



T ✓ PHYSIOLOGICAL ASPECTS OF SHOOT GROWTH REGULATION IN
JUVENILE AND ADULT *CITRUS SINENSIS* (L.) OSBECK ✓ /SR

by

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N.T. Thesis (Ph.D. ; Botany) - University of Natal
Pietermaritzburg, 1982.

N.G. 2 copies.

Submitted in partial fulfilment of the
requirements of the degree of

Doctor of Philosophy

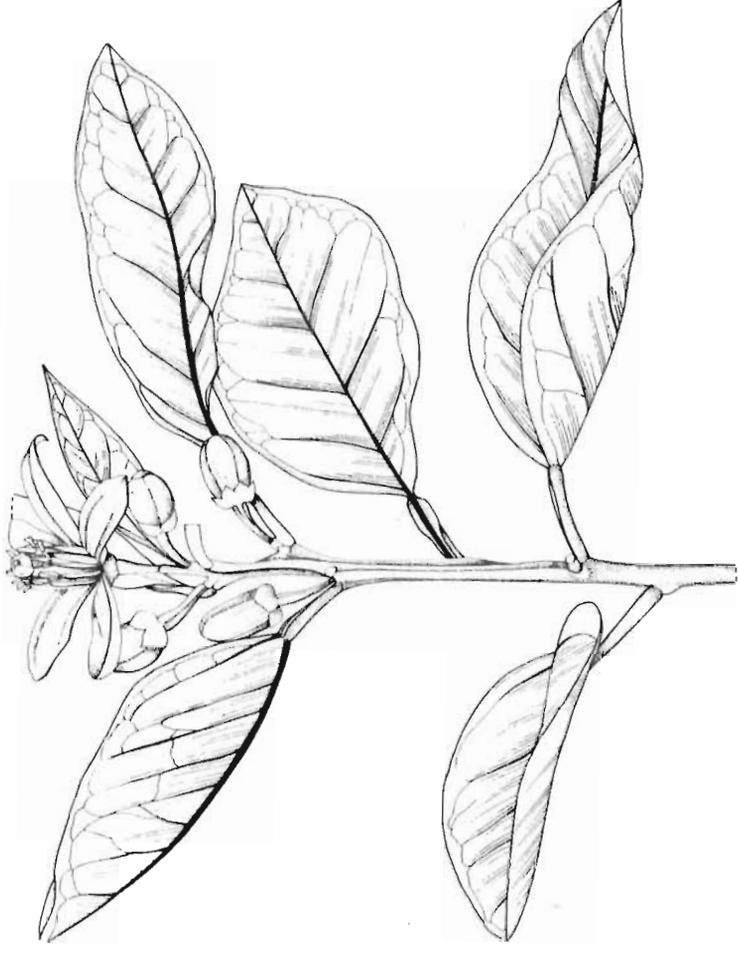
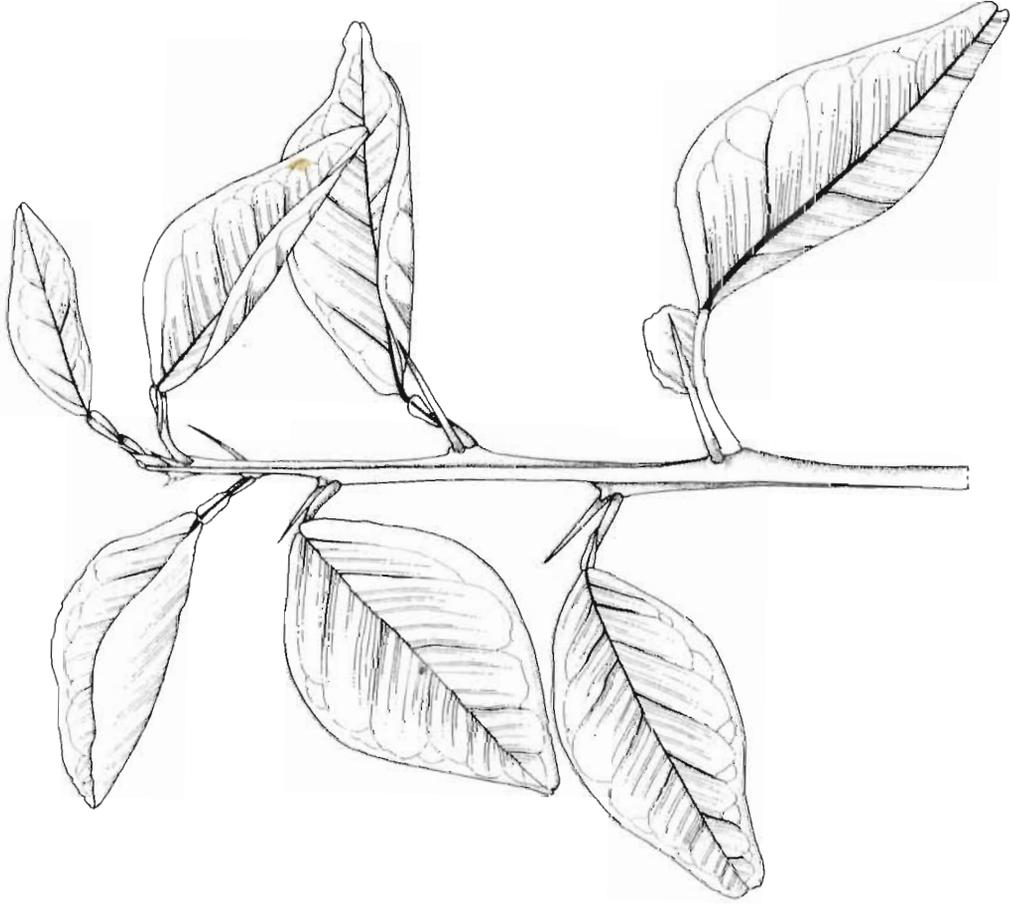
in the

Department of Botany

P University of Natal,

PP Pietermaritzburg :

D 1982.



Colinus sinensis (L.) Osbeck

PREFACE

The experimental work described in this thesis was carried out in the Department of Botany, University of Natal, Pietermaritzburg, from March 1980 to September 1982, under the supervision of Professor J. van Staden.

These studies represent original work by the author and have not been submitted in any form to another University. Where use was made of the work of others it has been duly acknowledged in the text.

NIGEL S. HENDRY
December 1982

ACKNOWLEDGEMENTS

I would like to record my appreciation for the supervision provided by Professor J. van Staden in the execution and documentation of my studies.

My parents provided assistance of many forms and were always encouraging and concerned with my endeavours.

Technical assistance from the following people is acknowledged and is greatly appreciated: Mr M.E. Gough of the Phytotron Unit; Mrs M.G. Gilliland and Messrs V. Bandu and M. Kundalram of the Electron Microscope Unit.

It is with gratitude that I acknowledge the proof reading and typing of this thesis by Sheila Niven and Ursula D'Arcy-Donnelly, respectively.

My colleagues and the academic and technical staff in the Department of Botany were a source of inspiration and assistance. My student years have been interesting and pleasant in their company.

Financial assistance in the form of a bursary from the Council for Scientific and Industrial Research, Pretoria; a research grant from the Department of Agriculture and Fisheries, Pretoria, and a graduate assistantship from the University of Natal, is gratefully acknowledged.

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INTRODUCTION

As the requirements for more efficient and economical crop production emerge, so the need for a better understanding of plant growth and development is felt. It is especially the need to understand means by which man may influence growth and development in order to achieve greater productivity, that requires attention. Observation and analysis of plant growth and the concomitant study of plant components, organs and chemicals, provides one of the research oriented approaches to gaining an understanding of the mechanism of growth control.

Ultimately, the extent of reproductive growth (fruiting) or the bearing capacity in all plants is governed by the volume and area of vegetative growth and the supply of substances required for growth produced by those vegetative organs. Productivity then, is governed by the efficiency of conversion of solar energy to chemical energy and by the relative ability to store this energy. Plant shoots play a fundamental role in both assimilation and storage of carbohydrates.

Given the same growing conditions, similar plants sometimes exhibit different rates of growth. For example, ontogenetically juvenile plants exhibit differences in form and vigour in comparison with ontogenetically adult plants. Although the genotype of such adult and juvenile plants may vary only in expression and not in constitution, there does appear to be some property in juvenile plants which induces the characteristic enhanced vegetative vigour. It is this property, amongst many others, which needs to be better understood, for it is a potential means of gaining greater yields from plants without alterations being made to the environment.

A high degree of organisation of growth processes must exist to account for the orderly development of plants. A few organic compounds that influence certain biochemical and physiological reactions have been isolated from the

wide array of organic and inorganic substances found in plant extracts. A growing body of evidence suggests that such growth regulatory compounds are ubiquitous in plants; are active at exceedingly low concentrations and are under genetic control. Plant hormones are almost certainly involved in directing or regulating plant growth and development.

The subject of the present research concerns the characterization of differences in shoot growth of juvenile and adult phases of a commercial *Citrus* cultivar and an investigation of the possible role which plant hormones may play in such differences. In addition to a comprehensive literature survey, three separate sets of experiments were conducted. In the first, an analysis of the components and parameters of the difference in vegetative shoot growth of adult and juvenile scions was carried out. Anatomical and ultrastructural comparisons were also made. In the second set of experiments the endogenous levels of gibberellins, cytokinins and inhibitors of buds and other tissues, which might play a role in the regulation of shoot flush growth, were examined. Specific extractions were performed and appropriate bioassay techniques were employed for the three hormone classes investigated. In the final set of experiments the effects of synthetic plant growth regulators on shoot flush growth were monitored. The growth regulators were applied by means of trunk injections or as droplets on shoot terminal buds.

These experiments were planned and executed with the above mentioned concepts and ideals in mind. The objective of the project was to contribute in some measure to the knowledge which will allow better use to be made of available natural resources.

CHAPTER I

LITERATURE REVIEW

The juvenility phenomenon

WAREING and PHILLIPS (1978) wrote that "the orderly succession of changes leading from the simple structure of the embryo to the highly complex organization of the mature plant presents some of the most fascinating and challenging outstanding problems of biology". One such "outstanding problem" is the unexplained difference in developmental behaviour of many seedling plant species and their adult progenitors. The fact that seedling plants only acquire the flowering and fruiting habit after the passage of a specific duration of growth probably has been observed from the time that man first cultivated fruit crops.

One of the more recent historical records of this observation is that of KNIGHT (1820) who noted: "When young trees have sprung from the seed, a certain period of time must elapse before they become capable of bearing fruit." An early researcher into the field of young, vegetative and older, generative plants was GOEBEL (1898), who defined hetero- and homoblastic forms of plants. He noted distinct juvenile and adult stages in the life cycle of woody plants (GOEBEL, 1900) and concluded that this heteromorphism, unlike sexual heteromorphism, exists as a sequence in the same organism. Research in this field is plagued by controversies even today, so it is noteworthy that at a very early date (JOST, 1907) an inconsistency in plant growth patterns was observed. Certain oak (*Quercus* L.) species, which normally flower for the first time at 60-80 years of age, were found to occasionally produce flowers in the first year only as seedlings.

As the limitations of existing fruit tree cultivars became apparent, so the need for breeding programmes became more urgent. Partly due to the need of tree breeders to induce early flowering, there was a period of an

awakening of interest in the juvenility phenomenon (WAREING, 1959). During this time many observations, definitions and theories were recorded. Terms such as ontogeny, topophysis, ageing, maturation and juvenile- and adult-phases were coined. FORTANIER and JONKERS (1976) distinguished ontogenetical ageing from chronological and physiological ageing, which are not as strictly related to the juvenility concept; ontogeny being the study of embryo development into an adult individual. During the ontogenesis of plants, vegetative somatic inheritance of characters takes place. This property of plants, which is termed the topophysis effect, was used by MOLISCH (1920) and ROBBINS (1964) to explain how a vegetatively propagated plant may retain the characters of that portion of the plant from which it came, be that juvenile or adult. Phrases such as "ripeness to flower" (KLEBS, 1918), "phase change" (BRINK, 1962) and "minimum leaf number" (PURVIS, 1934) have arisen from researchers' attempts to explain the change in behaviour of plants. All three phrases also represent attempts to define juvenility. Two aspects of development appear in most definitions of juvenility and these are firstly, the inability of seedlings to flower when very young and secondly, the vigorous vegetative growth of young plants relative to that of adult plants. LEOPOLD and KRIEDEMANN (1975) have described the juvenile state as a period when the plant is capable of exponential increases in size and when flowering processes cannot be readily induced. SCHWABE (1976) emphasized that it would be clearer to regard the plant as being in the juvenile condition when it fails to become reproductive even when exposed to environmental conditions, such as appropriate daylengths, which would normally induce flowering. The juvenile state, in addition, has a distinctive morphology of leaves, stems and other structures and it is displaced by a morphologically distinctive mature state (LEOPOLD and KRIEDEMANN, 1975). Although some of the definitions and descriptions of the juvenile condition allude almost solely to the lack of flowering ability (PASSECKER, 1949; CAMPBELL, 1961; BRINK, 1962; ZIMMERMAN, 1972; DE VRIES, 1976) it is now widely

accepted that many aspects of ontogeny may be related to phase change (DE MUCKADELL, 1954; WAREING, 1959, 1970; DOORENBOS, 1965; BORCHERT, 1976). These four authors adopt a very pragmatic approach to the problem, each appearing to be hesitant of stipulating hard and fast rules of definition, for as DE MUCKADELL (1954) stated, there are many stages of juvenility which are ill-defined and a great number of exceptions can be found among different plants. Besides which, it is evident that each of the characteristics that constitute the phenomenon of juvenility may, under certain circumstances, develop independently of the others (DOORENBOS, 1965). BORCHERT (1976) goes so far as to question the existence of one uniform juvenile state, based on the argument that each of the various components of juvenility changes at a different rate. The high correlation expected between the various phenomena associated with and implicitly considered to be indications of the juvenile state, is not always apparent. This suggests relatively independent control mechanisms for the various phenomena.

