelis-van Acker, 1994). Placing this bud on a medium without cytokinins caused the leaves that were already present to grow out (Figure 6.1A). Stem elongation and new-formation of leaves did not occur. Two small secondary buds were visible, of approximately 0.1 mg dry weight each. When 0.32 μM ZR was added to the medium, bud growth was comparable to the development of the axillary bud in vivo (Figure 6.1B). New compound leaves were formed and stem elongation, including the formation of prickles, took place. Addition of 3.2 μM ZR caused the outgrowth of two secondary shoots (Figure 6.1C). When the concentration of ZR was increased to 32 μM, tertiary shoots emerged in the axils of the secondary shoots (Figure 6.1D). The dry weight of the secondary and tertiary shoots was approximately equal to the dry weight of the primary shoot. When the cytokinin concentration in the medium increased from 0 to 32 μM, the leaves became smaller, stem elongation was reduced, and the percentage dry matter decreased from 13.6 in the absence of cytokinin to 9.2 at 32 μM ZR (data not shown). Bud break, expressed as the number of days until the first compound leaf was visible, was not affected by the presence of cytokinins (data not shown).

For experiment 1, the dry weight of axillary buds after 28 days of growth is depicted in Figure 6.2. When BA, BAR, Z or ZR in the medium were applied at 0.32, 3.2 or 32 μM, the dry weight of the total plant showed an optimum at 3.2 μM, whereas the dry weight of the secondary and tertiary shoots still increased from 3.2 to 32 μM. At 3.2 and 32 μM iP and iPAR in the medium, the dry weight of the secondary and tertiary shoots was significantly lower than when BA, BAR, Z or ZR were applied at the same concentration. (RS)DHZ or (RS)DHZR did not cause outgrowth of secondary shoots at 3.2 μM. The effect of 32 μM (RS)DHZ or (RS)DHZR was equal to that of 3.2 μM Z or ZR. The type of cytokinin affected the total number of secondary and tertiary shoots; significant differences in dry weight per secondary/tertiary shoot could not be observed (Table 6.1).

In the second experiment, the response of buds to DHZ and DHZR was further investigated (Figure 6.3). Z and ZR had a similar effect as in experiment 1, which allowed comparison between both experiments. Axillary buds on medium with 320 μM (RS)DHZ or (RS)DHZR showed a similar response as on 32 μM Z or ZR. Both S(-)DHZ and S(-) DHZR appeared to be more active than their cognate R-forms. The DHZ and DHZR forms induced the same number of secondary and tertiary shoots, although the dry weight per shoot was higher for the S-forms of DHZ and DHZR than for the R-forms (Table 6.2).

6.5 Discussion

Addition of cytokinins appeared to be necessary for an axillary bud to grow out to a fully developed shoot, isolated from the intact plant. Our results show that chemical modification of the side chain of cytokinins affected outgrowth of rose buds. Of the cytokinins tested, BA, BAR, Z and ZR were the most active ones. (RS)DHZ and (RS)DHZR were severalfold less active than Z and ZR, which is in accordance with the results of several bioassays (Matsubara *et al.*, 1977; Mok *et al.*, 1992). The double bond in the side chain of

Table 6.1 *Number of secondary and tertiary shoots and dry weight per shoot at 32 μM cytokinin (Experiment 1).*

cytokinin	number of shoots	dry weight per shoot (mg)
control	1.60	0.10
BA	7.83	2.62
BAR	4.40	3.35
Z	8.35	2.63
ZR	7.34	2.88
iP	3.65	3.13
iPAR	3.30	1.75
(RS)DHZ	2.96	2.84
(RS)DHZR	3.24	2.63
LSD (P=0.05)	1.98	1.65

Table 6.2 *Number of secondary and tertiary shoots and dry weight per shoot at 32 μM cytokinin (Experiment 2).*

cytokinin	number of shoots	dry weight per shoot (mg)
control	2.00	0.10
Z	5.15	4.31
ZR	5.40	3.91
(RS)DHZ	2.40	5.49
(RS)DHZR	3.05	3.15
R(+)DHZ	2.00	2.23
S(-)DHZ	2.15	5.59
R(-)DHZR	2.55	2.93
S(-)DHZR	2.70	6.76
LSD (P=0.05)	0.46	2.22

Z seems to be one of the structural features which confers such a strong cytokinin activity; saturation of the double bond reduces cytokinin activity (Matsubara, 1990). Our results show that substitution of the alkyl side chain by a benzyl group, as in BA and BAR has little effect on rose bud break and outgrowth. For ring-substituted aminopurines, of which BA is the most active one, similar structure-activity relationships are found as for 6-alkylaminopurines. Apparently, these two groups of cytokinins can exert similar activity, in spite of their structural differences.

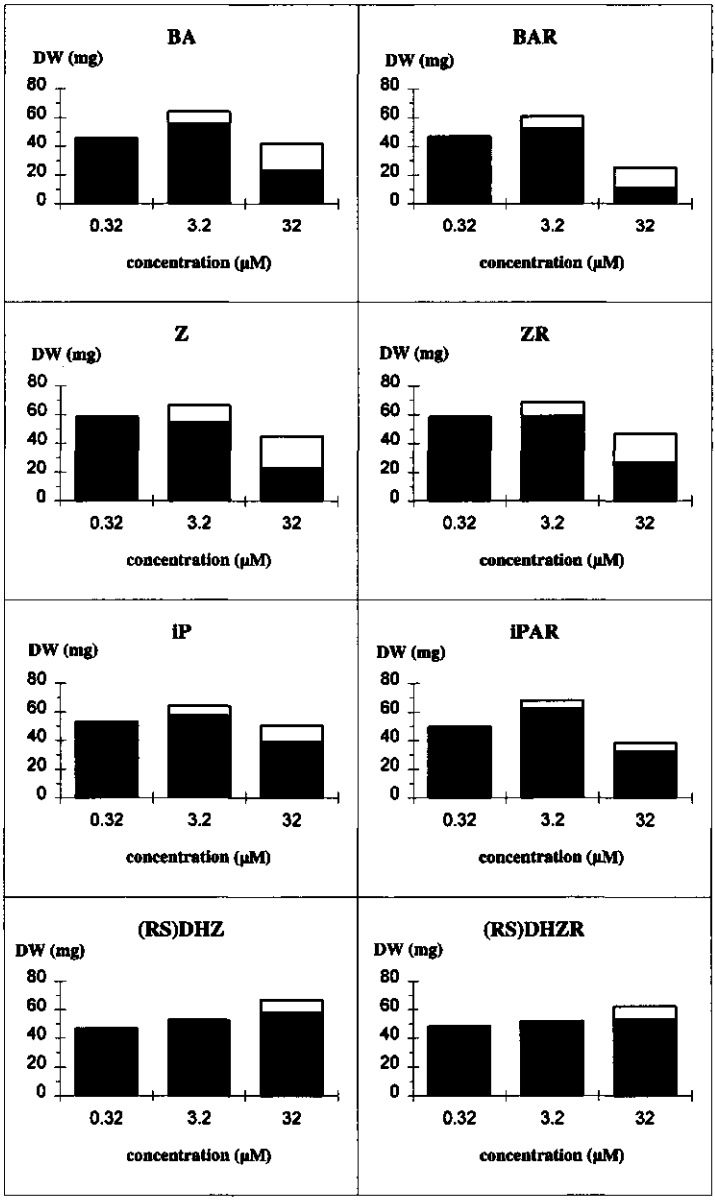


Figure 6.2 *Effect of BA, BAR, Z, ZR, iP, iPAR, (RS)DHZ and (RS)DHZR at 0.32, 3.2 or 32 µM on dry weight of primary (■) and secondary and tertiary (□) shoots of Madelon roses after 28 days of growth (Experiment 1). Dry weight control plants (no cytokinin) is 46.7 mg. LSD (P=0.05) primary shoot = 6 mg, LSD (P=0.05) secondary and tertiary shoots = 3 mg, LSD (P=0.05) total plant = 7 mg.*

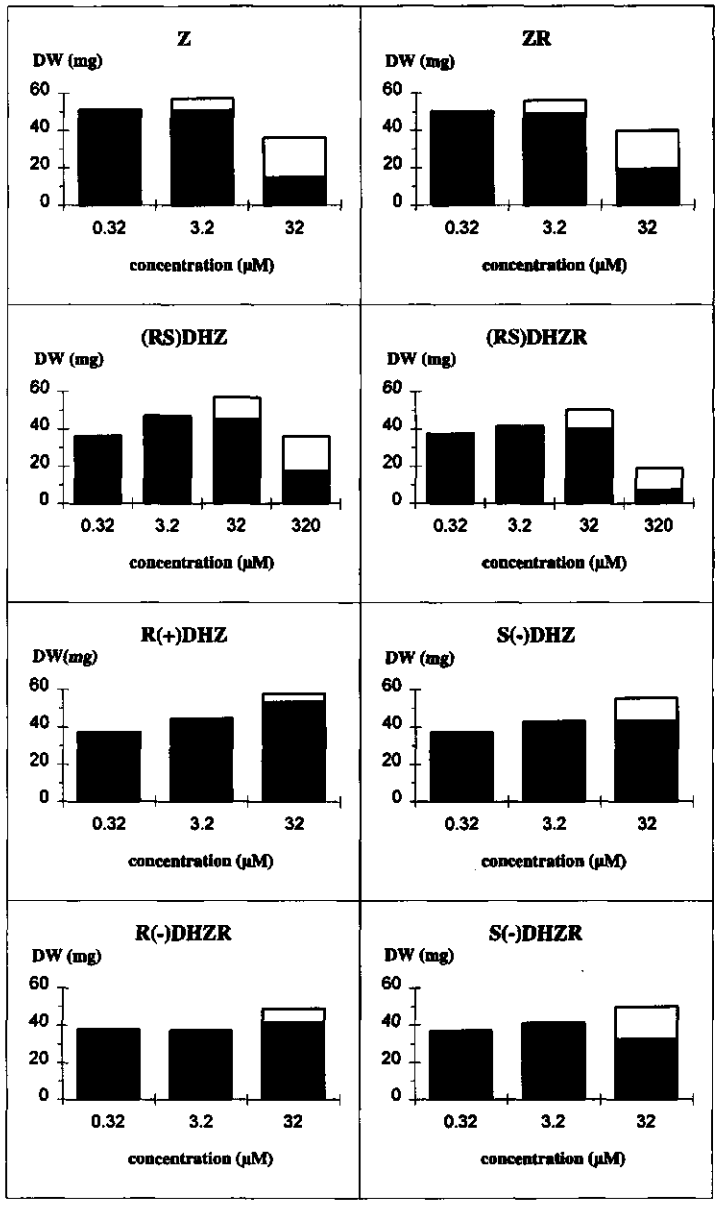


Figure 6.3 *Effects of (RS)DHZ, (RS)DHZR, R(+)DHZ, S(-)DHZ, R(-)DHZR and S(-)DHZR at 0.32, 3.2, 32 or 320 µM on dry weight of primary (■) and secondary and tertiary (□) shoots of Madelon roses after 28 days of growth (Experiment 2). Dry weight control plants (no cytokinin) is 39.4 mg. LSD (P=0.05) primary shoot = 6 mg, LSD (P=0.05) secondary and tertiary shoots = 3 mg, LSD (P=0.05) total plant = 4 mg.*

Our results show that cytokinin bases and ribosides are equally active in rose axillary bud growth, whereas in general, bases are more active than their corresponding ribosides (Matsubara, 1990). In some cases, even reversed activities of bases and ribosides were found (Mok *et al.*, 1985). When cytokinin bases are applied to plant tissues, the principal metabolites formed initially are the nucleotides and ribosides. Exogenous cytokinin ribosides yield bases and nucleotides. The nucleotides formed are rapidly further metabolized (Letham and Palni, 1983). The fact that cytokinin ribosides were equally active in rose bud growth as their cognate free bases might be explained in two ways. Firstly, the base and riboside might be metabolized with a similar efficiency to the nucleotide to be further metabolized via the same route. Secondly, the base might be converted to the riboside or the riboside to the base, yielding one 'active' cytokinin. Our results are not suitable to exclude either one of these metabolic conversions.