It is important to consider other changes occurring during ontogeny which are apparently of a different nature from the changes involved in the attainment of the adult condition (WAREING, 1959). Thus, for example, BORCHERT (1976) mentions that the most general aspect of ageing in trees, that is the gradual increase in size and complexity, has rarely been analysed or related to the phenomenon of juvenility. So although the transition to the flowering adult phase is usually sharp, there are indications that the conditions within the plant are gradually moving towards the mature state as indicated, for example, by gradients in leaf shape of some species (WAREING, 1959). Furthermore, caution needs to be exercised with respect to certain measurable parameters supposedly associated with maturation of seedlings. WAREING (1959) and BORCHERT (1976) also question the basis or theory of topophysis. The distinction is made between maturation or ontogenetical ageing (FORTANIER and JONKERS, 1976) and physiological ageing. The latter incorporates loss of vigour and senescence. The transition

from juvenile to adult involves a relatively stable change, whereas the latter effect involves changes which are easily reversed. Unlike adult characters, such as leaf shape and flowering habit, some features associated with ageing, such as the loss of vigour and tendency to negative geotropism, are not retained if grafted onto young stocks (WAREING, 1959; MOORBY and WAREING, 1963). The importance of this controversy lies in being able to distinguish features associated with maturation and those which are simply a part of the physiological ageing of a plant. Although there may be a gradation of some characters associated with the juvenile/adult phases, they are relatively stable and may be used as an index of the stage of maturity of the plant. Thus, DE MUCKADELL (1954) considered the morphological forms in trees to be better indices of juvenility than the ability of a tree to flower. Leaf and stem parameters have received more attention in this respect than any other organs of the plant.

SCHWABE (1976) considers that there are a number of selective advantages, from an evolutionary point of view, in features of juvenility. Young eucalypt (*Eucalyptus* L'Herit.) trees, for example, tend to produce long, thin stems in early years, followed by rapid strengthening of the trunk only after a certain height is attained. Long, thin stems or a whip-like growth is also evident in apple (*Malus* L.) (LEOPOLD and KRIEDEMANN, 1975) and *Citrus* L. (HENDRY and ALLAN, 1980). Wherever plants grow, light may be a limiting factor and adaptations are universally evident which enable plants to utilize available light more efficiently. Seeds of tree species often germinate under the canopy of their progenitors where light may limit full development. The long stem growth of trees and vines in the juvenile state seems to be an adaptive response by the plant to compete successfully for available light. The enhanced stem growth or elongation of juvenile plants appears to be a feature of greater internodal length and possibly also of a greater number of leaves being produced (WILLIAMS,

1961; STEIN and FOSKET, 1969; HENDRY and ALLAN, 1980). In *Hedera helix* L. internode lengths were always two to three times longer in juvenile plants (STEIN and FOSKET, 1969) and in *Citrus sinensis* (L.) Osbeck (Sweet orange) internodes were longer and more numerous (HENDRY and ALLAN, 1980). Stem diameter is thought to provide an index of comparative ontogenetical ageing and vigour in young seedlings. VISSER (1970), HEYBROEK and VISSER (1976) and VISSER, VERHAEGH and DE VRIES (1976) alleged that stem diameter appears to be negatively correlated with the length of the juvenile period in fruit and forest trees. However, the initially significant inverse relation became insignificant as the trees grew older due to retardation of growth when the seedlings became generative. This knowledge could be used for selecting seedlings with short juvenile periods without actually waiting for seedlings to commence flowering. ZIMMERMAN (1976) argued that seedlings could not be preselected for short juvenile periods using this criterion on the basis of his experiments with pear (*Pyrus* L.) seedlings. He found that these two criteria (juvenility and stem diameter) were not significantly correlated for all planting years, progenies or parents. They were subject to other unknown variables.

Two other differences sometimes noted between adult and juvenile growth forms may support the postulation of ecological advantage in juvenile characteristics. Rudimentary lateral branches which develop into spines are present on apple, pear, *Citrus*, *Gleditsia* and *Maclura* stems (DOORENBOS, 1965; ZIMMERMAN, 1972; LEOPOLD and KRIEDEMANN, 1975). These may act as deterrents to browsing animals and increase the chance of survival of the seedlings. A change in phyllotaxy (ZIMMERMAN, 1972) may be associated with light interception at different heights in forest strata. The shape of leaves and retention of leaves also differ in adult and juvenile forms and may play a role in providing advantages for survival in seedlings or bearing plants. It is well known that mature trees

produce two types of leaves, that is sun and shade leaves (MONSELISE, 1951). SCHRAMM (1912) reported that leaves from juvenile beech trees (*Betula L.*) resemble the shade leaves of adult trees and that juvenile plants produce only shade leaves. It was also found that leaves from the top of a tropical forest tree frequently differ in size and morphology from those of seedlings of the same species growing on the forest floor (LONGMAN and JENIK, 1974). These findings substantiate the hypothesis that there are graded levels of increasing maturity up the tree (PASSECKER, 1949). Thus plants grown from buds, scions or cuttings taken from the juvenile, basal zone develop into new plants which retain the juvenile characteristics such as leaf form, thorns, ease of rooting and tendencies to retain leaves during winter for certain periods, sometimes years after propagation (PASSECKER, 1949; DOORENBOS, 1965).

Leaves have been found to vary in many respects. The gradation of forms in some species represents as dramatic an effect as the sudden commencement of flowering. Some of the reported examples of plants showing leaf heteromorphism are *Acacia melanoxylon* and *Eucalyptus* (DOORENBOS, 1965), *Hedera helix* (LEOPOLD and KRIEDEMANN, 1975) and *Humulus lupulus L.* (WILLIAMS, 1961). Such dimorphic leaves have differences not only in shape (STOUTEMYER and BRITT, 1965) and thickness (ZIMMERMAN, 1972) but also in cellular contents. BORCHERT (1976) stated that, in general, such structural changes of leaves tend to be in the direction of greater xeromorphy, indicating probably that leaves growing on larger trees or late in the season develop under progressively higher water stress. Other differences in leaf characteristics include the production of higher anthocyanin pigment levels (STOUTEMYER and BRITT, 1961; DOORENBOS, 1965); increased carbohydrate and nitrogen levels in leaves (HEATH, 1957); lower RNA content and lower RNA/DNA ratios in *Pyrus* (ALI and WESTWOOD, 1966) and in *Hedera helix*, a higher total soluble protein fraction (GHOSH and MILLIKAN, 1970), in juvenile as opposed to

adult plants. Light saturated net photosynthesis (per unit leaf area) was found to be about 1,5 times higher in adult leaves than in juvenile ones from *Hedera helix* (BAUER and BAUER, 1980). The lower photosynthetic capacity in juvenile leaves was probably caused by the lower stomatal and residual conductance to carbon dioxide transfer. This corresponded to anatomical features of the leaves, that is lower stomatal frequency, fewer chloroplasts per cell and thinner leaves. It is interesting to note that although these leaves originated from the same light environment, they exhibited differences reminiscent of sun and shade leaves mentioned above.

Undoubtedly, tree age influences leaf characters and these characters are, consequently, closely related to readiness or ability to flower (MELCHIOR, 1976). Flowering then is related not only to leaf characters, but also, in some instances, to leaf numbers. In certain winter cereals (PURVIS, 1934) and in hops (THOMAS and SCHWABE, 1969) a specific number of leaves must be differentiated before the apex becomes generative. This situation may, however, be a special case of the juvenility phenomenon, as it almost certainly is not operative in many tree species where precocity is not related to leaf number or plant size.

There are two aspects of growth rate worthy of consideration in respect of plants undergoing phase change. One concerns the loss of vigour in ageing seedlings and the other is concerned with the relationship between precocity and seedling growth rate. The rate of growth of the whole tree assumes a sigmoid curve when the entire lifespan is considered (WAREING, 1959). During the early years of seedling growth there is an exponential phase of growth, and later as the plant reaches maturity, the curve flattens off. WAREING (1959) ascribes this loss of vigour to severe competition between various growth centres. Evidence for this theory comes from horticultural 'thinning out' or pruning which results in increased growth of the remaining shoots. This competition for nutrients may also

effect the loss of apical dominance and the gradual loss of geotropic orientation of stems. Loss of vigour, apical dominance and geotropism may therefore be related processes in ageing of trees (WAREING, 1970). None of these three features of ageing in trees is of a fixed nature, however, and each may be modified by, for example, grafting or pruning.

Many experiments have shown that vigour and precocity in seedlings is related (WAREING, 1959; VISSER, 1970; ZIMMERMAN, 1972). This relationship will be discussed later, along with other factors which affect the duration of the juvenile period.

It is worth noting an interesting fact concerning the somatic inheritance of the duration of the vegetative period. In vigorous apple cultivars flowering is frequently absent during the period of intense vegetative growth following grafting. Flowering does not commence until the vegetative vigour is reduced and a spur system is developed (WAREING, 1959). This vegetative period in grafted trees has been shown to be an echo of the original seedling clone in one decisive respect: the length of the vegetative period is clearly correlated with the length of the juvenile period (VISSER, 1965 ; VISSER and DE VRIES, 1970). This information is of importance for fruit growers, foresters and tree breeders in the selection of seedlings with a short juvenile period.

That the duration and other features of the juvenility phenomenon are genetically controlled and therefore heritable traits has been shown conclusively (ZIMMERMAN, 1972 with references). VISSER (1965) has conducted extensive research in this aspect of ontogenetic ageing. By categorising parent plants into groups with similar juvenile periods and by observing the duration of the juvenile period of the progeny, he was able to show that the offspring seedlings had similar periods of juvenility to the parents. Degree of juvenility also appears to be variable, as was demonstrated with apple seedlings categorised according to amount of thorniness

and general appearance of "wildness". VISSER (1965) reported a direct relationship between degree of juvenility and length of juvenile period. When grafted, however, the ability of apple and pear selections to flower is dependent on the association with the rootstock. In comparing graft combinations, VISSER and DE VRIES (1970) found that it was the combination which achieved greatest size at the season of flower initiation which flowered first. KHOURY and WHITE (1980) showed that in seedling cultivars of *Kalanchoe* Adans. Fam. cultivars, the juvenile phase varies considerably. In order to explain this variability in degree of expression of juvenility which, as mentioned above, occurs in apple crosses, VISSER (1976) proposed that polygenic inheritance of the juvenile period is most likely. He stated that the inheritance of the juvenile period will be determined by the inheritance of a number of factors governing the development of the seedling. It would, therefore, be feasible to breed for a shorter juvenile period by choice of the appropriate precocious progenitors (ZIMMERMAN, 1972).

Another difference between the adult and juvenile phase in plants is the greater propensity which the juvenile phase has for the formation of adventitious roots (ZIMMERMAN, 1972). Stem or root cuttings taken from young seedling plants or from the basal part of a tree will root much more readily than those taken from older plants or from more distal shoot tissues (HARTMANN and KESTER, 1975). SMITH and CHIU (1980) examined the seasonal effect of rooting in adult- and juvenile-derived pecan (*Carya illinoensis* Koch.) cuttings. Juvenile cuttings produced more roots and a larger percentage of rooted cuttings than adult cuttings. The lower percentage of rooting in defoliated cuttings from this deciduous species led these researchers to conclude that foliage was necessary for the synthesis of a root promoting substance. In similar-aged source trees, juvenile cuttings from vigorous, glasshouse-grown oaks rooted more easily than cuttings from ambiently grown parent trees (MORGAN, McWILLIAMS and PARR, 1980). They

also found an inverse linear relationship between rooting success and increasing seedling age. The role of rooting factors extracted from juvenile and adult tissues in rooting ability will be discussed later.

When the search begins for the site of ontogenetic control, the shoot apical meristem must be the most obvious starting point. At this stage only literature which reports on morphological or anatomical differences in adult and juvenile apices will be reviewed. The role of the apex in regulating or maintaining a particular phase will be discussed at a later stage.

A comprehensive anatomical study of the shoot apex of adult and juvenile *Hedera helix* has been provided by STEIN and FOSKET (1969). They present a number of major differences in these apices. Apices of the adult phase have a larger meristematic area consisting of smaller cells than do the juvenile apices, which have a larger subapical region where cell division continues for a longer period than in adult apices. This results in a delay of cell enlargement. In adult apices the subapical region is contracted so that internode elongation is delayed. The juvenile shoot apex is smaller but has larger cells than that of the adult. On the basis of this study the authors suggest firstly, that modifications of the rate of cell division as well as of division activity within the shoot are important in juvenile/adult phase differences, and secondly, that substances known to reduce cell division in subapical meristems are prime candidates for attempts to alter juvenile forms into the adult phase.

Earlier work with AMO-1618 (2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidene-carboxylate methyl chloride) and gibberellic acid, both of which have little effect on the apical meristem, showed that these two compounds have opposite effects on the subapical meristem (SACHS, LANG, BRETZ and ROACH, 1960). AMO-1618 inhibited subapical meristem activity

in *Chrysanthemum* L. and gibberellic acid applied simultaneously or after AMO-1618 overcame the inhibition. Flower growers make use of this compound to reduce the inflorescence stem length of potted plants. Gibberellin-like substances appear to regulate the activity of the subapical meristem and may thus be responsible for differences in stem and internode growth of adult and juvenile shoots.