In rose bud growth, the naturally occurring S(-)DHZ and S(-)DHZR appeared to be more active than their cognate synthetic R-forms. In contrast to this observation, R(+)DHZ and R(-)DHZR showed stronger cytokinin activities than the S-forms in other bioassays (Matsubara *et al.*, 1977), which might be explained by tissue-related stereoselectivity in inactivation of exogenously applied cytokinins.

All cytokinins tested appeared to be able to induce a similar maximal response, which suggests that these compounds bind to the same receptor. Cytokinin activity depends on the structure of the substituents on the purine ring and side chain, and on the stereochemical configuration of the asymmetric carbon atom in the side chain (Matsubara *et al.*, 1977). The configuration of the cytokinin affects the interaction between cytokinin molecule and its receptor site (Matsubara, 1990). Modification of the receptor can change its affinity to cytokinins, which will result in a shift in the dose-response curve along the X-axis, while the shape remains the same (Firn, 1986). Our results (Figures 6.1 and 6.2) indicate that the response of the buds to the different cytokinins is similar, but reaches a maximum at a cytokinin-dependent concentration. This suggests that cytokinins bind with different affinities to the same receptor. Another possibility is that the different cytokinins are metabolized to an 'active' cytokinin at a per cytokinin characteristic efficiency. This possibility cannot be excluded based on our results.

The single node system as a model system offers the possibility to study the effect of different cytokinins on processes involved in bud break and subsequent outgrowth at three levels: (1) Timing of bud break, expressed as the number of days until the first compound leaf was visible, which was not affected by the presence or the concentration of cytokinin in the medium. (2) Outgrowth of the primary shoot; its dry weight increased at increasing exogenous cytokinin concentrations. Depending on the type of cytokinin added, the optimum was reached at 3.2 μM or at a higher concentration. (3) Initiation and outgrowth of secondary and tertiary shoots. The number and dry weights of these shoots increased with increasing cytokinin concentration in the medium. Although this model system is rather complex, it has the advantage that growth and development are very similar to that of the plant *in vivo*.

Chapter 7

Uptake and Metabolism of ^{3}H-Zeatin Riboside and ^{3}H-Dihydrozeatin Riboside by Axillary Buds

7.1 Abstract

Comparing the response of axillary buds of *Rosa hybrida* Madelon for a number of cytokinins revealed that ZR was severalfold more active in inducing side-shoot formation than DHZR (Chapter 6). This might be explained by either a higher rate of uptake of ZR than of DHZR, or by differences in metabolism between ZR and DHZR, yielding different concentrations of 'active' cytokinins in the tissue. Feeding studies showed that uptake of ^{3}H-ZR and ^{3}H-DHZR by *in vitro* cultured axillary shoots did not differ significantly until day 14. As at that time, differences between ZR and DHZR in side-shoot formation were already visible, a causal relationship is not likely. Major metabolites of ^{3}H-ZR and ^{3}H-DHZR were ZOG and DHZROG, respectively. ^{3}H-ZR could be detected as the riboside up to day 3, whereas from day 1 on, ^{3}H-DHZR was metabolized, suggesting that growth of axillary buds might be affected by differences in metabolism between ZR and DHZR.

7.2 Introduction

Cytokinins are root-produced plant hormones that are transported via the transpiration stream to the above-ground plant parts where they stimulate growth and development. Most likely, cytokinins are involved in the induction of bud break and the subsequent outgrowth (Letham, 1994). Exogenous application of cytokinins to rose (Ohkawa, 1984) and bean (Pillay and Railton, 1983) plants broke the quiescence of axillary buds. In rose as well as in a number of other plant species, ZR is the predominant cytokinin in xylem exudate (Wagner and Beck, 1992; Letham, 1994; Chapter 2). The effects of various cytokinins on axillary bud break can be more easily studied when buds are isolated from the intact plant and are cultured in an *in vitro* system (Auer *et al.*, 1992a; Marcelis-van Acker and Scholten, 1995). In Chapter 6, the response of axillary buds of *Rosa hybrida* Madelon to a number of cytokinin free bases and ribosides was tested. ZR was found to be severalfold more active in inducing side-shoot formation than DHZR. This may be explained by either a higher rate of uptake of ZR than of DHZR, or by differences in metabolism between ZR and DHZR, yielding different concentrations of 'active'

cytokinins in the tissue. Studies in which ^{3}H-cytokinins were fed to plant tissues showed that a multitude of metabolites were formed, like adenine and adenosine (Singh *et al.*, 1992d; Sergeeva *et al.*, 1994), 7- and 9-glucosides (McGaw *et al.*, 1984, Van der Krieken *et al.*, 1988; Auer *et al.*, 1992b), ribosides (Palmer *et al.*, 1981) and O-glucosides (Jameson *et al.*, 1987; Sergeeva *et al.*, 1994).

In the present study, uptake of ^{3}H-ZR and ^{3}H-DHZR by axillary buds of *Rosa hybrida* Madelon and their further metabolism were studied. Our hypothesis is that the rate of uptake of ZR is higher than that of DHZR, leading to the formation of more shoots. Furthermore, an attempt was made to find out whether the observed differences in growth of axillary buds between ZR and DHZR are caused by differences in metabolism, resulting in the presence of more 'active' form(s) of ZR than DHZR in the tissue.

7.3 Materials and methods

7.3.1 Plant material

One cm long stem segments of *Rosa hybrida* Madelon bearing one quiescent axillary bud and a piece of petiole were cut. Segments belonging to the second, third and fourth five-leaflet leaf counted from the apex were sterilized by dipping in 70% ethanol and immersing in 1% (w/v) NaOCl for 20 minutes. After rinsing three times with sterile water, the buds were excised and each placed in test tubes on 15 ml MS medium (Murashige and Skoog, 1962), containing glycine (2 mg l^{-1}), thiamin, nicotinic acid, pyridoxine (each 0.5 mg l^{-1}), *myo*-inositol (100 mg l^{-1}) and glucose (45 g l^{-1}). The pH of the medium was adjusted to 5.7 with 1 M NaOH before 0.7% (w/v) BBL agar (Becton Dickinson, USA) was added. The medium was sterilized during 15 minutes at 115°C. For uptake studies ^{3}H-ZR (0.9 TBq mmol^{-1}) or ^{3}H-DHZR (1.1 TBq mmol^{-1}) in ethanol, specifically labelled at the C-2 position, was added to the medium to give an approximate activity of 0.1 kBq per ml. ^{3}H-ZR and ^{3}H-DHZR were obtained from Dr. M. Strnad, Institute of Experimental Botany, Czech Academy of Sciences, Olomouc, Czech Republic. The final concentration of ethanol in the medium is 0.07%, which does not affect the development of rose axillary buds (Dieleman *et al.*, 1995). Unlabelled ZR or DHZR (Apex Organics, Honiton, UK), dissolved in 0.07 M KOH, was applied filtersterile (Millex-FG, 0.2 µm, Millipore) to a final concentration of 32 µM. An equimolar amount of 1 M HNO_3 was added filtersterile (Acrodisc CR, 0.2 µm, Gelman Sciences) to maintain the pH. Explants were incubated at 20°C, at a photon fluence rate of 60 µmol m^{-2} s^{-1} and at a photoperiod of 16 hours.

7.3.2 Growth and uptake of ^{3}H-ZR and ^{3}H-DHZR

Uptake of ^{3}H-ZR and ^{3}H-DHZR was determined 1, 2, 3, 5, 7, 10, 14, 18, 23 and 28 days after the start of the experiment. Three explants per treatment were split into the stem segments, petioles and shoots (Figure 7.1). After weighing, each plant part was digested overnight in 2 ml of the tissue solubilizer Lumasolve (Lumac, Groningen, The Netherlands) at 50°C. Since the solution that resulted was too dark for accurate scintillation counting, it was bleached by adding 0.4 ml 2-propanol and 0.4 ml 30% hydrogen peroxide (Nordström and Eliasson, 1986). To measure radioactivity in the agar,

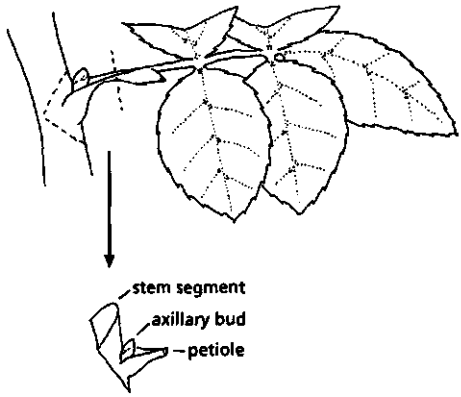


Figure 7.1 *Segment excised from the shoot of Rosa hybrida Madelon.*

the medium was heated to 80°C and 1 ml of the liquid medium was added to 2 ml of 30% hydrogen peroxide (Nordström and Eliasson, 1986). The agar was dissolved overnight at 50°C. To each vial, 15 ml of the scintillation counting solution Ultima Gold (Packard, Meriden, USA) was added. The samples were counted for 10 min in a Tri-carb 2100TR liquid scintillation analyser (Packard, Meriden, USA).

The experiment was arranged in a randomized block design, in which each harvest was represented by 3 replicates of 3 explants. Data were submitted to analysis of variance (ANOVA) by means of the statistical package GENSTAT 5. The significance of differences was determined by Student's t-test.

7.3.3 Metabolism of ^{3}H-ZR and ^{3}H-DHZR

To study metabolism, 130 axillary buds were placed on medium containing ^{3}H-ZR and 130 buds on medium containing ^{3}H-DHZR. After 1, 3 and 28 days, buds were collected, and split into stem segment, petiole and shoot (Figure 7.1). At day 1, 35 buds were combined, at day 3 21 buds and at day 28 9 buds. All samples were taken in duplo. As the fresh weight of the petiole did not increase and as it did not take up any ^{3}H-cytokinin after day 1, metabolism in the petiole was not studied. Stem segments and shoots were ground to powder in a mortar under liquid nitrogen and extracted in boiling ethanol 96% (10 ml g^{-1} fresh weight) for 10 minutes. After cooling down to room temperature, the extract was centrifuged (10 min, 4500 g) and the pellet re-extracted in 80% cold ethanol for 45 min. The combined supernatants were reduced by evaporation until free of ethanol. The extract was kept at -20°C overnight, thawed and centrifuged (10 min, 4000 g, 4°C). The supernatant was reduced to approximately 5 ml. An equal volume of 20 mM NH_4-formate buffer was added (final pH 3.7). The sample was passed through a column of polyvinylpolypyrrolidone (1 ml g^{-1}, Sigma, St. Louis, MO., USA). The columns were eluted with 10 column volumes of NH_4-formate and the eluate was evaporated to dryness.

For immunoaffinity purification, IAC columns (OlChemIm Ltd., Olomouc, Czech Republic) were used which were able to bind free bases, ribosides, 9-glucosides and nucleotides of Z, DHZ and iP. The samples were taken up in 15 ml PBS buffer (64.3 mM Na_2HPO_4, 25 mM NaH_2PO_4, 15 mM NaCl, pH 7.2) and passed through IAC columns three times. In the third run, a C_{18}-column (Analytichem Int., Harbor City, CA, USA) which had been primed by passing through 10 ml 96% ethanol and 10 ml PBS buffer, was coupled to the IAC column. The IAC/C_{18}-column combination was washed with 30 ml deionized water. The total flow-through, containing cytokinin break-down products like adenine and adenosine, was collected, evaporated to dryness, taken up in HPLC eluens and counted in a liquid scintillation analyser (Packard, Meriden, USA). The IAC/C_{18} columns were disconnected and the IAC columns were eluted with 5 ml ice-cold methanol. The eluate was evaporated to dryness and redissolved in HPLC-eluens. Immediately after elution, the IAC columns were washed with 20 ml deionized water and 20 ml PBS buffer for the next series of samples. The C_{18}-columns were eluted with 5 ml 96% ethanol, yielding O- and N-7 glucosides. The eluate was evaporated to dryness and redissolved in HPLC-eluens. Cytokinins eluting from IAC and C_{18}-columns were separated in individual runs on a μBondapak C_{18}-column (Waters, 30 x 0.39 cm) that was eluted with 20% methanol/10 mM NH_4-formate pH 4.0, flow rate 1.5 ml min^{-1} and column temperature 25°C. The first standard run, for cytokinins eluting off IAC columns, included ZMP (retention time of 8.1 min); Z9G (9.6 min); DHZ9G (10.8 min); Z (15.1 min); DHZ (16.8 min); ZR (29.7 min); DHZR (34.6 min) and iPMP (37.9 min). The second standard run, for cytokinins eluting off C_{18}-columns, included Z7G (7.1 min); DHZ7G (8.9 and 9.6 min, (+) and (-) enantiomer); ZOG (12.2 min); DHZOG (16.5 min); ZROG (21.5 min) and DHZROG (32.0 min). Fractions co-eluting with cytokinin standards and 2-min fractions in between were collected and counted in a liquid scintillation analyser (Packard, Meriden, USA). Peaks were only taken into account when the radioactivity was 3 times the background level.

7.4 Results

7.4.1 Growth and uptake of ^{3}H-ZR and ^{3}H-DHZR

When rose axillary buds were placed on medium containing ZR or DHZR, primary shoots were formed. After approximately 4 days, secondary shoots appeared. During the 28 days of culture, the shoots (primary and secondary shoots taken together) increased in fresh weight (Figures 7.2A, 7.3A); on DHZR its final weight was higher than on ZR ($P = 0.02$). As not all explants on medium with DHZR formed secondary shoots, the fresh weight of the secondary shoots on ZR is higher than on DHZR (datat not shown). The weight of the stem segment increased during the culture period, most likely due to water uptake and formation of callus. Explants on ZR formed more callus than on DHZR, which was reflected in a significantly heavier stem segment ($P < 0.001$). The fresh weight of the adjacent piece of petiole did not increase, irrespective of the cytokinin added.

After 28 days of culture, the explants had taken up 23% of the amount of ^{3}H-ZR present in the medium (Figure 7.2B) and 29% of the amount of ^{3}H-DHZR (Figure 7.3B). Pairwise comparison showed that differences in uptake between ^{3}H-ZR and ^{3}H-DHZR were significant from day 14. The major part, approximately 85%, of the cytokinins that

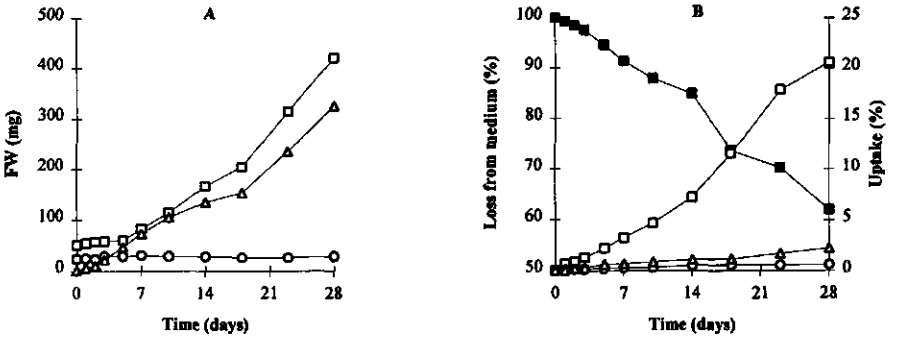


Figure 7.2 *(A) Fresh weight (mg) and (B) radioactivity (as % of total ^{3}H-ZR applied) in stem segment (□), petiole (○) and shoot (Δ) of Madelon bud explants, and the loss of radioactivity from the medium (■) during 28 days of growth (n=9).*

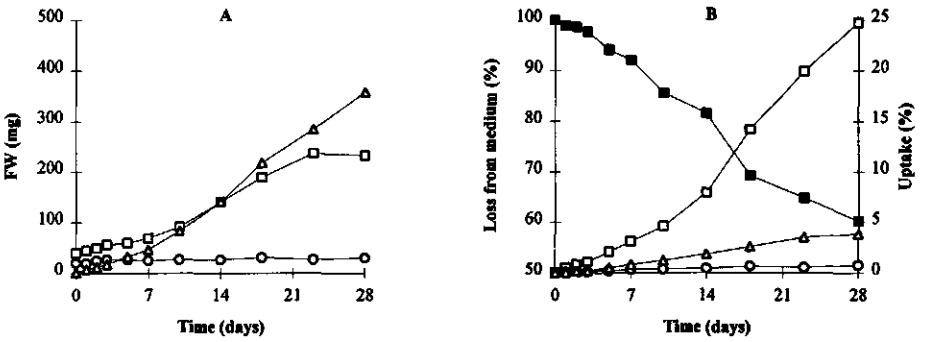


Figure 7.3 *(A) Fresh weight (mg) and (B) radioactivity (as % of total ^{3}H-DHZR applied) in stem segment (□), petiole (○) and shoot (Δ) of Madelon bud explants, and the loss of radioactivity from the medium (■) during 28 days of growth (n=9).*

were taken up, were found in the stem segment. The shoot and the petiole accounted for about 12 and 3%, respectively, of the final uptake. ^{3}H-ZR and ^{3}H-DHZR were found to disappear from the medium at an almost linear rate (Figures 7.2B, 7.3B) up to almost 40% of the initial activity. The amount of radioactivity that was lost from the medium was greater than the amount taken up by the explants.

7.4.2 Metabolism of ^{3}H-ZR and ^{3}H-DHZR

The metabolites that were formed after uptake of ^{3}H-ZR or ^{3}H-DHZR either bound to the IAC or C_{18}-column or did not bind at all. The latter fraction contained break-down

products of the cytokinin metabolism, amongst others adenine and adenosine. After 28 days, the radioactivity in this fraction was higher for stems and shoots grown on ^{3}H-DHZR than on ^{3}H-ZR (Table 7.1).

The major metabolites that could be detected in stem segments that received ^{3}H-ZR co-eluted with O-glucosides, predominantly ZOG (Table 7.2). At day 1 and 3, the riboside itself could be detected as well. Two unknown metabolites were found, 'A', which was found in the fraction also containing the Z7G standard, and 'B', eluting after 28-30 min, just before DHZROG. At day 1 and 3, uptake of ^{3}H-ZR by the shoots was too small to detect metabolites. After 28 days, peaks of radioactivity were found in the shoot co-eluting with the standard of DHZOG, and the unknown 'A'.

In the stem segments which were placed on the ^{3}H-DHZR medium, the riboside could not be detected (Table 7.3). The major metabolite found co-eluted with the DHZOG standard, and was already present after 1 day. Furthermore, radioactivity was found, co-eluting with the standard of DHZROG, the unknown 'A' and an unknown compound 'C', with a retention time of approximately 25 min, eluting shortly after ZROG. In the shoots, the same metabolites occurred.

Table 7.1 *Amount of radioactivity (dpm explant^{-1}) in the fraction that did not bind to the affinity columns and to the C_{18}-columns of stem segments and shoots of explants placed for 1, 3 or 28 days on medium containing ^{3}H-ZR or ^{3}H-DHZR (n=2). Values followed by different letters within either stem segment or shoot differ significantly (P=0.05).*

	stem segment			shoot		
day	1	3	28	1	3	28
^{3}H-ZR	95 c	126 c	1099 b	13 b	38 b	105 b
^{3}H-DHZR	109 c	146 c	2899 a	13 b	80 b	1153 a

Table 7.2 *Metabolites (dpm explant^{-1} ± SD) detected in stem segments and shoots of explants placed for 1, 3 or 28 days on medium containing ^{3}H-ZR (n=2).*

	stem segment			shoot		
day	1	3	28	1	3	28
ZR	7 ± 3	7 ± 1	-	-	-	-
'A'	-	-	814 ± 21	-	-	33 ± 7
ZOG	-	30 ± 6	1899 ± 94	-	-	-
DHZOG	-	95 ± 1	703 ± 54	-	-	20 ± 1
ZROG	-	12	626 ± 28	-	-	-
'B'	56 ± 8	66 ± 4	-	-	-	-

Table 7.3 *Metabolites (dpm explant^{-1} ± SD) detected in stem segments and shoots of explants placed for 1, 3 or 28 days on medium containing ^{3}H-DHZR (n=2).*

	stem segment			shoot		
day	1	3	28	1	3	28
'A'	-	14	1800 ± 109	-	-	131 ± 15
DHZOG	21 ± 3	180 ± 137	4855 ± 409	-	6	124 ± 35
DHZROG	-	-	679 ± 34	-	-	55 ± 16
'C'	-	33	1114 ± 35	-	-	73 ± 16

7.5 Discussion

7.5.1 Growth and uptake of ^{3}H-ZR and ^{3}H-DHZR

As was shown in Chapter 6, outgrowth of axillary buds was affected by the type of cytokinin in the medium, ZR being severalfold more active in inducing side-shoot formation than DHZR. We expected that in the present experiment the rate of uptake of ZR would be higher than that of DHZR, resulting in a higher endogenous concentration and, consequently, in the formation of more shoots. However, until day 14, uptake of ^{3}H-DHZR and ^{3}H-ZR did not differ significantly, whereafter uptake of DHZR was higher than that of ZR. As at that time, the differences between ZR and DHZR in side-shoot formation were already visible, a causal relationship is not likely. Differences in uptake might be rather a result than a cause of the differences in growth and development of the shoots.