A study was made of the intrinsic cellular physiological differences of the shoot apical meristems of juvenile and adult *Hedera helix* by POLITO and ALLIATA (1981). They found that with callus initiated from these organs the fresh weight growth index of juvenile-phase callus was approximately 3,5 times that of adult-phase callus. Microspectrophotometric analysis of nuclear DNA revealed no differences and no indication of endopolyploidy in juvenile and adult callus cells. These results indicated that shoot apical meristems possess inherent physiological differences which are expressed even in the absence of factors associated with subapical, differentiated tissues or organised meristem structure.

A number of observations have been made on callus cultured from juvenile and adult phase tissue of *Hedera helix*. These investigations throw some light on key aspects of factors suspected of operating in ontogenetical ageing. It is now well established that rejuvenation of adult cuttings of *Hedera helix* can be induced by applying gibberellic acid to shoots (ROGLER and HACKETT, 1975 a; WAREING and FRYDMAN, 1976). The findings of BANKS (1979) are of great interest as far as the actual process of cellular phase change is concerned. Using the rejuvenated tissue mentioned above and adult tissue, she showed that callus from the juvenile stems regenerated only shoots and callus from adult stems regenerated only embryos. Thus both types consistently regenerated different structures, presenting the possibility of an *in vitro* marker for phase change. It may be that since both juvenile reversion and mature derived callus gave

rise to regenerated structures that were juvenile, the *de novo* establishment or derivation of mature or adult meristems is not possible.

Both STOUTEMYER and BRITT (1965) and ROBBINS and HARVEY (1970) also reported that adult and juvenile derived tissue cultures consistently behaved differently. STOUTEMYER and BRITT (1965) found that stem callus from the juvenile form of *Hedera helix* had a higher proliferation rate and larger cells than adult derived stem callus. This finding is consistent with the report on callus growth from shoot apices as cited above (POLITO and ALLIATA, 1981). However, the stem callus growth has a bearing on cambial growth and may be related to differences of girth increment as discussed earlier. The growth rate of seedling derived callus was found to be similar to the juvenile reverted form used by STOUTEMYER and BRITT (1965) in exceeding the growth rate of adult derived stem callus (ROBBINS and HARVEY, 1970). This juvenile callus did, however, develop vigorous variants, which raised the question of the validity of extrapolating the findings of callus growth to the whole plant situation. BANKS-IZEN and POLITO (1980) reported that juvenile derived callus increased in volume at a faster rate than adult derived callus of *Hedera helix*. When chromosome counts were made with these calli, the proportion of non-diploid cells in juvenile derived callus was found to increase rapidly and then to level off. Conversely, in adult derived callus, the increase in the proportion of non-diploid cells was initially gradual and subsequently rapid. Taking into account the fact that an increase in ploidy levels of cells in culture is an often-described occurrence in herbaceous and woody plants, some doubt must again be expressed concerning the relation these findings present with the whole plant situation. Another doubtful point, in the context of tissue cultures using juvenile reverted tissue as opposed to young seedling material, was investigated by STOUTEMYER and BRITT (1969). In order to investigate whether or not the juvenile reversion shoots were physiologically identical to

seedlings which had never advanced to the stage of flowering, they cultured the tissue on different media and monitored habituation. In the absence of coconut milk or naphthalene acetic acid considerable growth was made by tissue cultures started from young seedlings and very little proliferation in cultures was started from juvenile reversion shoots. They concluded that seedlings produce rapid growing cultures less dependent on external auxin and on growth factors in coconut milk. Although they make no attempt to explain differences in whole plants based on their findings, these results are of use in offering criticism of some experiments conducted with juvenile reversion cuttings.

Experiments on chemical modification of juvenility open up the interesting possibility that growth substances are involved in the control of these developmental changes and that the gradual shift from juvenile to adult growth may be due to an alteration in the proportions of endogenous substances (LEOPOLD and KRIEDEMANN, 1975). The embryo-sac experience stimulates a rejuvenescence in the developing embryo, with the further possibility of a chemical stimulation to the juvenile condition. FORTANIER and JONKERS (1976) stated that not much is known about the hormonal regulation of phase change. They suggested that the ratio between growth promoting and growth retarding substances and/or the amount of a specific hormone are inductive for flowering only after a specific ontogenetical stage has been reached. Many lines of evidence also imply that the juvenile state is one in which there is a greater capacity for growth. It remains to be assessed to what extent the greater growth is genetically programmed or represents interactions of nutrients and hormones from various parts of the plant (LEOPOLD and KRIEDEMANN, 1975).

The practical benefits of being able to modify juvenility, both reversion and maturation, are manifold. It is therefore not surprising that a large number of experiments have been conducted which deal specifically with the

effects of chemical growth regulation of plants in the adult and juvenile phases of growth. Attempts to isolate one single hormone causing juvenility, flowering or good rooting of cuttings have been notoriously unsuccessful, probably because such attempts disregard the complexity of the factors determining the state of a growing shoot. Nevertheless, evidence is accumulating, some of which is presented below, which implicates gibberellins and antigibberellins in certain aspects of the expression of the stage of maturity.

In very few instances does the application of gibberellins to plants have a short term or immediate effect in reducing the juvenile period (BORCHERT, 1976). The gymnosperms do prove to be exceptions among the woody species. PHARIS and MORF (1967) were able to induce early staminate and ovulate strobili in two species of *Cedrus* Mill. and four species of *Cupressus* L. by spraying with gibberellins A₃ and a gibberellin A₄/A₇ mixture. Later it was reported that promotion of early flowering could be obtained with gibberellins A₄, A₅, A₇, A₉ and A₁₄ in at least one Cupressaceae species. Success with the polar gibberellin A₃ is ubiquitous through the Cupressaceae and Taxodiaceae, while only the polar gibberellins were effective in the Pinaceae. PHARIS and MORF (1967) came to two important conclusions. Firstly, that the differential efficacy of gibberellin may be related not only to familial differences in gibberellin metabolism and physiology, but also to differences between families and species in patterns and timing of sexual differentiation. Secondly, the possibility that endogenous levels of certain gibberellins are limiting factors in flowering of mature phase Pinaceae and juvenile phase Cupressaceae species, appears to be warranted as a working hypothesis.

Among the angiosperms, flowering may be induced in rosette plants by the application of gibberellins. In *Hyoscyamus niger* L. and *Samolus parviflorus* Nees. gibberellin application resulted in stem elongation (SACHS,

BRETZ and LANG, 1959). The zone of intensive meristematic activity was localized in the subapical region referred to previously as a site of difference between juvenile and adult plants. Gibberellins may substitute for light requirements in stimulating flowering of some herbaceous species. Flowering in *Bryophyllum diagremontianum* can be induced by transferring plants from long day to short day conditions or by applying gibberellins under short day conditions (ZEEVAART, 1969). Because application of gibberellin to mature leaves was more effective than to the youngest leaf pair and the tip, ZEEVAART (1964) suggested that the action of gibberellins in regulating flower formation is localized in the mature leaf. In *Pharbitis* Choisy, the inhibition of flower formation and stem growth with B-995 (N,N-dimethylamino succinamic acid) can be completely overcome by the application of gibberellic acid to the plumules (ZEEVAART, 1966).

For the most part, however, gibberellins have been found to prevent flowering or to cause a reversion to the juvenile state in angiosperms. This reversion in some fruit tree species may be detected by the appearance of thorns on bearing trees (ZIMMERMAN, 1972). *Acacia melanoxylon*, which shows leaf dimorphism in the juvenile and adult phases with intermediate leaf forms, can undergo reversion when treated with gibberellic acid (TRIPPI, 1963; BORCHERT, 1965). In *Mangifera indica* L. flowering in juvenile seedlings was induced in terminal shoot buds by spraying with ethephon [(2-chloroethyl) phosphonic acid] (CHACKO, KOHLI, DORE SWAMY and RANDHAWA, 1976). This effect was nullified by simultaneous applications of gibberellic acid, which also prevented flowering in adult plants when applied at the normal period of flower bud initiation. Similarly, both delayed flowering and inhibition of flowering after gibberellin treatment has been reported for a number of fruit tree crops (HULL and LEWIS, 1959; CRANE, PRIMER and CAMPBELL, 1960; GRIGGS and IWAKIRI, 1961). GUTTRIDGE (1962) reported a severe reduction in flower bud initiation following

gibberellin applications. The distinction must be made between juvenility effects, such as reversion to juvenile morphology, and interference with the flowering of plants. Without concrete evidence that this response to gibberellins is related to the control mechanism of juvenility no deductions concerning related effects can be made. It is tempting though, to think of the flowering inhibition response as having a similar physiological basis as the lack of flowering in juvenile plants.

FRANK and RENNER (1956) observed the morphological reversion of *Hedera helix* to the juvenile form after applications of gibberellic acid. Further research by ROGLER and HACKETT (1975 a) showed that this reversion effect is specific for gibberellins as a class but non-specific for a particular form of gibberellin. Gibberellins A₁, A₃ and A₄/A₇ caused reversion while indoleacetic acid, abscisic acid and ethephon did not. *Hedera helix* has the capacity to undergo spontaneous reversion to the juvenile form when subjected to low light intensity. This reversion could be prevented by applications of abscisic acid, ancymidol (5 pyrimidine methyl alcohol) and B-995 which stabilized the mature apex (ROGLER and HACKETT, 1975 b). Simultaneous application of abscisic acid and gibberellic acid only caused reversion when levels of the latter compound were relatively high. This implies that the relative and not the absolute amounts of gibberellin and abscisic acid are important in controlling the growth form. Whereas the synthetic growth retardant B-995 does have the effect of interfering with gibberellin biosynthesis, abscisic acid may also be effective in inhibiting gibberellin effects, although neither the site nor the mechanism of gibberellin-abscisic acid interaction is known. These findings support the hypothesis that the adult morphological form can be stabilized by regulating the effective level of gibberellins in the plant.

The growth and development of *Humulus lupulus* can be enhanced with gibberellic acid. Treated plants had increased numbers and lengths of

internodes as well as a larger total leaf area, although leaves were very pale green (WILLIAMS, 1961). The rate of meristematic development was thought to have been accelerated by the gibberellin application, and to have resulted eventually in larger plants.

An alternative approach to the investigation of gibberellin involvement in juvenility phenomena, is to observe the effect of specific anti-gibberellins on plant growth. Although the attainment of the ability to flower is the first sign of the adult phase, the end of the juvenile phase and the first appearance of flowers may not coincide. A transition phase may exist when flowering might not be induced (ZIMMERMAN, 1972). The transition period may for practical purposes be regarded as part of the juvenile phase and any treatment which eliminates it may be valuable in reducing the non-productive juvenile period in fruit trees, for example. Some cultural practices, to be discussed later, are effective in shortening the juvenile period through eliminating the transition period, but it is the synthetic growth retardants which may be of practical value in this respect. BRUINSMA (1966) found that B-995 sprays retarded growth of five-year old apple seedlings which had not yet flowered. The year following treatment, five of 23 treated seedlings flowered, while one of 23 untreated did so. Antigibberellins are often used on floricultural crops to reduce lankiness of flower stalks, but this effect has seldom been tested in fruit tree crops. CCC (2-chloro-ethyltrimethylammonium chloride) was found to inhibit growth of vigorously growing *Prunus* L. seedlings to a greater extent than in less vigorous seedlings. The usefulness of this treatment is in the potential of dwarfing whole plants, since it was found that the stunting effect was transferred to scions when subsequent budding took place on the treated stocks (SURANYI, 1976).

Another aspect of juvenility with practical value, which appears to be hormone moderated, has been linked to endogenous auxin requirements. This

is the rooting of cuttings. DAVIES and JOINER (1980) claimed that the difference in adventitious rooting between juvenile and adult cuttings may be partially attributed to endogenous auxin levels, since lower indole butyric acid application levels were required for optimal rooting in juvenile cuttings of *Ficus pumila* L. Other factors, such as auxin/cytokinin and auxin/gibberellic acid ratios and cofactors and inhibitors which have not been investigated, may also be involved. There does appear to be some endogenous substance(s) which promotes rooting in juvenile but not adult tissues, as is shown by the following two experiments. HESS (1957) found that juvenile tissue of *Hedera helix* contained at least two substances separable by chromatography, which in the presence of IAA stimulated rooting of cuttings from epicotyls of mung bean seedlings. These substances were not present in adult tissues; they were thermostable and soluble in water, ethanol, methanol and ether. HACKETT (1970) reported slightly different findings. Methanolic extracts of adult and juvenile *Hedera helix* shoot tissue were found to promote rooting in juvenile shoot apices of *Hedera helix*, but they had no effect on the rooting of adult apices. This juvenility phenomenon, the ease of rooting in juvenile cuttings, is well defined and well founded but seems to bear little relation to the gibberellin mediated effects or to the shoot meristem differences, apart from undergoing parallel ontogenetic change as other features of juvenility.

A series of publications by WAREING and FRYDMAN have established the importance of gibberellin in the juvenility phenomenon of *Hedera helix* (FRYDMAN and WAREING, 1973 a, b; 1974; WAREING and FRYDMAN, 1976). The observed morphological differences between the juvenile and adult shoots of *Hedera helix* and all other ontogenetically ageing plants, are clearly derived from differences in the structure and behaviour of their shoot apices. The endogenous gibberellin levels in apices and roots of juvenile

plants were found to be much higher than in adult plants. There were no significant differences in endogenous gibberellin levels of adult and juvenile leaves. Application of gibberellin to adult plants caused reversion to the juvenile phase. This effect of exogenous gibberellin suggests that the juvenile condition may be associated with high endogenous gibberellin levels. FRYDMAN and WAREING intimated that the prostrate ivy vines growing on the soil surface remain juvenile because of the proximity of nodal adventitious roots to shoot apices and because of the maintenance of high gibberellin levels in the apices. One of the conditions for phase change to the adult state may be the establishment of a certain distance between the roots and shoot apices, with an associated decline in endogenous gibberellin levels in shoot apices. As evidence for this hypothesis they noted that rooted cuttings of *Hedera helix* show a tendency to produce juvenile shoots from buds which arose below the soil and consequently close to the roots. When roots were excised from seedlings and cuttings of the juvenile vines the level of extractable gibberellins was reduced. This suggested that at least part of the gibberellins in shoot apices are supplied by the roots, which means that the higher endogenous gibberellin levels in juvenile apices may be a cause and not a result of the juvenile condition. WAREING and FRYDMAN formulated the hypothesis that once phase change had occurred, a stable condition is established in the cells of the meristem, which is not controlled by the prevailing gibberellin levels. Evidence is provided by the retention of adult characters in an adult rooted cutting. These experimental results and interpretations undoubtedly explain the gibberellin-juvility relationship to a great extent in *Hedera helix* but this may be too rigid a model for juvility in forest trees (LONGMAN, 1976) and also in fruit tree crops.

Reports in the literature indicate that a phenomenon very similar to the juvenile condition may occur in plant material taken as cuttings from older

stock plants which had been flowering for many years. This pseudo-juvenile phase intervenes annually in species such as *Ribes nigrum* L. which does normally flower annually but where flower bud formation cannot be induced in leaf axils of shoots less than about 20 nodes in length (SCHWABE, 1976). This failure to flower in cuttings or aerial shoots on mature plants represents a size effect very similar to that which supposedly operates in some instances of juvenility (LONGMAN and WAREING, 1959; ROBINSON and WAREING, 1969). SCHWABE and AL-DOORI (1973) established the following facts for flowering of *Ribes nigrum* cuttings:

1. Adult rooted shoots with less than 20 nodes cannot be induced to initiate flowers by short day treatment;
2. Aerial rooting on long adult cuttings prevents flower initiation;
3. Gibberellin applications prevent flowering in long adult cuttings;
4. Extractions reveal the presence of gibberellin activity in roots and lower parts of stem, while none could be detected in tops of long shoots and
5. Abscisic acid and CCC application gave some promotion of flowering in long shoots under inductive conditions but not in short shoots. They traced the root effect to gibberellin-like activity coming from roots and travelling upwards, apparently for only a limited distance. They claim to have established that the very proximity of the tip of the shoots to the roots controls whether the morphogenetic changeover to the initiation of floral primordia can be induced or not. They further suggest that the correlation between shoot length and gibberellin activity may be decisive in determining pseudo-juvenility in *Ribes* and possibly in other species.

Both the work of WAREING and FRYDMAN (1973 a, b; 1974) and of SCHWABE and AL-DOORI (1973) implicate root supplied gibberellins in phase change, but both pairs of researchers throw no light on the fact of the stability of the adult condition as embodied in the shoot apex.

The controversy over effective translocatable factors influencing phase change has not been resolved, mainly because there is evidence both for and against the existence of such a substance or substances. Studies of reciprocal grafting of juvenile and adult tissues have been made in order to determine whether a mobile stimulus or inhibitor is involved in controlling phase change (CLARK and HACKETT, 1980). Extensive experiments with *Citrus* indicate that grafting does not have any significant effect on inducing phase change. These will be discussed later. No hastening of flowering ability was obtained by grafting juvenile seedlings onto adult seedlings, even though the older seedlings continued to form flower buds after grafting onto young seedlings (ZIMMERMAN, 1971). It appears that the age of the juvenile seedling is important in these grafting experiments. The transition phase (ZIMMERMAN, 1972) might be eliminated by grafting juvenile scions of Japanese and European larch onto adult rootstocks only if the juvenile plants were approaching the adult condition (ROBINSON and WAREING, 1969). With apples there is evidence that grafting onto a dwarfing rootstock does shorten the juvenile period by one to three years (ZIMMERMAN, 1972). On the other hand VISSER (1973) claims that under optimal conditions an apple seedling on its own roots may flower as soon as, for example, when grafted onto the dwarfing M IX rootstock. He is of the opinion that the grafting or budding operation checks growth and delays flowering. WAY (1971) reported similar findings in apple to those reported above by ROBINSON and WAREING (1969). He stated that the greatest reduction in juvenile period was obtained with the greatest age of juvenile scions at grafting time. MAURER (1957) provides important evidence for the existence of a translocatable influence. Apple seedlings were approach grafted to the trunks of 10-year old trees. By three years after grafting 11 percent of grafted trees had flowered while none of the original seedlings had. Aspen seedlings were induced to early flowering by grafting into the crowns

of precocious types (HEIMBURGER, 1958), thus providing evidence that a translocatable factor may exist and that precocity is a heritable factor.

There is overwhelming evidence for the fact that a rapid growth rate of seedlings will result in a shorter juvenile period than in a restricted or inhibited one (DOORENBOS, 1955; LONGMAN and WAREING, 1959; WAREING, 1961; VISSER and DE VRIES, 1970; SAURE, 1970). The experiments of TYDEMAN (1937) seem to indicate that these conditions for rapid growth might apply only to shoot growth. He found that apple seedlings with roots restricted in pots flowered more quickly than seedlings of the same age growing in the open.

Juvenility in *Citrus*

As far as the juvenile and adult stages of growth are concerned, the situation in *Citrus* appears to be essentially similar to that of a number of other kinds of woody plants (ROBBINS, 1957). Juvenile characters in *Citrus* are prominent and may exist for a long time. They include thorniness, a vigorous and upright habit of growth, slowness to fruit, alternate bearing in early years and physiological differences in fruit character (CAMERON and FROST, 1968). These authors imply that the existence of clear juvenile characters (e.g. thorniness) and the evidence for associated, slower-changing characters (e.g. flowering and fruiting developments), make it conceivable that continuous physiological ageing, as opposed to an abrupt change, can take place. Alternately, they suggest that the transition phase from juvenile to adult is extended. Extensive evidence exists for a gradual change of phase in woody plants, during which they become more and more susceptible to induction of flower initiation (FURR, COOPER and REECE, 1947; PASSECKER, 1949; PHARIS and MORF, 1967; WAY, 1971).

There is good reason for selecting *Citrus* or *Mangifera* species for a comparative study of juvenile and adult phases in woody plants, as both genera

have cultivars exhibiting various degrees of nucellar embryony (FROST, 1938 ; HODGSON and CAMERON, 1938; CAMERON, SOOST and FROST, 1959; CHACKO, KOHLI, DORE SWAMY and RANDHAWA, 1976). FROST and SOOST (1968) estimated that in *Citrus sinensis*, cultivar Valencia, 85 percent of embryos are of nucellar origin. This means that it is possible to make direct comparisons of juvenile and adult characteristics of such nucellar, juvenile and parental, old line plants with a reasonable degree of accuracy and without having to account for extraneous variability due to genetic diversity. Developing nucellar embryos recapitulate the embryonic and juvenile morphology of zygotic embryos (SWINGLE, 1943). There is no reason to believe that in nucellar seedlings the juvenile period is different from that of the original zygotic seedlings from which the cultivar originated (FURR, COOPER and REECE, 1947). Comparisons with zygotic seedlings from the same seed revealed no differences either. SWINGLE (1943) found that thorniness may be just as great in the nucellar seedlings as in zygotic seedlings with which they are associated. Considered from the point of view of the topophysis phenomenon, nucellar embryos could be regarded as a type of vegetative reproduction analogous to a bud or cutting from a juvenile seedling (ROBBINS, 1957).

Although nucellar embryony has value in the study of juvenility and the study of juvenility may be useful for plant breeders, nucellar embryony is of little direct benefit in evaluating crosses. It is sometimes only possible to distinguish hybrid (zygotic) seedlings from nucellar seedlings after fruiting (FURR, COOPER and REECE, 1947). This problem adds further justification to the study of ageing in plants. Nucellar embryony has been used extensively in variety improvement programmes. Nucellar selections, which are true-to-type, have been found to be more healthy, more vigorous, longer-lived and higher yielding even though problems associated with juvenile characteristics still remain in some cultivars

(CAMERON and FROST, 1968). MARLOTH, BASSON and BREDELL (1964) found that over the initial eight-year period of fruiting, nucellar Olinda and nucellar Frost Valencia selections averaged greater yields than the old budline. However, both cultivars showed marked alternation in bearing initially and low total soluble solids. In evaluating Washington Navel orange cultivars, it was found that up to the fourteenth year from planting, the nucellar selections were among the highest yielders and produced some of the biggest trees (GARDNER and REECE, 1960). It is uncertain whether any of the more persistent differences between nucellar *Citrus* selections and their seed parents reflect a physiological ageing in the parent clones beyond that associated with sexual maturity (CAMERON and FROST, 1968). Nevertheless, worldwide use is made of nucellar selections in an effort to improve local citrus industries. By 1968 most citrus propagated in California and increasing amounts in South Africa, Central and South America and various Mediterranean countries was of nucellar selections (CAMERON and FROST, 1968).

Comparisons of nucellar selections and their parental old budlines are most often performed after grafting onto uniform seedling rootstocks as in commercial citrus growing. OLSON, WUTSCHER and SCHULL (1969) and MAXWELL, BURNS and WUTSCHER (1973) found that the initial deficit in yield of young, nucellar grapefruit selections, compared with the parental budline, was made up within ten years from budding. They concluded that juvenility limits production in the early years of trees and that phase change is, therefore, not an on/off mechanism, but persists for many years after flowering is possible, in nucellar trees. CAMERON and SOOST (1952) found that not only did 90 percent of budded *Citrus* nucellar seedling lines yield more, but that their growth was 33 - 108 percent in excess of the parental, old lines. Growth was assessed as average cross-sectional area of the trunk. Using the same parameter of growth, FROST (1952) found

that although growth of young, nucellar seedling selections was greater after three years from budding, the tendency to flower was much lower. Many differences also exist in fruit characters and yield in newly matured nucellar selections compared with old lines. These differences do subsequently become modified as seedling selections age, so that the characters eventually resemble those of the parental old line (CAMERON and FROST, 1968).

It has been suggested that a possible reason for the retarded growth of old budlines is virus infection (CALAVAN and WEATHERS, 1959; TUBBS, 1973 a, b). The expression or effect of virus infections is subject to many variables, the most important being scion or rootstock susceptibility and climatic conditions, which may enhance or reduce symptoms of infection (ROISTACHER, 1976). ALLEN and HILGEMAN (1972) tested the effect of inoculating old line Campbell Valencia, budded on Sour orange, with tristeza virus. Three years after inoculation tree mass was 58 percent lower than in uninfected control trees. It had been reported previously, however, that Sour orange is highly susceptible to decline caused by tristeza virus (WEBBER, 1943). Thus, it is obvious that changes due to age are difficult to analyse in view of the number and variability of virus symptoms in *Citrus* (CAMERON and FROST, 1968).

Many of the original concepts of the juvenility phenomenon were first reported after work was conducted on *Citrus* plants (SWINGLE, 1932; FROST, 1938; HODGSON and CAMERON, 1938). Differential ageing of proximal and distal portions of a plant was reported by FROST (1943). He found that the trunk of a thorny *Citrus* seedling and the proximal portion of its main branches retain for a long time the ability to produce thorny shoots. At the same time shoots from the uppermost branches show a tendency towards progressive reduction in thorniness and an increase in propensity for flowering. The juvenile nature of the basal portion of flowering seedling trees was also demonstrated by CAMERON, SOOST and FROST (1959) in nucellar

Lisbon lemon and by FURR (1961) in grapefruit. A lower percentage of early flowering was recorded in trees propagated from lower parts of these trees as compared with propagation from the upper parts.

Normally *Citrus* seedlings flower for the first time at five to ten years of age. *Citrus sinensis* (L.) Osbeck, the sweet orange, for example, initiates the first flowers when grown on its own roots after six to seven years (FURR, COOPER and REECE, 1947). Anomalous flowering does sometimes occur in very young, juvenile *Citrus paradisi* Macf. after it has been subjected to normal dormancy. A single terminal flower is borne on the main axis of the plant. Thereafter vegetative growth continues until the normal juvenile period has passed (FURR, COOPER and REECE, 1947). It was subsequently confirmed that flower induction in one-year-old grapefruit seedlings appears to be favoured by low temperature situations, although these same conditions did not induce flowering in older seedlings (HIELD, COGGINS and LEWIS, 1966). These one-year-old seedlings only flowered if six or more nodes were present on the seedlings at the time of dormancy induction. With the current state of knowledge of the juvenility phenomenon, no reasonable explanation may be offered for such early flowering of grapefruit seedlings.