Uptake of ^{3}H-cytokinins by rose explants was about 25-30% in 28 days. Suppose rose explants do not discriminate between ^{3}H-cytokinins and unlabelled cytokinins, the uptake of ZR is 0.11 µmol per explant (23% of 15 ml of medium containing 32 µM ZR) and of DHZR 0.14 µmol. Nordström and Eliasson (1986) found that apple shoots of about the same weight as the rose shoots took up 63% of the BA in the medium in 36 days. The concentration of cytokinin in their medium was approximately 10-fold lower than in our experiment, so the uptake in µmol per apple explant is severalfold lower than in the rose explants.

In our system, the amount of radioactivity recovered from the explants was less than the amount lost from the medium. Loss of ^{14}C-BA from the medium was also observed by Biondi *et al.* (1984), Nordström and Eliasson (1986) and Blakesley (1991). According to Biondi *et al.* (1984), this might be due to loss of ^{14}C as $^{14}CO_2$, formed by gradual degradation of purine via urea. However, Nordström and Eliasson (1986) were not able to detect $^{14}CO_2$ in the atmosphere of the culture vessels. Jameson *et al.* (1987) found that after feeding [8-^{3}H]-ZR via the xylem to blue lupin plants, only 45% of the ^{3}H supplied was recovered. They suggested that conversion of the purine ring of adenine and adenosine into compounds such as allantoin (Beevers, 1976) would release the ^{3}H at C-8. This ^{3}H would not be recovered during extraction, which might explain the incomplete recovery. In our system, the loss of ^{3}H might be explained by the formation of $^{3}H_2O$ via [2-^{3}H]-adenine (Beevers, 1976). The $^{3}H_2O$ might be lost via transpiration.

7.5.2 *Metabolism of ^{3}H-ZR and ^{3}H-DHZR*

Given the results obtained (Table 7.2), ZR is likely to be both glucosylated to ZROG and deribosylated to Z. Then, Z can be glucosylated to ZOG and hydroxylated to DHZ which can yield DHZOG (see Figure 1.2). The conversion of ZR to DHZOG was also reported to occur in intact blue lupin plants (Jameson *et al.*, 1987). An enzyme capable of converting Z to DHZ was found in *Phaseolus* embryos (Martin *et al.*, 1989). Our results show that feeding ^{3}H-DHZR to axillary buds resulted in the formation of DHZROG. Furthermore, DHZR was deribosylated to DHZ, which was then glucosylated to DHZOG. O-glucosides are considered to be storage forms of cytokinins, which are stable but may be readily metabolized into biologically active cytokinins when required (McGaw and Burch, 1995). Since in the present experiment, shoots were still growing actively, ^{3}H-ZR and ^{3}H-DHZR might be metabolized to these cytokinin storage forms rather than to inactive forms, like 7- and 9-glucosides, which are found to be the major metabolites in a number of other plant species (Van der Krieken *et al.*, 1988; Blakesley, 1991; Auer *et al.*, 1992b).

After feeding both ^{3}H-ZR and ^{3}H-DHZR, an unknown metabolite 'A' was formed (Tables 7.2 and 7.3). 'A' was found in the fraction of Z7G. However, this peak can not be pure Z7G, as it is also found after feeding ^{3}H-DHZR, and to the best of our knowledge, the conversion of DHZ to Z was never reported (see Figure 1.2).

Addition of ^{3}H-ZR and ^{3}H-DHZR led furthermore to metabolites that did not bind to the IAC and C_{18}-columns, possibly adenine and adenosine. Inactivation of the surplus of uptake of exogenous applied cytokinins by side-chain cleavage was also described by Singh *et al.* (1992b) and Sergeeva *et al.* (1994). Like the formation of N-glucosyl conjugates, side chain cleavage leads to the irreversible loss of cytokinin activity and may be important in the deregulation of cytokinin activity (McGaw and Burch, 1995).

One serious criticism of the present experiment is that the identification of cytokinins is based only on the retention time of the added cytokinin standards. However, since in every run cytokinin standards were included and their absorbance recorded, the chances of shifting of peaks without noticing was minimal. Nevertheless, identification of the HPLC peaks by GC-MS or LC-MS would have notably improved the reliability of the results.

Our results show that metabolism of ^{3}H-ZR and ^{3}H-DHZR takes place according to more or less the same pattern, yielding primarily the O-glucosides of the free bases. The observed differences in activity between ZR and DHZR with respect to the fresh weight (this Chapter) and the number (Chapter 6) of secondary shoots might be explained by the fact that ^{3}H-ZR could be detected as the riboside until day 3, whereas from day 1 on, ^{3}H-DHZR was converted to DHZOG. This suggests that ZR might be present in an 'active' form for a longer period than DHZR, which fits in with the finding that the amount of radioactivity in the fraction that did not bind to the IAC or C_{18}-columns was higher for DHZR than for ZR. DHZR was more rapidly metabolized to O-glucosides and degraded, possibly to adenine and adenosine than ZR.

Chapter 8

General Discussion

8.1 Introduction

As was stated in the General Introduction, the aim of this thesis is to establish a correlative relationship between cytokinins and the development of the shoot of rose combination plants. In this General Discussion, the results of the foregoing chapters are integrated to ascertain to what extent this aim has been achieved. The first part of this study focussed on the cytokinin pattern of rose plants during their development, and is discussed in paragraph 8.2. In paragraph 8.3, the use of bleeding sap as a means to quantify the export of cytokinins from the roots to the shoot is accounted for. In paragraph 8.4, an attempt is made to correlate this cytokinin export with shoot development. Although no experimental data on endogenous auxins in rose were obtained, they were incorporated in a model in which the correlative inhibition was described. Furthermore, we tried to relate the cytokinin production of a rootstock genotype to its vigour. Finally, some practical consequences of this study are discussed in paragraph 8.5.

8.2 Cytokinins in plant tissues

Cytokinin biosynthesis in the roots starts with the synthesis of iPMP from AMP and Δ^2-iPP (Letham and Palni, 1983). IPMP is rapidly hydroxylated yielding ZMP, which is then dephosphorylated to ZR. ZR is transported from the roots to the shoot via the xylem, and can be metabolized to form the nucleotides, free bases and the 7, 9 and O-glucosides of ZR and DHZR (see Figure 1.2). Based on the results of Chapter 2, in which endogenous cytokinins were quantified, an overview of cytokinin metabolism and transport in roses is given in Figure 8.1.

In young leaves, iP-type cytokinins could be detected (Chapter 2). As iP-compounds were not detectable in bleeding sap, and, to the best of our knowledge, dehydroxylation of Z-type into iP-type cytokinins has never been reported in plants, young leaves must be capable of *de novo* synthesis of cytokinins. *De novo* synthesis of cytokinins in leaves was reported earlier for sunflower (Salama and Wareing, 1979), pea (Chen *et al.*, 1985) and *Perilla* (Grayling and Hanke, 1992). Our results show that the concentration of iP-type cytokinins in the leaves remained approximately constant when the leaves were maturing (Tables 2.3-2.5). Assuming that all cytokinins in the shoot are subjected to degradation

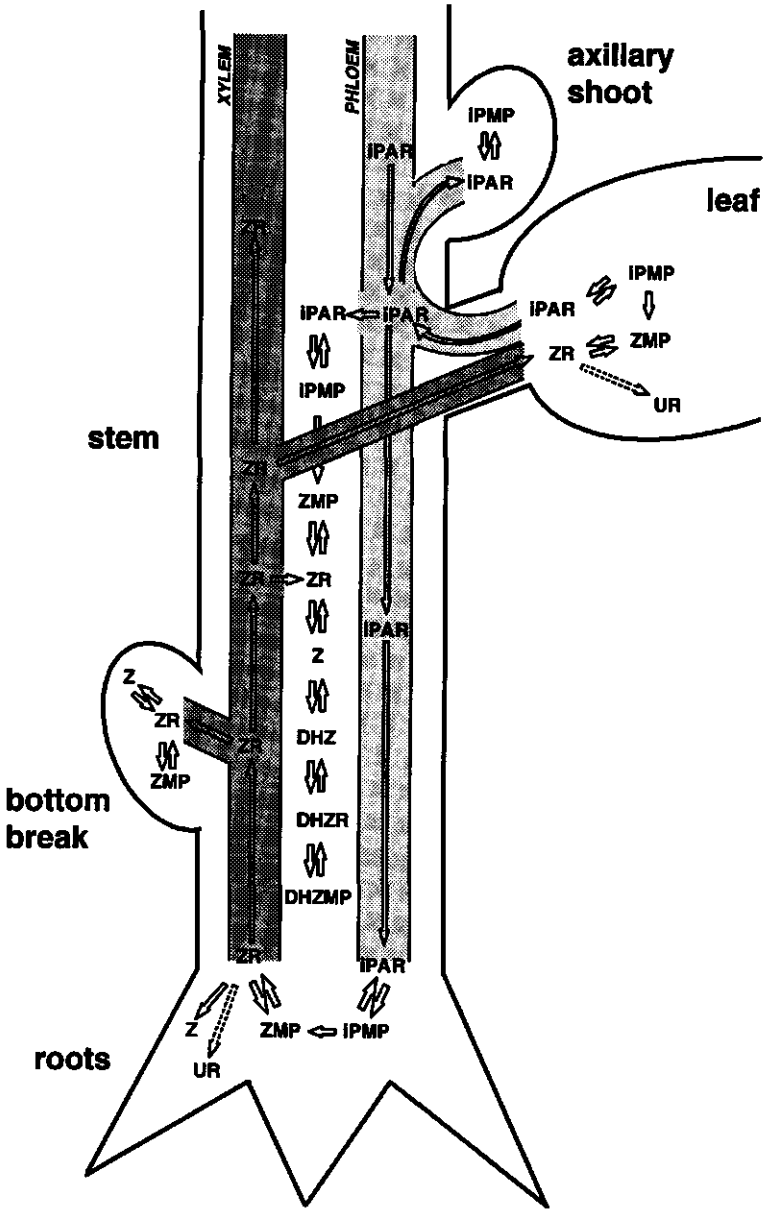


Figure 8.1 *Schematic representation of cytokinin metabolism and transport in roses. Solid arrows: metabolic steps; dotted arrows: putative metabolic steps; arrows in xylem and phloem denote transport of ZR and iPAR, respectively.*

(Chapter 3), the concentration of iP-type cytokinins was expected to decrease in older leaves. Since this did not occur, cytokinin biosynthesis in the leaves must continue when the leaves age. As, in general, levels of cytokinin free bases and ribosides decline in senescing leaves (Badenoch-Jones *et al.*, 1987; Singh *et al.*, 1992a), cytokinin synthesis in rose leaves may have a role in suppressing their own senescence, as was previously suggested for tobacco (Singh *et al.*, 1992b).

For their outgrowth, axillary buds most likely depend on cytokinins that are imported via the phloem. Due to their limited transpiration rate, import ZR via the xylem will be minimal. Cytokinins were found in phloem sap of a number of plant species (Vonk, 1979; Jameson *et al.*, 1987; Hoad, 1995). In *Ricinus communis* (Komor *et al.*, 1993), *Perilla* (Grayling and Hanke, 1992) and *Sinapis alba* (Lejeune *et al.*, 1994), iP-compounds were the main phloem cytokinins. Van Staden (1982) demonstrated that after decapitation of rose plants, cytokinins moved from the subtending leaf to the axillary bud via the phloem. Whether the axillary bud will actually grow out, depends on its state of inhibition, which is controlled by apically produced and basipetally transported IAA (King and Van Staden, 1988; Tamas, 1995). Pillay and Railton (1983) found that a single application of Z to inhibited buds of *Pisum sativum* caused their elongation, whereas a similar treatment with iP resulted in only limited outgrowth. They suggested that inhibited lateral buds are unable to hydroxylate the 'inactive' iP to the active Z. Apparently, a reduction of auxin export from the apex stimulates the conversion of inactive cytokinins to active ones. Since the buds forming the bottom breaks do not have subtending leaves, for their cytokinin supply they depend either on import via long distance phloem transport, or on transport via the xylem. As the basal buds primarily contain ZR and ZMP (Table 2.8), and ZR is the major translocation form in the xylem, the latter is most probable.

Rose stems were found to contain high concentrations of cytokinins (Table 2.6). Jameson *et al.* (1987) found that if ^{3}H-ZR was supplied exogenously into the transpiration stream of *Lupinus angustifolius*, a considerable portion moved directly from the xylem into the bark. Both the lateral movement of cytokinins into stem tissues, and the ability of the stem to metabolize cytokinins (King and Van Staden, 1990; Letham, 1994) may explain the presence of high concentrations of various cytokinins in the stem. DHZR and DHZMP were only detected in the stem and in young bottom breaks which primarily consist of stem tissue. Most likely, ZR originating from the xylem is converted into DHZR and DHZMP via the reduction of Z to DHZ (see Figure 1.2). The ribosylation of DHZ yielding DHZR is likely to occur rapidly, as DHZ could not be detected. Komor *et al.* (1993) found that if cotyledons of *Ricinus* seedlings were incubated with ZR, the concentration of Z and ZR, and of DHZ and DHZR in phloem sap increased. Although their exact function remains a matter of conjecture, DHZR and DHZMP might be involved in the redistribution of cytokinins in plants, possibly via the phloem.

When rose explants took up ^{3}H-ZR and ^{3}H-DHZR from the tissue culture medium, O-glucosides were found to be major metabolites (Chapter 7). The explants of approximately 700 mg fresh weight took up 0.10-0.15 µmol ZR or DHZR in 28 days, resulting in an endogenous cytokinin concentration about 1000 times higher than that *in vivo* (Tables 2.2-2.8). O-glucoside formation in *in vitro* grown explants is probably the result of an unphysiologically high concentration of endogenous cytokinins; it does not necessarily imply that O-glucosides are major metabolites of xylem-derived ZR *in vivo*. In intact plants, endogenous O-glucosides were not determined (Chapter 2).

8.3 Bleeding sap

To quantify the export of cytokinins from the roots to the shoot, the cytokinin concentration in xylem sap has to be known. As it is not possible to sample the transpiration stream directly (Jackson, 1993), measurements must be made indirectly, using root exudate obtained after pressurizing the root system (Beck and Wagner, 1994) or using bleeding sap (Heindle *et al.*, 1982). The advantage of using sap is that it can be collected relatively easily and that it needs little purification before actual analysis of cytokinins in comparison to plant tissues. The disadvantage is that the plant must be destructed to collect sap, and that before conclusions can be drawn, it has to be ascertained whether the concentration of cytokinins in the sap collected is representative of that of intact plants. Since root pressure exudate is likely to be contaminated by the contents of cells damaged by the pressure applied, in the present study, bleeding sap was used.

The length of the period of collection of bleeding sap differs considerably in various studies, from 1-2 h (Badenoch-Jones *et al.*, 1996), 6-8 h (Heindle *et al.*, 1982), 12 h (Jameson *et al.*, 1989) to 48 h (Baker and Allen, 1992). During this time root metabolism will have changed by shortage of assimilates from the shoot, with inevitable consequences for cytokinin production (Jackson, 1993). To obtain a realistic estimate of the cytokinin concentration actually present in the transpiration stream as it enters the shoots of intact plants, Zhang and Davies (1990) collected the first droplets of sap after removing the shoot, as they considered this to be captured transpiration sap. In contrast, Heindle *et al.* (1982) and Ferguson (1980) discarded these first few drops, believing them to be contaminated by solutes from severed sieve elements or damaged cells at the cut surface. In rose, we collected bleeding sap for 30 min, after discarding the first drop of exudate that appeared at the surface of the cut stump after shoot removal (Chapters 2-5). Na_2MoO_4 was added to the vial to inhibit phosphatase activity. Our results show that the volume flow rate of bleeding sap was about 5% of the daytime transpiration rate (Chapter 4). In general, concentrations of hormones in slowly flowing sap from de-topped plants cannot be equated with those of the intact plant and will overestimate the true concentration present in the transpiration stream (Jackson, 1993). However, when the volume of the collected sap is small, it might be considered to be captured transpiration sap in transit to the shoot. This situation probably holds in our experiments, since in Chapter 4 the ZR concentration in bleeding sap of roses collected for 30 min was found to be about the same as in sap collected at flow rates of intact transpiring plants. Therefore, in contrast to the general rule, in the present experiment the export of cytokinins from the roots can be quantified by multiplying the cytokinin concentration in bleeding sap with the transpiration rate.

The only cytokinin that could be detected in bleeding sap of rose was ZR, suggesting it to be the major translocation form, as was found for several other plant species (Jameson *et al.*, 1989; Wagner and Beck, 1992). In tissues, ZR is also present in relatively high concentrations. The results of the experiments described in Chapter 6 show that from the ribosides ZR, iPAR and DHZR, ZR is the most active cytokinin. These facts point at a key role for ZR itself, or for its immediate metabolite, which might be, in rose, the free base Z. Since Z and ZR are equally active in inducing side-shoot formation (Chapter 6), the question which cytokinin is the active one remains open.

8.4 Cytokinins in bleeding sap in relation to bud break

In the present study, an attempt was made to describe the role of endogenous cytokinins in plant development. To that end, the concentration of ZR in bleeding sap of plants in various stages of development around bud break of axillary shoots and bottom breaks was determined. When measuring cytokinins in bleeding sap collected at 2- and 3-weekly intervals, no significant differences could be found (Chapter 2), probably because the intervals between the successive harvests were too large to detect a relationship between plant development and cytokinin concentration in bleeding sap. The same held true for the experiment described in Chapter 5, in which bleeding sap was collected weekly. However, as reported in Chapter 4, where bleeding sap was collected at 2- and 3-daily intervals, the ZR concentration increased before bud break of axillary shoots and bottom breaks, and decreased thereafter. Based on the results of Chapter 2, the export of cytokinins from the roots and their degradation rate in the shoot were estimated (Chapter 3). As the transpiration rate increased linearly during plant development, the results of Chapter 4, combined with those of Chapters 2 and 3 do suggest a quantitative relationship between cytokinin export from the roots and shoot development.

8.4.1 Correlative inhibition within a scion/rootstock combination plant

The apex of the primary shoot exerts apical dominance over the axillary buds along the shoot, until the terminal flower bud is visible. As at that time, the auxin export from the apex decreases, the inhibition of the uppermost 2-4 axillary buds is released, resulting in bud break. Axillary bud break is attended by a high ZR concentration in bleeding sap. As

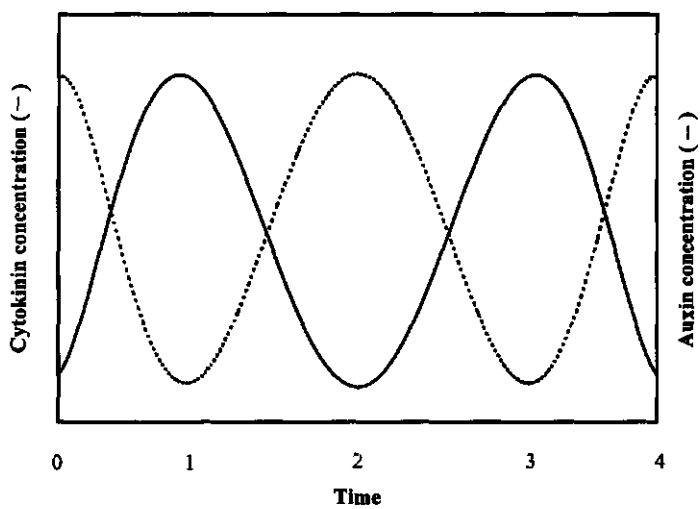


Figure 8.2 *Simplified model of auxin and cytokinin concentrations during plant development. Time = 0, flower bud of the primary shoot is visible; time = 1, axillary bud break; time = 2, axillary shoots inhibit bud break further down the stem; time = 3, basal bud break; time = 4, dominance of bottom breaks over future shoots.*

found by Bangerth (1994) for bean, a decrease in basipetal auxin flow is the signal that leads to an increase of the cytokinin concentration in xylem sap. That increase is reported to coincide with or precede the beginning of bud outgrowth in bean (Bangerth, 1994) and apple (Tromp and Ovaa, 1990). Later, the young axillary shoots will take over apical dominance and inhibit bud break further down the stem by their auxin production. When the terminal flower buds of the axillary shoots become visible, apical dominance is reduced again, leading, following the same reasoning as above, to basal bud break. It remains a matter of conjecture why the basal buds break, instead of the buds in the axils of the middle leaves of the primary shoot. In Figure 8.2, the foregoing is visualized in a simplified model.

8.4.2 Correlative inhibition within a scion/rootstock combination plant under the influence of environmental factors

8.4.2.1 Season

When Madelon is grafted onto the rootstock Multic, after 7-8 weeks the uppermost 2-4 axillary shoots of the primary shoot break (Table 8.1). Later, at the base of the primary shoot, one or more bottom breaks appear. When the outgrowth data for the bottom breaks for the various experiments are compared, it appears that the time between bud break of axillary and basal buds varied widely (Table 8.1). Obviously, that time is affected by the moment of grafting.