Methods to accelerate or delay flowering could be of considerable advantage in *Citrus* breeding and commercial production. Treatments tested for promotion of early flowering in *Citrus* include ringing, heavy top or root pruning, confining root systems in small pots, stunting the plant by withholding water and nutrients, frequent transplanting, artificially controlling photoperiod, subjecting plants to low temperature, grafting onto dwarfing stocks and inarching seedlings to adult plants. FURR, COOPER and REECE (1947) found that the effects of these different treatments were somewhat conflicting. They were concerned particularly with the inability of juvenile plants to flower and designed their grafting and

ringing experiments to test two assumptions: Firstly, that leaves may produce some substances necessary for flower formation, but leaves of juvenile plants may not normally produce these and, secondly, that these flower forming substances can move across the graft union from adult to juvenile plant or scion. Three important effects were reported in these attempts to manipulate plant development. Firstly, it was found that the only treatment to hasten flowering in juvenile seedlings was ringing. Secondly, this ringing was found to be less effective in three-year-old than in seven-year-old seedlings and, thirdly, that seedlings inarched into adult trees grew even more vigorously. No early flowering was obtained from grafting juvenile buds into the crown of adult trees, nor from subjecting plants to severe drought and nutrient deficiency over a trial period of four years. In essence the experiments performed by WAY (1971) on apples, indicated that these evergreen and deciduous species are subject to the same ontogenetic control mechanism. TUBBS (1973 a, b) mentioned the difficulty of evaluating the interactions between rootstocks and scions of different physiological age, but he attached some importance to the scion's dependence on and response to the input received from the rootstock. This dependence may be illustrated clearly in the use of dwarfing stocks in *Citrus* for hastening bearing of cultivars worked on them (SWINGLE, 1943).

The mere fact that grafting and especially girdling was found to have an effect on phase change, prompted some researchers to investigate translocatable factors which may be involved. FURR, COOPER and REECE (1947) mentioned that there is extensive evidence that leaves are essential for flower initiation. As has been mentioned earlier, work with *Hedera helix* implicates leaf translocated factors in affecting the ontogenetic expression of the plant. The most obvious unexplored field deserving of attention at this stage, with regard to a possible regulation by a

translocatable factor is endogenous plant growth regulators or hormones. As with other plants, gibberellin-like substances appear to play a prominent role in regulating both morphological and reproductive ontogenetical differences. It was claimed that increased seedling growth was obtained in *Citrus* seedlings after applications of gibberellic acid (MARTH, AUDIA and MITCHELL, 1956; RANDHAWA and SINGH, 1959; NISHIURA and IBA, 1964). On the other hand, gibberellic acid was found to break natural quiescence in mature *Citrus* trees and to initiate shoot growth during periods between spring, summer and winter growth flushes, without improving the total seasonal growth as compared with untreated trees (COOPER and PEYNADO, 1958). They reported that these hormone applications caused treated shoots to develop long thorns and abnormally narrow leaves, the former trait being similar to those found on juvenile shoots. Watershoots arising from the basal part of plants were found to contain fairly high levels of gibberellin A₇ (KAWARADA and SUMIKI, 1959), thus providing some evidence of natural gibberellin differences in juvenile and adult shoots.

The work of MONSELISE and HAYLEVY (1962, 1964) indicates that although gibberellins may be involved in certain aspects of physiological differences between adult and juvenile *Citrus* plants, there are many differences which remain unaltered by artificial regulation of gibberellin levels. Six-month-old Sweet lime seedlings sprayed with gibberellic acid showed progressively increased shoot and internode length, no difference in number of leaves and a decrease in leaf area (MONSELISE and HALEVY, 1962). The dry mass of shoots from treated plants was higher, while the dry mass of leaves and roots was lower and the dry mass of the whole plant in relation to leaf area or leaf mass was higher. This suggests that the gibberellin caused a change in relative sink strength and also increased the synthetic efficiency of leaves, respectively. Gibberellic acid applications also resulted in a decreased chlorophyll content of leaves. In subsequent

experiments it was shown that gibberellic acid applications also inhibited flowering of orange trees (MONSELISE and HAYLEVY, 1964). COGGINS and HIELD (1968) also reported a delay in flowering of lemon trees following gibberellic acid treatments. Whether these effects of gibberellins are related to alternate bearing in young nucellar selections and other juvenility phenomena in *Citrus* and other woody plants remains to be investigated.

The long delay before flowering is initiated in *Citrus* means that extensive tracts of land are required for long periods of time when seedlings are to be examined or evaluated for some specific purpose, during their growth. Research into early flowering and the vegetative vigour of juvenile *Citrus* plants would, therefore, be of benefit to breeding programmes and to early commercial production.

The locus of juvenility

Ultimately, after the current fund of knowledge concerning juvenility phenomena has been assimilated and once the various theories of cause and effect have been considered, a number of possible theories may be adopted and followed in the process of delving into this frontier of studies in plant growth and development. On one score only does there appear to be general consensus: juvenile or adult tissues emerge from and are therefore controlled by the shoot apical meristem (ROBBINS, 1957; DOORENBOS, 1965; PIERIK, 1967; ROBINSON and WAREING, 1969; BORCHERT, 1976). This immediately leads to the question of whether a meristem is a responding or a directing region (ROMBERGER, 1976). DE MUCKADELL (1954) proposed that the maturity gradient up a tree may be attributed to progressive ageing of the meristem as it grows. Subsequently, however, he proposed that the description of the passage from the juvenile to the adult condition as a function of the overall growth of the meristem does not make it easier to understand how or why some of the differences do exist (DE MUCKADELL, 1959).

It does not explain the establishment of juvenility in seeds, nor that plant size or translocatable factors may be involved, nor the topophysis or inherent persistence phenomenon, nor the sudden transition to the ability to induce flowering, as well as many other differences which are not understood.

The juvenile phase begins with the germination of seed, but SWINGLE (1932) proposed that it was a persistent effect of special conditions occurring during the development of the embryo, which initiates juvenile characters in seedlings. The embryo-sac experience stimulates a rejuvenescence in the developing ovary, with the further possibility of a chemical stimulation to the juvenile condition (LEOPOLD and KRIEDEMANN, 1975). Thus the meristem-ageing concept of DE MUCKADELL (1954) may represent a gradual dilution of a juvenility stimulus introduced at the embryo stage of development. The embryo-sac experience was absent from the embryoids of *Citrus* developed in culture from single cells by BUTTON (1978). He found that such embryoids gave rise to thornless seedlings, a development which may confirm SWINGLE's theory, in that the shoot apices of such seedlings may not have been rejuvenated. So, while some may argue that phase change involves the accumulation of differentiated tissue and a concomitant increase in complexity, the stability when grafted, of the extant phase of a particular apex, indicates that the locus of gradual change in the plant is the apex. This implies that the change in behaviour of the apical meristem is not occasioned by change in external conditions, but must be brought about by some endogenous mechanism (WAREING, 1959). SUSSEX (1976) also questioned whether there might be some external signal to the meristem to initiate the developmental phase change response or whether the meristem behaves independently of the remainder of the plant. He postulated the existence of a time-measuring system within the cell or the organ that is used to specify the time of occurrence of phase change. Both BRINK (1962)

and ROBINSON and WAREING (1969) suggested that this developmental time-measuring system is associated with the cell cycle and that in some way the cell can maintain information on the number of divisions it has undergone. SUSSEX (1976) believes that phase change, like all other differentiatonal events, must ultimately be specified in terms of gene activity and further that any model based on gene mutation or irreversible gene inactivation can be discounted. Thus the only differentiatonal mechanisms permitted within the meristem are those that involve selective gene activity. Scant evidence is provided for change being brought about by a gradual change in the genetic programme in the meristem, however. Examination of the nucleic acid levels in these tissues revealed that higher levels of DNA may be found in the nuclei of adult bud and leaf cells (SCHAFFNER and NAGL, 1979).

Applications of gibberellins to plants can induce phase change, either through reversion (ROGLER and DAHMUS, 1974) or through maturation (PHARIS and MORF, 1967). Gibberellins have been shown to lead to the initiation of gene activity and abscisic acid has been shown to block this activation (VARNER, 1964). Hormones are also known to cause changes in the rate of RNA synthesis (SUSSEX, CLUTTER and WALBOT, 1975). Thus, hormones are candidates for the activators of the genetic mechanism of phase change. Endogenous levels of the gibberellin-like hormones have been found generally to be higher in juvenile shoot apices and this may be significant, but it is still not known whether the differences are a cause or effect of phase change (WAREING and FRYDMAN, 1976). Once phase change has occurred, a stable condition is established in the cells of the meristem which is not directly controlled by the prevailing gibberellin levels, so that even a rooted adult cutting is able to retain adult characters. In supporting the hypothesis that higher gibberellin levels result from the juvenile condition, LUCKWILL (1970) suggested that the rapid growth characteristics of juvenile trees bring about a high level of endogenous gibberellin. It appears that

meristematic cells of adult shoot apices become intrinsically different in their responses from juvenile apices as a result of phase change, but whether these differences include changes in gibberellin synthesis and metabolism remains to be established (FRYDMAN and WAREING, 1974). From the experimental and observational evidence available, it appears that both hormonal and nutritional factors can be involved in the control of phase change, but that a specific juvenile or ageing hormone is not involved (HACKETT, 1976).

Discussion

The study of juvenility in plants has become very broad and may now encompass flowering, growth and development, anatomy, histochemistry, ecology, biochemistry and many other aspects. When the state of knowledge on the subject is assimilated, the most obvious question to ask is whether or not all the different features associated with the juvenility phenomenon are linked genetically or physiologically. There is no evidence to link all of these features to a central controlling mechanism and doubts have been expressed over the validity of the concept of juvenility (BORCHERT, 1976). In fact, LONGMAN (1976) proposed that evidence is accumulating which suggests that many forest trees are simply more 'reluctant' to flower during the early years of life, rather than being incapable of so doing. In spite of the debate which may exist over definitions and concepts of juvenility in herbs and woody plants, there is a usefulness for the researcher in accepting that a strict definition of juvenility is neither feasible nor practical. Plants do, nevertheless, behave differently when young and old and these differences may be exploited in the form of providing suitable material for the investigation of plant growth and development. Much has been written on the phase change in *Hedera helix*. Woody species such as fruit trees have been the subject of some investigations but they are deserving of further research in this field.

CHAPTER II

MATERIALS AND METHODS

In order to make comparisons of the physiological properties of plants in relation to ontogenetical ageing, all differences other than age should be eliminated or reduced to a minimum. Such differences may be brought about by genetic inconsistency, variation in gross morphology and by the size of the organism, by differences in disease status and by dissimilar environments in which the plants are growing. In the following account of materials and methods employed in this study, the prime requirement for uniformity (except of plant age) in such a comparative study has been borne in mind throughout.

The evergreen *Citrus sinensis* (L.) Osbeck, cultivar Pickstone Valencia orange which is of subtropical origin (CHAPOT, 1975), was used to provide the adult and juvenile scion material in these experiments. Eighty-five percent of embryos produced by this cultivar are of nucellar origin (FROST and SOOST, 1968). This means that there is a reasonable degree of certainty that seedling selections are true to parental type, as zygotic seedlings for the most part are weaker in genetic constitution than the corresponding nucellar seedlings (FROST and SOOST, 1968). The probability of variation being due to genotypic differences is therefore assumed to be slight. The subtropical origin of the genus meant that the ambient environment of Pietermaritzburg (30°E 30°S) was adequate for growing the plants when a glasshouse was not being used.

Plant establishment and maintenance

Germinated Rough lemon (*Citrus limon* Burm.) seedlings were selected for uniformity and transplanted to 10 l black plastic bags from seedling trays. The potting medium consisted of sand: sawdust: peat moss (2:1:1 v/v). The following fertilizers were incorporated into the soil mix: 1,44 kg m⁻³ single super phosphate; 2,23 kg m⁻³ dolomitic lime; 0,746 kg m⁻³ calcium carbonate;

84 g m⁻³ copper sulphate; 27 g m⁻³ zinc sulphate; 27 g m⁻³ manganous sulphate; 46 g m⁻³ ferrous sulphate; 0,8 g m⁻³ boric acid and 0,4 g m⁻³ ammonium molybdate. The ingredients were essentially the same as those recommended by NAUER, ROISTACHER and LABANAUSKAS (1968) for *Citrus* propagation. In order to promote vegetative growth, the plants were kept in a glasshouse under a 16 h daylength and a 30⁰/25⁰C diurnal temperature regime (REUTHER, 1978). Supplementary lighting was provided by five 300 Watt Hg-vapour lamps suspended two metres above the plants.