In all our experiments, grafting and bud outgrowth occurred under the same controlled conditions. However, the plant material used for grafting originated from the greenhouse and has grown under environmental conditions that varied throughout the year. The lateral bud that was taken for grafting already contained a number of leaves, of which the lowermost scale-like ones enclosed basal buds, the future bottom breaks (Marcelis-van Acker, 1993). The inhibitory state of these basal buds is controlled by the physiological condition prevailing in the 'mother bud' (Zamski *et al.*, 1985), that in turn is dependent on variations in daylength and temperature in the greenhouse. After grafting, the bud was released from apical dominance, and formed the rest of the leaves and axillary buds, and the terminal flower bud (Marcelis-van Acker, 1993) under controlled conditions, which explains why axillary shoots appeared in all experiments after a more or less constant period of 7-8 weeks (Table 8.1). However, the basal buds, which form the bottom breaks, were initiated in the greenhouse under varying environmental conditions, which may have led to the observed variation in the moment of basal bud break. Broadly speaking, the formation of bottom breaks occurs when due to some growth retarding stimulus apical dominance weakens (Kool, 1996), and auxin export from the apex decreases, followed by a resumption of favourable growing conditions. In experiments 2, 4, 5 and 7, 'mother' buds were taken from plants growing at adverse conditions of decreasing temperature, photoperiod and light intensity in the greenhouse, and were transferred to the high temperature and light intensity in the growth room. The basal buds in these 'mother' buds might be less inhibited than those of the 'mother' buds originating from vigorously growing plants (Experiments 1, 3 and 6), and would thereby respond more rapidly to an increase in cytokinin supply when the apical dominance from the axillary shoots is released, resulting in a rapid basal bud break.

Table 8.1 *Seasonal effects on the number of weeks between grafting and axillary bud break and between bud break of axillary buds and bottom breaks.*

experiment	date of grafting	number of weeks between grafting and axillary bud break	number of weeks between bud break of axillary buds and bottom breaks
1	Sept. 1, 1993	7	3
2	Jan. 11, 1994	7	1
3	Jun. 13, 1994	7	4
4	Nov. 24, 1994	8	1
5	Mar. 28, 1995	8	1
6	Aug. 14, 1995	8	4
7	Dec. 27, 1995	8	1

8.4.2.2 Root temperature

An increase in root temperature was shown to accelerate axillary bud break (Chapter 5). According to the model of an interaction between auxin and cytokinin concentration, as described in section 8.4.1, this acceleration might be caused by either an earlier or a more rapid reduction of auxin export. As a consequence, the cytokinin supply to the shoot is advanced, which favours emergence of the bottom breaks. Unfortunately, due to the large variation in plant material, and the long intervals between successive samplings of bleeding sap, this line of thought cannot be substantiated by the data obtained.

8.4.3 Correlative inhibition as affected by rootstock genotype

When Madelon was grafted onto the rootstock genotypes Multic and Vivaldi, in both combinations axillary shoots emerged after approximately 8 weeks (Chapter 4). The fact that axillary bud break was not affected by the genotype of the rootstock is a further indication that, according to the model presented in section 8.4.1, axillary bud break is controlled by the genotype of the scion, more precisely by the auxin production of the apex. It may be assumed that the decrease in auxin export was equal for both combinations; it must have caused the cytokinin concentration in xylem sap to increase. However, as can be seen in Figure 4.3, the ZR increase was severalfold higher in Madelon/Multic than in Madelon/Vivaldi, resulting in an earlier appearance of the bottom breaks for the former. As the shoot fresh weights, and thereby leaf areas, of Madelon/Multic and Madelon/Vivaldi were approximately equal, their transpiration rates must be equal as well. As a consequence, the amount of ZR imported into the shoot must have been much higher for Madelon/Multic than for Madelon/Vivaldi, most likely due to a higher cytokinin production in the former.

The effect of the genotype of the rootstock on the performance of the shoot does not occur via altering the sensitivity of the axillary buds for root-produced cytokinins. This became clear from unpublished results from our institute. Axillary buds of the primary shoot of Madelon/Multic, Madelon/Vivaldi and Madelon/Madelon combination plants

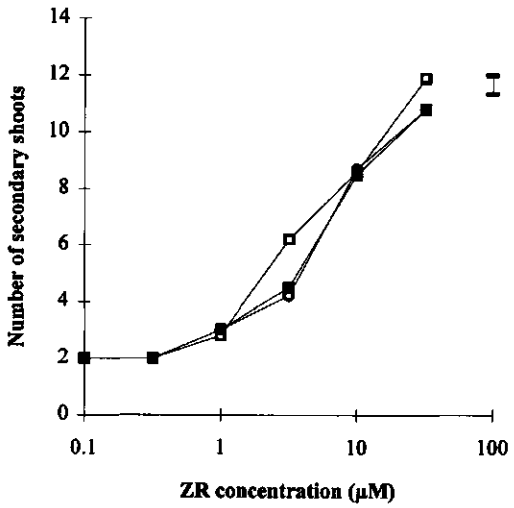


Figure 8.3 *Number of secondary shoots formed by Madelon axillary buds originating from Madelon/Vivaldi (■), Madelon/Multic (□) and Madelon/Madelon (○) combination plants. Axillary buds were grown on medium with 0-32 µM ZR for 28 days at 20°C, a photoperiod of 16 h and photosynthetically active radiation of 60 µmol m^{-2} s^{-1} (n=10). Vertical bar indicates LSD (P=0.05).*

were placed on a tissue culture medium containing ZR concentrations ranging from 0 to 32 µM (as described in Chapter 6). As can be seen in Figure 8.3, the number of secondary shoots was more or less the same, irrespective of the rootstock used. Obviously, the rootstock genotype did not alter the sensitivity of the axillary buds for cytokinins.

8.5 Practical consequences

All experiments described in this study were performed under controlled conditions, resulting in axillary bud break 7-8 weeks after grafting. However, appearance of bottom breaks was more variable and was found to be affected by the season the plants were grafted (Table 8.1). Unlike axillary buds which were formed under the controlled conditions in the climate rooms, basal buds forming the bottom breaks were initiated in the greenhouse under varying environmental conditions, leading to the variation in basal bud break. It is obvious that for the selection of scion and rootstock material the season that is best suited for grafting should be considered, in order to obtain vigorous combination plants with homogeneous bud break.

The results obtained in Chapter 4 indicate that the genotype of the rootstock affects growth and development of the scion, thereby confirming earlier experimental work. Until now, rose breeders usually recommend certain cultivar/rootstock combinations based on greenhouse trials that take several years, as the production of a rootstock in its

first year is not alway representative for its production throughout the years (Kool and Van de Pol, 1996). A rootstock characteristic that can be used as an early selection criterion might accelerate the selection process of rootstocks considerably. The results presented in this thesis show that the pattern of cytokinin concentrations in bleeding sap is correlated with bud break of axillary shoots and bottom breaks and with the vigour of the rootstock. Therefore, the concentration of cytokinins in bleeding sap may be used as an early selection criterion for the vigour of rose rootstocks. As cytokinin analyses are still laborious and therefore expensive, they can be applied for rootstocks that have passed a first screening successfully, based on characteristics which are easier to be determined.

As was shown in Chapter 4, exogenous application of cytokinins can advance the emergence of bottom breaks, expecially of scions grafted onto less vigorous rootstocks. Nowadays, cytokinins are applied in greenhouse rose growing. This development has to be delt with with care, since at the moment of cytokinin application sufficient supply of carbohydrates should be warranted to let the bottom breaks grow out into shoots of a high quality. Large quantities of inferior shoots are of no value. However, sensible use of cytokinins in a rose crop can be a good means to stimulate bud break, especially in cultivars having a strong apical dominance.

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Summary

In the Netherlands, the rose is the most important glasshouse cut flower. Approximately 50% of the glasshouse roses are combination plants, consisting of a scion cultivar and a rootstock of a different genotype. Although a considerable amount of rootstock research has been done, the way rootstocks affect the physiology of the scion is still little understood. Rootstocks inducing differences in the vigour of the scion are suggested to differ in cytokinin production and export to the shoot, thereby affecting bud break and outgrowth of the scion. In the present study, the course of the endogenous cytokinins in rose combination plants is determined, which should ultimately lead to a better understanding of the role of cytokinins in the relationship between rootstock and scion. The knowledge obtained might be used to determine if cytokinins can be used as an early selection criterion for rose rootstocks.

To assess the role of endogenous cytokinins in the development of rose plants, their concentrations in bleeding sap and roots, stem, leaves, axillary shoots and bottom breaks were quantified in three stages of development (Chapter 2). The major translocation form in the xylem was found to be ZR. In all mature tissues, cytokinins of the Z-type were predominant, amounting to 80-90% of the total cytokinin concentration. The stems contained high concentrations of cytokinins, which might be explained by both the lateral movement of ZR from the xylem to adjacent stem tissue and the ability of the stem to metabolize cytokinins. In young leaves, the contribution of iP-type cytokinins to the total cytokinin pool was about 50%. Since iP-compounds were not detected in bleeding sap and dehydroxylation of Z-type into iP-type cytokinins has never been reported in plants, young leaves are probably capable of *de novo* synthesis of cytokinins. The iP-type cytokinins might be transported via the phloem from the leaf into the axillary bud. Whether the axillary bud will grow out, depends on its state of inhibition, which is controlled by apically produced and basipetally transported auxin. Inhibited buds are suggested to be unable to hydroxylate the 'inactive' iP to the 'active' Z. This inhibition can be overcome by a reduction of auxin export from the apex. Since the buds forming the bottom breaks do not have subtending leaves, for their cytokinin supply they depend either on import via long distance phloem transport, or on transport via the xylem. As the basal buds primarily contain ZR and ZMP, the latter is most probable.

In the present study, we tried to establish a relationship between cytokinin export from the roots and the development of the shoot. The export of cytokinins was estimated based on the assumption that the cytokinin concentration in bleeding sap is representative for the cytokinin concentration in xylem sap *in situ*. Although the flow rate of bleeding sap was found to be about 5% of the daytime transpiration rate, the ZR concentration of bleeding sap was indeed about the same as in sap collected at flow rates of intact transpiring plants (Chapter 3). Therefore, it is justified to calculate the cytokinin export from rose roots by multiplication of the cytokinin concentration in bleeding sap with the transpiration rate. Experimental results discussed in Chapter 4 show that the ZR

concentration in bleeding sap of Madelon/Multic combination plants was correlated with bud break; the concentration increased before bud break and decreased thereafter. As the transpiration rate of the plants increased linearly during plant development, the results of Chapters 2-4 suggest a quantitative relationship between cytokinin export from the roots and shoot development.

Grafting the scion Madelon on six rootstock genotypes revealed that the root and shoot dry weight, and the number of bottom breaks were affected by the genotype of the rootstock (Chapter 4). If rootstocks would mediate scion growth via their cytokinin production, it might be possible to improve the performance of the rootstock by exogenous application of cytokinins. Indeed, BA application to the stem stump was found to increase the number of bottom breaks, especially when a weak rootstock was used. The hypothesis that a rootstock that induces earlier bud break of the scion supplies larger quantities of cytokinins to the shoot, was tested by measuring, during plant development, the level of ZR in bleeding sap of Madelon grafted onto Vivaldi and onto the vigorous rootstock Multic. The timing of axillary bud break was not affected by the genotype of the rootstock, but bottom break emergence was advanced when Madelon was grafted onto Multic. The ZR concentration in bleeding sap increased before bud break and decreased thereafter. ZR in bleeding sap of Madelon/Vivaldi plants was considerably lower than that of Madelon/Multic plants, but showed more or less the same pattern.

Based on the foregoing results an attempt was made to describe the correlative inhibition in rose. The apex of the primary shoot is thought to exert apical dominance over the axillary buds along the shoot, until the terminal flower is visible. As at that time, the auxin export from the apex decreases, the inhibition of the uppermost 2-4 axillary buds is released, resulting in bud break. Axillary bud break is attended by a high ZR concentration in bleeding sap. Later, the young axillary shoots will take over apical dominance and inhibit bud break further down the stem by their auxin production. When the terminal flower buds of the axillary shoots are visible, apical dominance is reduced, leading, following the same reasoning as above, to basal bud break.

Apart from variation in growth of the scion induced by the genotype of the rootstock, growth of the shoot might also be influenced by environmental factors, like root temperature. Although root temperature in the range of 11 to 26°C changed root morphology, root dry weight was not affected (Chapter 5). Higher root temperatures advanced axillary and basal bud break. However, due to the large variation in plant material and the long intervals between successive samplings of bleeding sap, the effects of root temperature could not be correlated reliably with the cytokinin export from the roots to the shoot.

In experiments done *in vitro*, the effect of a number of cytokinin free bases and ribosides on axillary bud break and outgrowth of Madelon roses was studied (Chapter 6). With respect to secondary shoot induction, the sequence of activity was as follows: Z(R) $\geq$ BA(R) >> iP(AR) >> DHZ(R). These results, together with the fact that ZR is the major translocation form in the xylem, points at a key role for ZR or its immediate metabolite, which might be the free base Z, in growth and development of rose plants. The difference in effectivity of the various cytokinins may be due to differences in rate of uptake or in metabolism. Studies in which ^{3}H-ZR and ^{3}H-DHZR were fed to axillary buds showed that their uptake did not differ significantly before differences in side-shoot formation were visible. Therefore, a causal relationship between the two is not likely. Major metabolites of ^{3}H-ZR and ^{3}H-DHZR were ZOG and DHZOG, respectively. Since ^{3}H-ZR could be detected unmetabolized in the tissue for a longer period than ^{3}H-DHZR, it is most likely

that the outgrowth of axillary buds might be affected by differences in the metabolism of ZR and of DHZR.

Finally, some practical consequences of this study are discussed. The major result is that the concentration of cytokinins in bleeding sap may be used as an early selection criterion for rose rootstocks.

Samenvatting

De kasroos is in Nederland de belangrijkste snijbloem. Ongeveer 50% van de kasrozen zijn combinatieplanten, die bestaan uit een ent en een onderstam van een verschillend genotype. Combinatieplanten hebben vaak een hogere bloemproductie dan een snijrooscultivar op eigen wortels. Hoewel er veel onderzoek naar is gedaan, is het nog steeds onduidelijk hoe onderstammen de groei van de ent beïnvloeden. Een van de mogelijkheden is dat de door de onderstam geïnduceerde verschillen in groei en vertakking van de ent berusten op verschillen in de productie van cytokininen. Cytokininen zijn plantenhormonen die in de worteltopjes worden gevormd. Via de transpiratiestroom worden de cytokininen naar de bovengrondse delen getransporteerd, waar ze een belangrijke rol spelen bij het uitlopen van okselknoppen. In dit proefschrift wordt het verloop van de endogene cytokininen gevolgd gedurende de ontwikkeling van de rozenplant. Dit zou uiteindelijk moeten leiden tot een beter begrip van de rol die cytokininen spelen in de relatie tussen onderstam en ent. De kennis die hiermee verkregen wordt, kan gebruikt worden om te bepalen of de cytokininenproductie gebruikt kan worden als een eigenschap op basis waarvan rozenonderstammen geselecteerd kunnen worden.

Om de rol van endogene cytokininen in de ontwikkeling van rozen vast te kunnen stellen, werden hun concentraties in bloedingssap en in wortels, stengels, bladeren, zijscheuten (de zogenaamde 'pluizen') en grondscheuten in drie ontwikkelingsstadia van de rozenstruik bepaald (Hoofdstuk 2). In het xyleem bleek ZR de voornaamste transportvorm te zijn. In alle volwassen weefsels waren de cytokininen van het Z-type de belangrijkste; ze vormden 80-90% van de totale hoeveelheid. De stengels bevatten hoge concentraties cytokininen, hetgeen zowel verklaard kan worden door zijwaarts transport van ZR vanuit de xyleemvaten naar aanliggende weefsels als door het vermogen van de stengel cytokininen om te zetten. In jonge bladeren vormden iP-cytokininen ongeveer 50% van de totale hoeveelheid cytokininen. Omdat in bloedingssap van roos geen cytokininen van het iP-type voorkomen en omdat de omzetting van cytokininen van het Z- naar het iP-type in planten voor zover we nu weten niet plaatsvindt, zijn jonge bladeren waarschijnlijk in staat tot nieuwvorming van cytokininen. Via het floeem kunnen de iP-cytokininen vanuit het blad naar de bijbehorende okselknop getransporteerd worden. Of de okselknop dan uit zal lopen hangt af van de mate waarin deze knop geremd is. De mate van remming wordt waarschijnlijk bepaald door auxine, dat in de apex van de groeiende hoofdscheut wordt geproduceerd en van daaruit naar beneden wordt getransporteerd. Verondersteld wordt dat geremde knoppen niet in staat zijn het 'inactieve' iP te hydroxyleren naar het 'actieve' Z. Pas wanneer de export van auxine vanuit de apex afneemt, wordt de remming opgeheven en treedt knopuitloop op. In tegenstelling tot de hoger gelegen knoppen die de zijscheuten vormen, hebben de knoppen die de grondscheuten leveren geen bijbehorend blad en bevatten voornamelijk

ZR en ZMP, wat betekent dat ze voor hun cytokininenvoorziening waarschijnlijk afhankelijk zijn van de wortels.

In dit proefschrift hebben we geprobeerd de ontwikkeling van de scheut te relateren aan de export van cytokininen vanuit de wortels. Om een inschatting te maken van de export van cytokininen hebben we gebruik gemaakt van bloedingssap, waarbij verondersteld werd dat de cytokininenconcentratie in bloedingssap representatief is voor de concentratie in xyleemsap in de intacte plant. Hoewel de stroomsnelheid van bloedingssap ongeveer 5% van de transpiratiesnelheid bleek te zijn, was de ZR-concentratie in bloedingssap en in sap bemonsterd bij stroomsnelheden van intacte verdampende planten inderdaad ongeveer gelijk (Hoofdstuk 3). De hoeveelheid cytokininen die uit de wortels van de roos geëxporteerd wordt, mag daarom berekend worden door de ZR-concentratie in bloedingssap te vermenigvuldigen met de verdampingssnelheid. De resultaten besproken in Hoofdstuk 4 laten zien dat de ZR-concentratie in bloedingssap van Madelon/Multic combinatieplanten gecorreleerd was met knopuitloop; de ZR-concentratie nam toe vóór de knopuitloop en nam daarna weer af. Omdat de verdamping van de planten lineair toenam gedurende de ontwikkeling van de plant bleek behalve de ZR-concentratie ook de export van cytokininen uit de wortels gecorreleerd met knopuitloop.

In Hoofdstuk 4 werd het effect van zes onderstamgenotypes op de groei van de ent Madelon beschreven. Het drooggewicht van de wortels en de scheut, en het aantal grondscheuten werden beïnvloed door het genotype van de onderstam. Om te onderzoeken of bij deze sturing cytokininen wellicht een rol spelen, werd het effect van exogene toediening van cytokininen aan combinatieplanten bestudeerd. Toediening van BA aan gedecapiteerde planten bleek inderdaad een groter aantal grondscheuten op te leveren, voornamelijk bij de zwakkere onderstammen. De hypothese dat een onderstam die vroege knopuitloop bij de ent induceert een grotere hoeveelheid cytokininen naar de scheut transporteert, werd getoetst door de ZR-concentratie in bloedingssap van Madelon geënt op de zwak groeiende Vivaldi en de groeikrachtige onderstam Multic te bepalen gedurende de ontwikkeling van de plant. Het moment van uitlopen van de bovenste okselknoppen (pluizen) werd niet beïnvloed door het genotype van de onderstam, maar grondscheuten liepen eerder uit als werd geënt op Multic. De ZR-concentratie in bloedingssap nam toe voorafgaande aan de knopuitloop en nam daarna af. De ZR-concentratie in bloedingssap van Madelon/Vivaldi was enkele malen lager dan van Madelon/Multic planten, maar vertoonde ongeveer hetzelfde verloop. De resultaten verkregen in Hoofdstukken 2-4 wijzen op een kwantitatieve relatie tussen de export van cytokininen uit de wortels en de ontwikkeling van de scheut, en tussen het genotype van de onderstam en de cytokininenconcentratie in bloedingssap.

Met behulp van bovenstaande gegevens werd een poging gedaan de correlatieve remming in rozen te beschrijven. De apex van de primaire scheut oefent dominantie uit over de okselknoppen, totdat de bloem van de primaire scheut zichtbaar is. De auxine-export vanuit de apex neemt dan af, en de remming van de bovenste 2-4 okselknoppen wordt opgeheven met als gevolg dat de knoppen uitlopen. Deze knopuitloop gaat samen met een hoge ZR-concentratie in het bloedingssap. Later nemen de jonge zijscheuten de apicale dominantie over, en remmen door hun auxineproductie het uitlopen van de lager gelegen okselknoppen. Als de bloemen van de pluizen zichtbaar zijn, neemt hun apicale dominantie af, hetgeen volgens hetzelfde mechanisme als hierboven beschreven staat leidt tot het uitlopen van de grondscheuten.

Behalve de onderstam kunnen ook omgevingsfactoren zoals worteltemperatuur de groei van de ent beïnvloeden. Hoewel de worteltemperatuur in het traject van 11 tot 26°C de morfologie van de wortels wel beïnvloedde, bleef het drooggewicht van de wortels gelijk (Hoofdstuk 5). Bij hogere worteltemperaturen liepen pluizen en grondscheuten eerder uit, maar als gevolg van de grote variatie in het plantmateriaal en de lange tijdspanne tussen de opeenvolgende bemonsteringen van het bloedingssap, kon geen betrouwbaar verband tussen de worteltemperatuur en de export van cytokininen vanuit de wortels naar de bovengrondse delen vastgesteld worden.