The scion material was obtained from two sources. The original adult budwood was obtained from trees which had been vegetatively propagated for over 50 years (POHL, 1979). Juvenile budwood was obtained from one-year-old seedlings. Seed for these plants was obtained from fruits of the above adult plants. It was found that the adult bud source plants showed positive symptoms of tristeza virus infection when indexed with West Indian lime (*Citrus aurantiifolia* Swing.), while the juvenile bud source plants did not. In order to eliminate tristeza and other viruses and viroids which may have been present, both adult and juvenile plants were subjected to a shoot-tip grafting procedure (MURASHIGE, BITTERS, RANGAN, ROISTACHER and HOLLIDAY, 1972; NAVARRO, ROISTACHER and MURASHIGE, 1975), which may be up to 98 percent successful in providing disease-free material. Defoliation of *Citrus* plants results in bud sprouting along the stem (DE LANGE, 1980). The small shoots which arose from sprouting buds on defoliated plants were used for the shoot-tip grafting procedure. They were sterilized by placing them in a 0,3 percent solution of sodium hypochlorite to which a drop of TWEEN 20 (polyoxyethylene sorbitan mono-laurate) had been added as a surfactant, for ten minutes. The following steps of the procedure were performed under sterile conditions on a laminar-flow bench. The shoots were rinsed with autoclaved, distilled water and sections including only the apex plus two leaf primordia were dissected from the shoot-tip and placed onto two week old decapitated Troyer citrange

(*Poncirus trifoliata* (L.) Raf. X *Citrus sinensis* (L.) Osbeck) etiolated seedlings grown on Murashige and Skoog medium (Table 2.1) to which no hormones had been added. This rootstock cultivar was selected because any adventitious shoots arising from the stem would be distinguishable from those of the implanted Pickstone Valencia bud by the trifoliate (as opposed to the monofoliate nature of leaves produced by these two cultivars, respectively). Grafted plantlets were transferred to liquid Murashige and Skoog medium (Table 2.1) where they were supported by filter paper bridges. After about fourteen days, two or three leaves had developed from the implanted apex. The plantlets were decapitated with a slanting cut for the purpose of grafting these small shoots onto well established rootstocks. This procedure was recommended by DE LANGE (1978) for shortening the period before buds could be collected from shoot flushes of virus-free plants and for increasing the percentage of survival by eliminating the critical stage of establishing seedlings in soil medium from the tissue culture conditions. A number of plants were budded with shoots which had been successfully shoot-tip grafted. These were grown until at least one shoot flush had developed fully. The plants were all placed in a controlled environment cabinet (Controlled Environments, London, England) for 28 days at high temperatures as recommended by NYLAND and GOHEEN (1969). They found that heat therapy, consisting of 40°C constant for 28 days, would render unviable those heat labile infectious microorganisms such as citrus greening, infectious variegation and tristeza virus. These heat treatments have two disadvantages: Firstly, plants may not survive such treatment. A number of plants did in fact wilt and eventually become totally necrotic. Secondly, plants can undergo mutations which may not be immediately apparent (DE LANGE, 1980).

Of the plants which did survive, the most healthy juvenile and adult plants were selected and used as a source of buds for budding onto large and vigorous Rough lemon rootstocks. Two rootstocks each were used for adult and juvenile

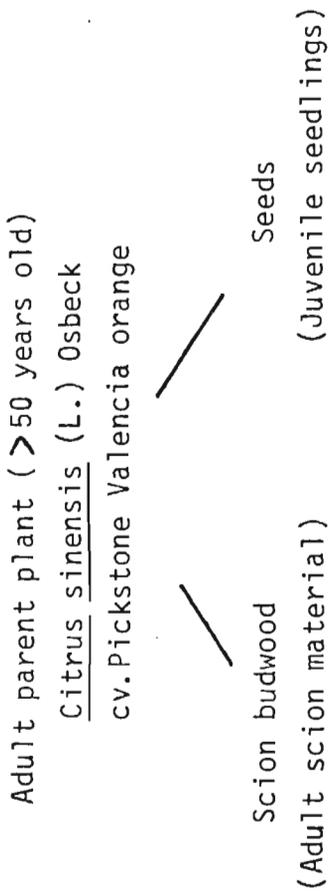
Table 2.1 Murashige and Skoog basic medium (adapted from HARTMANN and KESTER, 1975) which was used as a gel-type medium with agar or in the liquid state without agar, in the shoot-tip grafting procedure

| Chemical | mg l ⁻¹ culture medium | Chemical | mg l ⁻¹ culture medium |
|--|--------------------------------------|--------------------------------------|--------------------------------------|
| NH ₄ NO ₃ | 400 | ZnSO ₄ .7H ₂ O | 2,7 |
| Ca(NO ₃).4H ₂ O | 144 | KI | 0,75 |
| KNO ₃ | 80 | thiamin | 0,1 |
| KH ₂ PO ₄ | 12,5 | nicotinic acid | 0,5 |
| MgSO ₄ .7H ₂ O | 72 | pyridoxine | 0,5 |
| KCl | 65 | glycine | 2,0 |
| NaFeEDTA | 25 | myo-inositol | 100 |
| H ₃ BO ₃ | 1,6 | Casein hydrolysate | 1 000 |
| MnSO ₄ .4H ₂ O | 6,5 | sucrose | 20 |

optional: agar 1%

budding. At this stage the possibility still existed that tristeza virus or some other unidentified micro-organism might be present in the tissue of either juvenile or adult plants. Since most of these infectious diseases may be transmitted by grafting, any difference in disease status was eli-

Outline of generalised procedure for the generation of juvenile- and adult-budded experimental plants



they were sprayed fortnightly with

Table 2.2 Hoaglands nutrient solution which was used to fertilize plants at weekly intervals (adapted from BONNER and GALSTON, 1967)

| Stock solution | Chemical | g l ⁻¹ stock solution | ml stock solution per l medium |
|----------------|---|----------------------------------|--------------------------------|
| Stock 1 | Ca(NO ₃) ₂ | 236 | 5 |
| Stock 2 | KNO ₃ | 101 | 5 |
| Stock 3 | MgSO ₄ | 246,5 | 2 |
| Stock 4 | KH ₂ PO ₄ | 136 | 1 |
| Stock 5 | NaFeEDTA | 5,6 | 1 |
| Trace Element | H ₃ BO ₃ | 2,86 | 1 |
| | MnCl ₂ ·4H ₂ O | 1,81 | |
| | ZnSO ₄ ·7H ₂ O | 0,22 | |
| | CuSO ₄ ·5H ₂ O | 0,08 | |
| | H ₂ MoO ₄ ·H ₂ O | 0,02 | |

precautions red spider mite (*Tetranychus cinnabarinus* Boisd.) infestation occurred and orange-dog caterpillars (*Papilio thoas thoas* L.) attacked the leaves. Infected plants were rejected and only used after new growth flushes had matured and replaced damaged tissues.

Valencia orange trees normally produce three growth flushes during the growing season in South Africa (GILFILLAN, 1977). Although generative buds may and do arise following the winter dormant period the latter two flushes are usually entirely vegetative. Since hormone concentrations and ratios are known to change dynamically during dormancy, quiescence and growth changes (ALTMAN and GOREN, 1974; WAREING and PHILLIPS, 1978), it was necessary to synchronize the growth/quiescence cycles of all plants in order to carry out simultaneous sampling for hormone comparisons of plant organs from all plants. Both drought and cold, together or separately, are known to induce dormancy in *Citrus* species (REUTHER, 1977). Attempts to effect controlled drought were not successful, whereas dormancy was readily induced by manipulating temperatures.

Temperatures of 13°C and below are sufficient to induce cessation of shoot extension growth in *Citrus* (REUTHER, 1977). Plants were, therefore, placed in controlled environment cabinets for 28 days and held at a 13°/9°C (day/night) diurnal temperature regime with an eight-hour photoperiod of 7,1 $\mu\text{W nm}^{-2}$. Light measurements were recorded with a plant growth photometer (Model IL 150; International Light Inc., Newburyport, Massachusetts). Relative humidity was maintained at 60/40 percent for day and night conditions, respectively. When plants were removed from these conditions they were once again placed in the glasshouse under the conditions specified above (30°/20°C diurnal temperature cycle; 16 h daylength). Weekly irrigation and fertilizer practices were continued. A fairly well synchronized flush grew and reached maturity in about eight weeks after removal of plants from the cold conditions.

The distinction must be made between quiescent and dormant plants. Between the production of growth flushes during the growing season, stem tissues are regarded as being in the quiescent state. This means that there is no active production of new stem and leaf material from shoot apices. While quiescent, plants may exhibit low metabolic rates and no active differentiation of new shoot material. Dormancy is most often induced in subtropical plants by adverse climatic conditions such as cold or dry winter conditions. The apical buds of sprouting or flushing plants are in a phase of active cell division and differentiation and the shoot tissues are in a state of active metabolism. Although the impact of these different phases of growth on the hormone status of specific plant organs is not fully understood at present, it is important to note such details for the plant material which is being analysed.

Plants were selected randomly according to the number required for all experiments. Sampling in some cases, as for example for buds, left the plants intact. In these cases, the plants continued to grow normally and produced shoot growth flushes. They were subsequently used for further experimentation. The timing and method of sampling will be described individually for each experiment.

Hormone analyses

Gibberellins were extracted and partitioned according to the method described by REEVE and CROZIER (1978), with small alterations. Gibberellins were extracted from the *Citrus* tissues by homogenizing in a Waring blender with 80 percent ethanol (five millilitres per gramme fresh mass of material). Bud samples, which consisted of a very small mass of material, were macerated in a small glass mortar and pestle with 80 percent ethanol (a minimum of 50 millilitres was used per sample). The homogenate was allowed to stand for 24 hours at 10°C. The extract was filtered *in vacuo* through Whatmans No. 1

filter paper disks and the filtrate was reduced to the aqueous phase *in vacuo* at 35°C. The aqueous residue was diluted at least two-fold with pH 8,0; 0,5 mole millilitre⁻¹ phosphate buffer (Table 2.3). The buffer phase volume was always made up to 100 millilitres. The buffer phase was first partitioned five times against half volumes of petroleum spirit (boiling point 60-80°C) and then purified further by slurrying with insoluble PVP (polyvinylpyrrolidone) before acidification to pH 2.5 with 1N hydrochloric acid and extraction with five times two-fifths volumes of redistilled ethyl acetate. GLENN, KUO, DURLEY and PHARIS (1972) suggested the use of PVP as a means of purifying plant extracts, especially of phenolic compounds. The partition coefficients at pH 2,5 are such that the bulk of free gibberellins will be removed by the ethyl acetate (DURLEY and PHARIS, 1972). Only gibberellin A₃₂ and the bulk of the conjugated gibberellins are retained by the aqueous buffer phase, although some conjugated gibberellins do migrate into the ethyl acetate phase. The remaining conjugated gibberellins and gibberellin A₃₂ were extracted from the acidified aqueous phase with *n*-butanol. The acidic ethyl acetate and acidic *n*-butanol soluble fractions were taken to dryness *in vacuo* at 35°C. The residue was taken up in 6 ml of methanol and separated chromatographically using half sheets of Whatmans No. 1 chromatography paper. The solvent used consisted of *iso*-propanol: 25% NH₄OH: H₂O (10:1:1 v/v).

Dried paper chromatograms were divided into ten equal R_f strips and each R_f strip was eluted separately with 10 ml of methanol. The methanolic eluates from the paper separations were then taken to dryness at room temperature in a stream of air. These dried fractions were either used for bioassay immediately or stored at -20°C until used in a bioassay.

The dwarf rice microdrop bioassay for gibberellins (MURAKAMI, 1968; 1970) was used with slight modifications. The dwarf cultivar Tan-ginbozu of *Oryza sativa* L. was used because it is particularly sensitive, responds to

Table 2.3 Phosphate buffer solutions used in the extraction of gibberellin-like extracts and in the wheat coleoptile bioassay for abscisic acid (adapted from MACHLIS and TORREY, 1959)

| Stock solution | g l^{-1} stock solution | ml stock solutions for pH 7,4 buffer | ml stock solutions for pH 8,0 buffer |
|---------------------------------|--|---|---|
| 0,07M KH_2PO_4 | 9,08 | 39 | 11 |
| 0,07M Na_2HPO_4 | 9,47 | 161 | 189 |

a wider spectrum of gibberellins than other cultivars and has no endogenous gibberellin-like activity (SUGE and MURAKAMI, 1968; MURAKAMI, 1972). Helminthosporol and some of its derivatives are the only non-gibberellins or non-gibberellin precursors known to be slightly active in this assay (KATO, KATSUMI, TAMURA and SAKURAI, 1968). The test is sensitive to 0,1-1000 ng gibberellin A₃ per rice seedling and produces a linear curve for a log dose-log response relationship in this range.

Seed of the Tan-ginbozu cultivar was kindly provided by Dr Y. Murakami (National Institute of Agricultural Sciences, Tsukuba, Japan). Excess seed was partially sterilized by soaking for ten minutes in a 0,3 percent sodium hypochlorite solution to which a drop of TWEEN 20 had been added as a surfactant. The seed was then thoroughly washed with tap water before being soaked in distilled water in a covered beaker for two days at 32^oC constant. By this time the coleoptile had emerged and germinated seeds were selected for uniformity. The repli-dishes described by EEUWENS and SCHWABE (1975) were filled with 0,9 percent Bacto agar (DIFCO Laboratories, Detroit, Michigan) which had been dissolved by autoclaving for 15 minutes at 105 kPa. Ten seeds were planted per R_f strip and five sets of standards in the range 0,1 to 1000 ng plant⁻¹ were included with each bioassay. Repli-dishes were placed in a tray which was sealed over with clear plastic so as to maintain high humidity. Seedlings were treated with test solution two days after planting. The dry extracts obtained from each R_f strip were taken up in 0,3 ml of 50 percent aqueous acetone. Two microlitres of this solution was deposited in the axil of the first leaf of each seedling using a microsyringe. Three days later the length of the second leaf sheath was measured.

Tissues were analysed for inhibitors in a further investigation of the hypothesis that differences in growth of juvenile and adult plants may be due to a difference in the ratio of gibberellins and inhibitors (FORTANIER and

JONKERS, 1976). The inherent problems of comparing only gibberellin:inhibitor ratios in plants were recognized and no great attempt was made to resolve the question of the importance of this balance of promoters and inhibitors in ontogenetical behaviour. (See, for example, CORCORAN, 1975; LETHAM, 1978; SAUNDERS, 1978a). Nevertheless, inhibitors were analysed in either crude extracts or those extracted specifically for abscisic acid. Purification of plant extracts was carried out according to the method of SAUNDERS (1978). Plant material was extracted for 24 hours at 10°C in 80 percent ethanol after maceration in a glass pestle and mortar. The extract was then filtered through Whatmans No. 1 filter paper *in vacuo* and reduced to the aqueous phase *in vacuo* at 35°C after adjusting to pH 8,0. The pH was then adjusted to 3,5 with 1N hydrochloric acid before partitioning three times against equal volumes of diethyl ether. Non-acidic substances were removed by partitioning three times against one third volumes of sodium hydrogen carbonate. The combined sodium hydrogen carbonate extract was adjusted to pH 3,5 with 1N hydrochloric acid and partitioned with three equal volumes of diethyl ether. The ether extracts were combined and dried *in vacuo* at 35°C. The residue was dissolved in five millilitres of methanol and applied to silica gel F₂₅₄ plates for thin layer chromatography. The solvent system used was toluene: ethyl acetate: acetic acid (40:5:2 v/v). Plates were developed to 150 mm in three successive runs. The plates were divided into ten equal R_f strips and each R_f strip was scraped off and eluted with six millilitres of methanol. The eluates were dried in repli dish compartments (EEUWENS and SCHWABE, 1975) which contained strips of Whatmans No. 1 chromatography paper.