In weefselkweekexperimenten werd nagegaan wat de invloed van een aantal vrije basen en ribosiden op de uitloop en uitgroei van Madelon okselknoppen is (Hoofdstuk 6). Wat betreft de vorming van zijscheuten was de volgende volgorde van activiteit zichtbaar: Z(R) $\geq$ BA(R) >> iP(AR) >> DHZ(R). Deze resultaten, samen met het feit dat ZR de transportvorm van cytokininen in het xyleem is, duidt op een sleutelrol voor ZR of voor de vrije base Z, die direct uit ZR gevormd kan worden, in de groei en ontwikkeling van de roos. Het verschil in effectiviteit van de onderzochte cytokininen zou kunnen berusten op een mogelijk verschil in de mate waarin ze opgenomen worden door het weefsel of in het metabolisme. Experimenten waarin ^{3}H-ZR en ^{3}H-DHZR werden aangeboden aan okselknoppen toonden echter aan dat de opname van deze cytokininen niet significant verschilde voordat verschillen in de zijscheutvorming zichtbaar werden. Daarom is een oorzakelijk verband tussen opname van cytokininen en hun effect op zijscheutvorming niet waarschijnlijk. De voornaamste metabolieten van ^{3}H-ZR en ^{3}H-DHZR bleken respectievelijk ZOG en DHZOG te zijn. Omdat ^{3}H-ZR gedurende een langere periode in het weefsel te vinden was dan ^{3}H-DHZR wordt de uitgroei van okselknoppen hoogstwaarschijnlijk beïnvloed door verschillen tussen het metabolisme van ZR en dat van DHZR.

Tenslotte wordt een aantal praktijktoepassingen van dit onderzoek besproken. De belangrijkste hiervan is dat onze resultaten laten zien dat het mogelijk is cytokininen te gebruiken in het selectieproces van rozenonderstammen.

Account

Parts of this thesis and related work have been or will be published elsewhere:

Chapter 2

Dieleman JA, Verstappen FWA, Nicander B, Kuiper D, Tillberg E, Tromp J (1997) Cytokinins in *Rosa hybrida* in relation to bud break. *Physiol. Plant.* 99: 456-464

Chapter 3

Dieleman JA, Verstappen FWA, Perik RRJ, Kuiper D (1997) Quantification of the export of cytokinins from roots to shoots of *Rosa hybrida* and their degradation rate in the shoot. *Physiol. Plant.* 101: 347-352

Chapter 4

Dieleman JA, Verstappen FWA, Kuiper D (1998) Bud break and cytokinin concentration in bleeding sap of *Rosa hybrida* as affected by the genotype of the rootstock. Accepted by *J. Plant Physiol.*

Chapter 5

Dieleman JA, Verstappen FWA, Kuiper D Root temperature effects on growth and bud break of *Rosa hybrida* in relation to cytokinin concentrations in xylem sap. Accepted by *Scientia Hort.*

Dieleman JA, Perik RRJ, Kuiper D (1995) Effect of ethanol on the growth of axillary rose buds in vitro. *J. Plant Physiol.* 145: 377-378.

Curriculum vitae

Anja Dieleman werd op 13 juni 1968 geboren in Kommerzijl. In 1986 behaalde zij het VWO-diploma aan de C.S.G "Lauwers College" te Buitenpost en begon de studie Tuinbouwplantenteelt aan de Landbouwuniversiteit in Wageningen. Tijdens haar studie liep zij stage op het Vågønes Forskningsstasjon te Bodø, Noorwegen en op het Instituut voor Agrotechnologisch Onderzoek (ATO-DLO) te Wageningen. In 1992 slaagde zij voor het doctoraalexamen. In datzelfde jaar werd zij aangesteld als assistent in opleiding (AiO) bij de vakgroep Tuinbouwplantenteelt en permanent gedetacheerd op de afdeling Biochemie en Celfysiologie (later Plantenfysiologie) van het Centrum voor Agrobiologisch Onderzoek te Wageningen (CABO-DLO, later Instituut voor Agrobiologisch en Bodemvruchtbaarheidsonderzoek, AB-DLO). De aanstelling betrof het project 'De rol van het cytokininemetabolisme in de regulatie van de bovengrondse groei van de roos, o.i.v. verschillende onderstammen'. Dit project werd gefinancierd door het Produktschap voor Siergewassen. Van maart tot en met juni 1995 was zij gastmedewerker bij de vakgroep Växtfysiologi (Plantenfysiologie) van de Sveriges Lantbruksuniversitet te Uppsala, Zweden. Sinds 1 januari 1997 werkt zij als regelingsontwikkelaar voor bos- en natuursubsidies bij LASER (Landelijke service bij regelingen) Zuidoost in Roermond.

Nawoord

Vier jaar ben ik er mee bezig geweest. Maar nog steeds is het mij niet duidelijk waarom een plant zo veel verschillende cytokininen nodig heeft. Dat een plant één groep cytokininen bevat, kan ik begrijpen en dat er binnen die groep verschillende vormen zijn, namelijk een actieve vorm, een transportvorm en verschillende opslagvormen, daar kan ik me ook nog wel iets bij voorstellen. Maar waarom behalve Z-type cytokininen dan ook nog iP- en DHZ-type cytokininen? En waarom het bijzondere verloop van knopuitloop bij rozen? Eerst loopt een aantal knoppen boven in de plant uit, en een aantal weken later een groep knoppen onder in de plant. Waarom juist zo? Ik weet het niet. En juist dit soort vragen, waarom is een plant zoals hij is en doet hij zoals hij doet, maken het doen van plantenfysiologisch onderzoek zo machtig mooi.

Met heel veel plezier heb ik vier jaar aan cytokininen en rozenonderstammen gewerkt. En veel geleerd over de groei van rozen, het bepalen van cytokininengehaltes in bloedingssap en in plantenweefsels, het samenwerken in kleinere en grotere groepen, het uitdenken van de juiste onderzoeksvragen en de bijbehorende proeven, en het rapporteren van verwachtingen, experimenten, uitkomsten en bijgestelde verwachtingen in artikelen. Gelukkig heb ik dit niet allemaal alleen hoeven doen. De mensen die hierbij betrokken zijn geweest en die mij veel geleerd hebben, wil ik hier bedanken.

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Ettelijke grote rozenproeven hebben we in het klimaatgebouw gedaan. De medewerkers van de Proeftechnische Dienst wil ik bedanken voor het feit dat de klimaatkamers draaiende werden gehouden en dat de proeven ook in het weekend in de gaten werden gehouden. In het bijzonder Kees Jansen wil ik bedanken voor het verzorgen van onze proeven. Bedankt Kees, dat onze rozen er altijd zo mooi bij stonden en dat het al die jaren is gelukt om ze ziektevrij te houden. Het stenten van de rozen hebben we in het begin op het CPRO-DLO en later bij de vakgroep Tuinbouwplantenteelt gedaan. Voor het

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In een van de eerste proeven die ik gedaan heb, werd de groei van rozen bij verschillende worteltemperaturen gevolgd. Met het realiseren van die worteltemperaturen wist de Technische Dienst wel raad. Zij hebben in korte tijd hiervoor een opstelling gebouwd. Ook voor het uitvoeren van de grotere en kleinere klussen die daarna kwamen, wil ik Coen Holleman, Maas van den Born, Bart IJkhout en Cees Bongaards bedanken. Het spijt me dat twee van hen dit niet meer mogen meemaken.

Al een halfjaar nadat ik begonnen was, kwam de eerste student, René Perik. Op zijn eigen, onverstoorbare wijze deed hij de weefselkweekproeven, en breidde ze nog een beetje uit. De uitwerking hiervan heeft uiteindelijk tot het 'ethanol-artikel' geleid. René kwam later nog een keer terug, en legde het fundament voor hoofdstuk 3. Bedankt René. Ook Liesbeth van den Herik, Maria Kladou, Yvonne Bogaard, Ard Kragten en Wendy Deijkers wil ik bedanken voor hun inbreng in het rozenonderzoek.

In 1995, I was fortunate enough to be able to work at another laboratory for some time, to enlarge my knowledge of and experience with the determination of endogenous cytokinins. I would like to thank Professor Elisabeth Tillberg of the Department of Plant Physiology of the Swedish University of Agricultural Sciences in Uppsala, Sweden for offering me the possibility to work at her department for three months. Dr. Björn Nicander taught me the use of immunoaffinity columns and the interpretation of the UV spectra of the HPLC. Thanks Björn, especially for coming to the lab at one of the last evenings, when the needle of the HPLC crashed. I would like to thank PeO Björkman for GC-MS analyses of some of my samples. For me, it was very special to work at a lab where a number of people knew what cytokinins are and how to determine them, and to join a group discussing articles concerning auxins and cytokinins. I would like to thank Núria Massot, Håkan Strid, Tinna MacKey, Salme Timmusk, Russell Hammerton, Jolanta Borowitz, Marit Lenman, Ulf Ståhl, Asha Yahya, Hauke Hansen and all the others of 'Växtfysiologi' for making my stay in Sweden one that I will always remember.

De voortgang van ons project en de ontwikkelingen in het rozenonderzoek in Nederland werden halfjaarlijks besproken in de klankbordgroep. Ik heb de discussies met Lidwien Dubois, Dik de Vries, Peter van de Pol, Michaël Kool, Cor Vonk Noordegraaf en Hendrik Jan van Telgen altijd boeiend en verhelderend gevonden. Verder wil ik hen bedanken voor het feit dat ik altijd welkom was om een beroep te doen op hun kennis en ervaring. Vanzelfsprekend wil ik ook het Productschap voor Siergewassen bedanken voor het financieren van het rozenonderstammenproject.

De figuren 2.1, 6.1 en 7.1 zijn op vakkundige wijze getekend door Dorothée Becu. Voor de eerste figuren hebben we uitgebreid alle details van de planten zitten te bestuderen. Maar hierna raakte je zo bedreven dat figuur 7.1 na een telefoontje getekend kon worden. Bedankt, jouw tekeningen zijn zowel erg mooi als verhelderend geworden.

Ik was op het CABO/AB niet de eerste rozenAiO. Helen van Reenen ging mij voor in het onderzoek naar de rol van koolhydraten bij bloemknopopening van rozen. Bedankt Helen, je hebt voor mij het pad geëffend, meer dan veel mensen zich zullen realiseren. Met mijn kamergenote Iris Kappers heb ik alle stadia van het AiO zijn doorlopen. Planten oogsten, monsters verwerken, samen op congres, het eerst artikel geaccepteerd. Bedankt Iris, voor het delen van deze en een heleboel andere ervaringen. Door de inbreng van Bob Veen en Guus Broekhuijsen zijn de berekeningen in hoofdstuk 3 aanzienlijk verbeterd,

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Als 'groentje' op het lab moesten mij dringend nog een aantal practische zaken worden bijgebracht op het moment dat ik voor het eerst de labs op het AB betrad. Naast Francel is er nog een aantal mensen geweest dat zich dat aantrok. Marcel Visser deed dat op het weefselkweeklab, Jan Kodde op het isotopenlab, Simon Ribôt op het lab boven en Evert Davelaar op het EIA lab. Bedankt allemaal. Verder wil ik Janneke Abbas en Rina Kleinjan-Meijering bedanken voor hun hulp bij de opmaak van het proefschrift.

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Tot op 20 mei.