The wheat coleoptile bioassay described by EEUWENS and SCHWABE (1975) was used to assess the level of abscisic acid in the extracts. Wheat caryopses (*Triticum vulgare* L. cultivar Scheepers) were thoroughly leached before being incubated in the dark on moist filter paper at 25°C for 20 hours. After this time the bulk of the endosperm was separated from the embryo by a slanting

cut. Embryos were then placed in the cubes of the repli-dishes which contained the dried extracts and chromatography paper strips. The paper had been moistened with two millilitres of 0,07 M phosphate buffer (pH 7,4; Table 2.3). Six embryos were placed in each cube and three cubes contained equal amounts of extract from a particular R_f strip. The length of the coleoptiles was measured after 48 hours of incubation in the dark at 25°C.

Although a fairly extensive investigation of cytokinins in juvenile and adult Pickstone Valencia orange has been undertaken (HENDRY, VAN STADEN and ALLAN, 1982 a, b), certain aspects of their involvement in growth differences were considered to be deserving of further investigation. This is particularly relevant when the disease status of plants used in the previous study is noted adult but not juvenile plants were infected with tristeza virus.

Cytokinins were extracted from the plant material by homogenizing in 80 percent ethanol in a glass pestle and mortar and allowing the homogenate to stand for 24 hours at 10°C. The homogenate was filtered *in vacuo* through Whatmans No. 1 filter paper and the filtrate was adjusted to pH 2,5 with 1N hydrochloric acid. The acidified extract was passed through Dowex 50W-X8 cation exchange resin (J.T. Baker Chemical Company, Phillipsburg, New Jersey; H⁺ form; 20-50 mesh; 25x250 mm columns). Twenty-five grammes of resin was used per extract and the extract was eluted through the resin at a flow rate of 100 millilitres per hour. The column was then washed with 100 millilitres of 80 percent ethanol, after which the cytokinins were eluted from the column with 100 millilitres of 5N ammonium hydroxide. The ammonia eluate was concentrated to dryness *in vacuo* at 40°C. The residue was either taken up in one millilitre of 10 percent methanol for column chromatography or in six millilitres of 80 percent ethanol for paper chromatography.

The technique of using cation exchange resins for the purification and recovery of cytokinins from ethanolic plant extracts has been employed extensively (ENGELBRECHT, 1971; HEWETT and WAREING, 1973; LORENZI, HORGAN

and WAREING, 1975; VAN STADEN 1976 a, b; WANG, THOMSON and HORGAN, 1977). Most of the known cytokinins may be recovered from acidified plant extracts when Dowex 50W-X8 cation exchange resin is used.

Where separation was to be achieved using paper chromatography, resuspended residues were strip-loaded onto a 10 mm band at the base of a sheet of Whatmans No. 1 chromatography paper. The chromatograms were run with *iso*-propanol: 25% NH₄OH: water (10:1:1 v/v). The solvent front descended about 0,3 m in nine hours. Chromatograms were then oven-dried for 24 hours at 25⁰C. Dried chromatograms were cut into ten equal R_f strips which were placed into 25 millilitre erlenmeyer flasks and assayed for cell division activity.

Column chromatography was used as an aid in the determination of the cytokinins present in adult and juvenile *Citrus* extracts. The technique used was based on that of ARMSTRONG, BURROWS, EVANS and SKOOG (1969) with the modifications of HUTTON and VAN STADEN (1981). The column (25x900 mm) was packed with Sephadex LH-20 which had been swollen with 10 percent aqueous methanol. It was eluted with 10 percent aqueous methanol at a flow rate of 15 millilitres per hour. Forty millilitre fractions were collected. Columns were kept at constant temperature (17⁰C) in incubators. The elution volumes of adenine, zeatin, ribosylzeatin, dihydrozeatin, *iso*-pentenyl adenine and *iso*-pentenyl adenosine were provided by HUTTON and VAN STADEN (1981). The fractions collected were dried in 25 ml erlenmeyer flasks under a stream of air at 40⁰C.

The fractions obtained as a result of paper and column chromatography were assayed for cytokinin activity using the soybean callus bioassay (MILLER, 1963; 1965). The soybean callus bioassay has been used extensively as the range of concentrations over which a linear response is obtained is greater in this assay than in the tobacco pith callus bioassay (FOX, 1969; SKOOG and ARMSTRONG, 1970). Furthermore, the cell division assays are considered

to be superior to the chlorophyll retention bioassays (KENDE, 1971). Callus was obtained from the cotyledons of *Glycine max* L., cultivar Acme, according to the procedure described by MILLER (1963; 1965) and was maintained by three-weekly subculture.

Four stock solutions were prepared and the nutrient medium was made up as outlined in Table 2.4. Fifteen millilitres of medium was added to each of the 25 millilitre flasks which contained 0,15 g of agar. The flasks were stoppered with non-absorbent cotton wool bungs, which were then covered with aluminium foil. The flasks were then autoclaved at a pressure of 105 kPa for 20 minutes, before being transferred to a sterile transfer-chamber, where they were left for about six hours under ultra-violet light. Three pieces of callus of approximately 20 mg were then placed on the basal medium. Thereafter the flasks were incubated in a growth room where conditions of constant temperature (27°C) and continuous low light intensity (cool white fluorescent tubes) were maintained. After 21 days the mass of the three pieces of callus in each flask was determined.

All extractions were duplicated and all results presented are averages of two bioassay determinations. In all three bioassays described above the growth response parameter was plotted relative to the control value. The significance limit at the 0,05 percent level was calculated and all regions significantly different from the control were indicated on the histograms by shading. Standards were included with each bioassay. In order to estimate gross levels of hormone activity in particular extracts, the response curve from the graded concentration series was used to interpolate equivalent hormone activity for the significant peaks of response. Results were expressed as gibberellin A₃- or kinetin-equivalents, respectively, depending on the bioassay in use.

Table 2.4 Basal Medium for Soybean Callus Bioassay
(adapted from Miller, 1963, 1965)

| Stock Solution | Chemical | g l ⁻¹ stock solution | ml stock solution per litre medium |
|------------------------------|--|-------------------------------------|------------------------------------|
| Stock 1 | KH ₂ PO ₄ | 3,0 | |
| | KNO ₃ | 10,0 | |
| | NH ₄ NO ₃ | 10,0 | |
| | Ca(NO ₃) ₂ ·4H ₂ O | 5,0 | 100 |
| | MgSO ₄ ·7H ₂ O | 0,715 | |
| | KCl | 0,65 | |
| | MnSO ₄ ·4H ₂ O | 0,14 | |
| Stock 2 | NaFeEDTA | 1,32 | |
| | ZnSO ₄ ·7H ₂ O | 0,38 | |
| | H ₃ BO ₃ | 0,16 | |
| | KI | 0,08 | 10 |
| | Cu(NO ₃) ₂ ·3H ₂ O | 0,035 | |
| | (NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O | 0,01 | |
| Stock 3 | myo-inositol | 10,0 | |
| | nicotinic acid | 0,2 | |
| | pyridoxine HCl | 0,08 | 10 |
| | thiamine HCl | 0,08 | |
| Stock 4 | NAA | 0,02 | 10 |
| Additional | Sucrose | | 30 g l ⁻¹ medium |
| | Agar | | 10 g l ⁻¹ medium |
| pH adjusted to 5,8 with NaOH | | | |

Column separation of gibberellins and abscisic acid

The most accurate and precise detection of hormones in plant extracts is only achieved when highly purified extracts are used. Sephadex LH-20 has been shown to be a useful tool for the separation of cytokinins (ARMSTRONG, BURROWS, EVANS and SKOOG, 1969; CARNES, BRENNER and ANDERSEN, 1975; HUTTON and VAN STADEN, 1981) and gibberellins (MACMILLAN and WELS, 1973). According to SLOMINSKI, REJOWSKI and NOWAK (1979) the gibberellin A₃ and abscisic acid components of partially purified plant extracts can be separated on a Sephadex LH-20 column. This method was modelled on that of STEEN and ELIASSON (1969) who claimed to be able to separate indole-3-acetic acid and abscisic acid by gel filtration on a Sephadex LH-20 column. SLOMINSKI, REJOWSKI and NOWAK (1979) eluted the column with 80 percent ethanol containing 10⁻⁴M hydrochloric acid at a flow rate of eight millilitres per hour. Two millilitre fractions were collected. No further details were provided concerning band spreading or elution volumes of the abscisic acid and gibberellin A₃.

The procedure had the potential for providing a direct comparison of gibberellin and abscisic acid levels in the same extract. A column (25x900 mm) was therefore packed with Sephadex LH-20 which had been swollen in 80 percent ethanol containing 10⁻⁴M hydrochloric acid. A mixture of authentic *cis/trans* abscisic acid and gibberellin A₃ was applied to the column. The column was eluted at 7,5 ml h⁻¹ and five millilitre fractions were collected. The fractions collected were analysed as follows: a) the presence of abscisic acid was tested for by recording ultra-violet (260nm) absorbance for each fraction; b) the wheat coleoptile bioassay was performed to test the fractions for the presence of abscisic acid and c) the dwarf rice microdrop bioassay was performed in order to test for gibberellin A₃ in each fraction. Twenty microlitres of each fraction were used for the gibberellin determination and the remainder was used for the abscisic acid detection.

When the column was operated under the conditions stipulated above, separation of the compounds was inadequate (Figure 2.1). The wide band spreading of the gibberellin A_3 and especially of the abscisic acid did not permit recovery of uncontaminated compounds. Several modifications were implemented in an attempt to increase the number of theoretical plates on the column so as to improve the separation of the compounds. The column length was doubled, the flow rate was decreased and the solvent ratios were altered. Sephadex G-10 was also tested. The best separation of abscisic acid and [^{14}C]-gibberellin A_3 which was finally achieved is shown in Figure 2.2. The column (25x900 mm) was eluted at a flow rate of 3 ml h^{-1} , two millilitre fractions were collected and the solvent was 100 percent ethanol which had been adjusted to pH 8,0. Elution volumes of abscisic acid were detected by ultra-violet (260nm) absorbance and gibberellin was detected by counting disintegrations of the isotope.

The procedure did not provide suitable separation of abscisic acid and gibberellin A_3 and was abandoned at this stage in favour of using separate extractions of plant material for abscisic acid and gibberellin determinations.

Microscopy techniques

Dormant and sprouting buds from adult and juvenile plants were collected and fixed at 4°C in six percent gluteraldehyde, buffered at pH 7,2 with 0,05 Molar sodium cacodylate, for 12 hours. Although only the apices and surrounding tissues were of interest in this study, it was found to be simpler to handle whole buds and not to dissect out the very small shoot apices for fixation. The fixed material was washed three times for periods of 30 minutes each in 0,05 M sodium cacodylate buffer. It was then post-fixed in two percent osmium tetroxide, buffered at pH 7,2 with 0,05 M cacodylate. After four hours of fixation it was washed three times in 0,05 M cacodylate buffer. The material was dehydrated in an alcohol graded series,

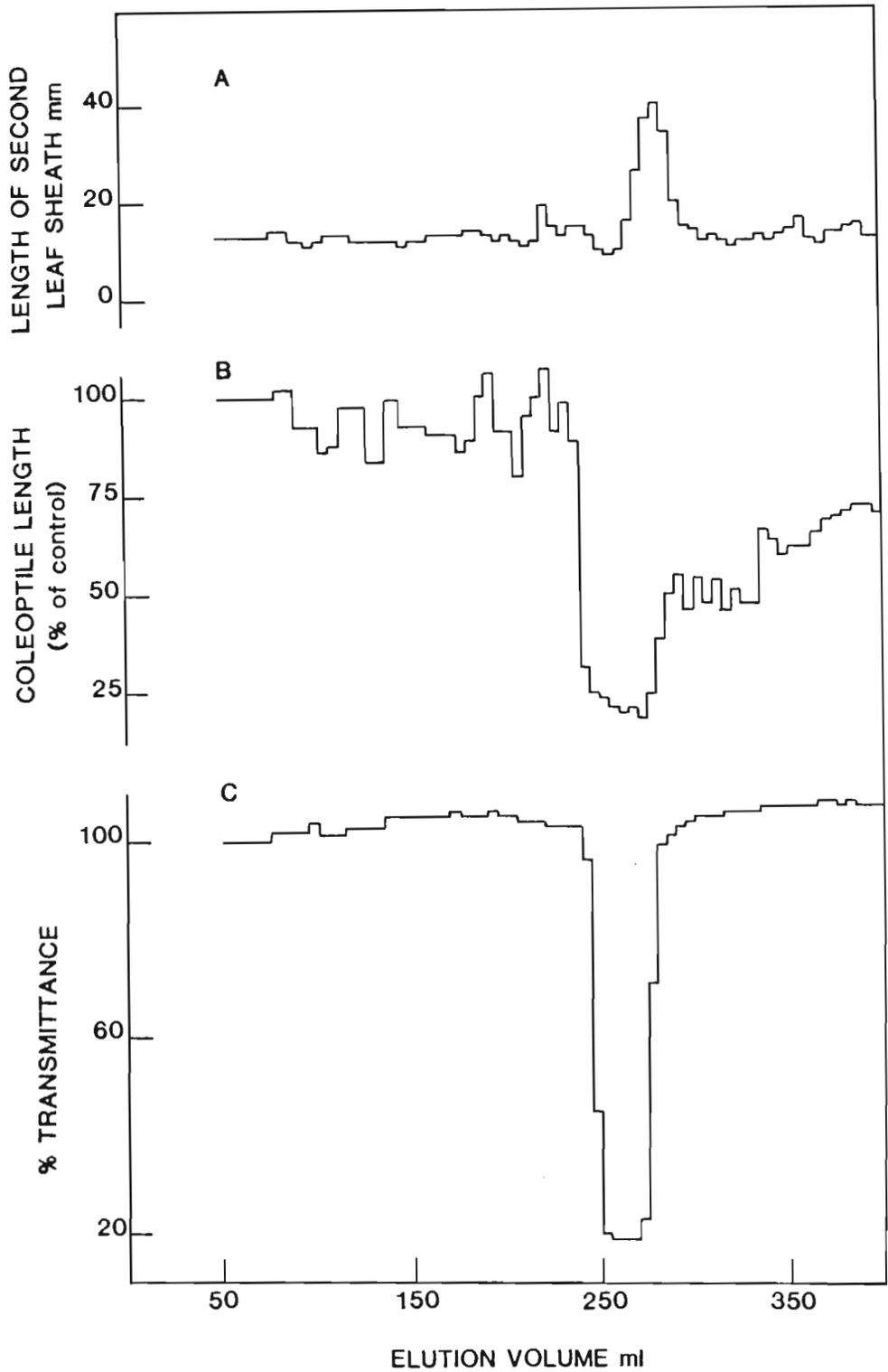


Figure 2.1 The separation of gibberellin A_3 and abscisic acid on a Sephadex LH-20 column which was eluted with acidic 80 percent ethanol at a flow rate of $7,5 \text{ ml h}^{-1}$. Five millilitre fractions were collected and assayed for gibberellin-like activity using the dwarf rice microdrop bioassay (A) and for abscisic acid-like substances using the wheat coleoptile bioassay (B) and by measuring absorbance of ultra-violet light (260 nm) (C).

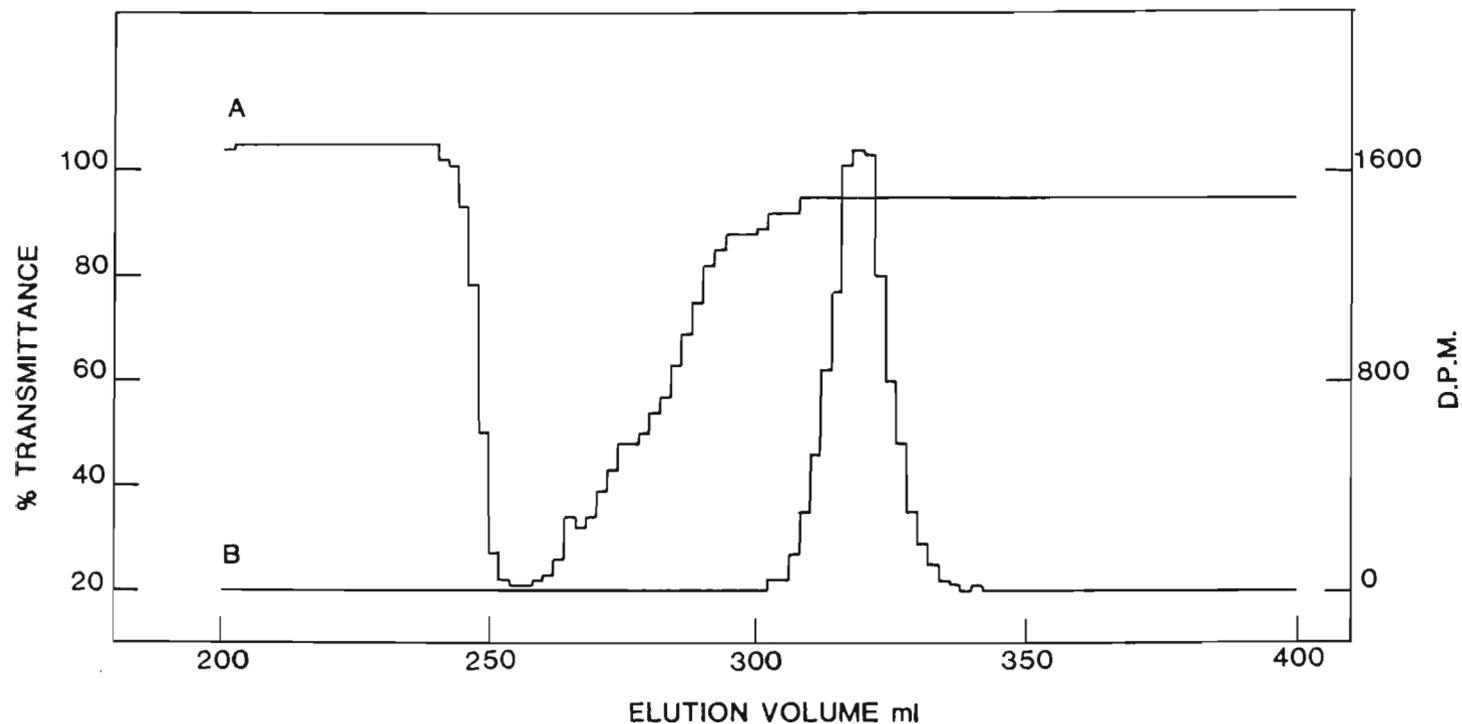


Figure 2.2 The separation of ^{14}C -gibberellin A_3 and abscisic acid on a Sephadex LH-20 column which was eluted with absolute ethanol (pH 8,0) at a flow rate of $3,0 \text{ ml h}^{-1}$. Two millilitre fractions were collected and the elution volumes of abscisic acid were determined by measuring ultra-violet absorbance at 260 nm (A). The elution volumes of ^{14}C -gibberellin A_3 were determined by counting the disintegrations (D.P.M.) of the radioactive isotope in each fraction (B).

followed by immersion in propylene oxide. It was then embedded in araldite resin. Polymerisation lasted for 48 hours at 70°C. Sections for light and electron microscopy were cut with a glass knife and a diamond knife respectively, on an LKB microtome. The sections were stained for electron microscopy with uranyl acetate and lead citrate as described by REYNOLDS (1963). Sections were examined and photographed using a JEOL JEM-100CX electron microscope at an accelerating voltage of 80 kV.

Monitor sections of each apex were cut and stained using one percent toluidine blue in one percent borax: one percent pyronin Y (1:1 v/v). They were examined and photographed using a Zeiss photomicroscope.

Starch determinations

Material to be analysed for starch content was first oven dried at 100°C to constant mass (24 h) and then ground to a fine powder. The method of ADAMS, RINNE and FJERSTAD (1980) was adapted for determination of starch deposition in the leaves of adult and juvenile Pickstone Valencia orange plants. Samples were moistened with five millilitres of distilled water before adding 25 ml of hot 80 percent ethanol. Extraction of free sugars was allowed to proceed for 10 minutes, after which the mixture was centrifuged at 3000 g for 10 minutes. The supernatant was discarded. The extraction of free sugars in this way was repeated three times. The pellet was finally dissolved in five millilitres of 0.1 M sulphuric acid and incubated at 100°C for 40 minutes in order to hydrolyse the starch. The extract was then filtered, using Whatmans No. 1 filter paper and the residue was rinsed with distilled water. The rinse and filtrate were combined, adjusted to pH 6.5 and made up to 20 ml.

Five millilitres of this solution, containing the hydrolysed starch, was added to a commercially available combined enzyme-colour reagent solution (Sigma Chemical Co., St Louis, Missouri). The procedure is based upon the

CHAPTER III

COMPARISONS OF GROWTH AND MORPHOLOGY OF SHOOTS AND ANATOMY OF BUDS

Introduction

It has been declared that juvenile plants are more vigorous than adult plants (BORCHERT, 1976; HEYBROEK and VISSER, 1976). Certain anatomical and morphological features also distinguish the two phases of growth. Before a complete understanding can be gained of the processes regulating changes accompanying ontogenetical development in plants, the inherent differences in growth, morphology and anatomy should be fully documented. The complex array of plant characteristics which exhibit variation linked to ontogenetical age has caused some researchers to doubt whether one central controlling mechanism directs all aspects of ontogenetical ageing (BORCHERT, 1976; LONGMAN, 1976).

It is probably sensible to view differences in juvenile and adult shoot growth from an ecophysiological point and to assess the ecological advantages of specific features of the one or other phase of growth.

The inherent problems of measuring growth and morphological features in woody perennial plants have been recognised and discussed extensively (WATSON, 1947; MONSELISE, 1951 a; BARLOW, 1970; EVANS, 1972). Such plants are slow growing, they generally have greater size and morphological complexity than annual crops and they have a high degree of individual variability. Greater accuracy and precision of data can most often be gained by using large quantities of plants. However, the time and cost of producing and maintaining plants and the large areas of glasshouse space required, does often limit the numbers of experimental plants which can be used in a comparative study.

In the experiments described below, comparisons of the dimensions of specific shoot organs were made within and between juvenile- and adult-budded plants. Various anatomical components were also compared in these two plant tissues.

Experiment 1 Parameters of growth and morphological features
of juvenile and adult shoots

It would be of dubious value to compare rates of shoot growth and even the shoot morphology of relatively small, juvenile seedling plants with large fully matured trees. Such a comparison would reveal how shoot growth changes not only with ontogenetical and chronological ageing, but also with increasing complexity of the whole plant's structure. The inherent differences between the growth and differentiation of adult and juvenile stem tissues are stable during somatic regeneration. Differences which may be due to positional competition or other effects in shoot growth may be eliminated by grafting adult and juvenile buds onto uniform rootstocks. When these budded plants are cultured under identical conditions, direct comparison of the inherent differences is possible.

Experimental procedure

The adult- and juvenile-budded plants used in these determinations were propagated and maintained as described in Chapter II. Only three branches were allowed to develop on each outgrowing scion. All of the comparisons detailed in the current experiment were made with plants which had produced three flushes of growth since budding was performed. Individual growth flushes may be distinguished from each other by short, swollen internodes at the beginning and end of each flush (SCHNEIDER, 1968). Each flush is at a slight angle to the previous one because each new flush originates from an axillary bud. Only the growth of the terminal shoot flushes on the three branches of adult- and juvenile-budded plants was compared. Basic numbers, dimensions and mass data of leaf and stem material were recorded and ratios of certain parameters were calculated.

A distinct pattern in leaf size and internode lengths is apparent in individual mature flushes of *Citrus* plants. The first leaf to emerge from a sprouting bud is small. The second and third leaves to appear are larger

and leaf areas of individual leaves seem to gradually decrease towards the tip of the flush. Internode lengths appear to follow a similar pattern (see frontispiece). As this developmental pattern may be related to hormone activities during the growth of a flush, it was considered worthy of detailed examination. The variation of leaf area, leaf mass and internode lengths over the length of a shoot flush was, therefore, recorded. The distribution of leaves along the length of a shoot flush was calculated. The variation of leaf dry mass as a percentage of fresh mass and leaf width : length ratios, as they varied along the length of a stem flush were also calculated.

Dry mass of leaves and stems was determined after drying the material to constant mass (24 h) in an oven held at 100°C. Dried material from ten mature leaves of plants which were quiescent (as defined in Chapter II) were analysed for starch deposition prior to the emergence of a new growth flush. The method of starch extraction and detection was described in Chapter II.

Stomatal density was compared on abaxial surfaces of leaves of adult and juvenile scions. The adaxial surfaces of *Citrus* leaves are virtually without stomata (ERICKSEN, 1968). Stomata are difficult to discern when whole leaves are examined under a light microscope, due to background shading from mesophyll and other cells. Very accurate counts can be made of stomata on the negative imprint of a thin plastic layer painted onto the abaxial surface of leaves. The method was suggested by LOTTER (1978), who prepared the plastic by dissolving a plastic petri dish in a mixture of 20 ml of benzene and 30 ml of toluene. The solvents evaporate very rapidly after application to the leaf and the layer can be peeled off and examined under a light microscope. Stomatal density varies over a leaf, but COWART (1935) suggested that an area adjacent to the leaf midrib and extending towards the leaf margin is most representative. Twenty adult and 20 juvenile leaves were sampled, each leaf being counted over an area of 10 mm².

Relative levels of chlorophyll were estimated in mature leaves of adult and juvenile scions. Four circular disks (10 mm diameter) were punched from each of ten adult and ten juvenile leaves and the disks from each leaf were extracted in 10 ml of absolute methanol for 24 h in the dark at room temperature. Disks were then removed and the absorbance at 440 nm and 660 nm was recorded for the aliquot from each leaf. The mean absorbance was calculated for juvenile and adult leaves.

The rooting ability of juvenile and adult plants was also investigated. Leaf cuttings, both with and without buds attached, from juvenile and adult scions were treated with the commercial rooting powder Seradix (active ingredient 0,8 percent 4(3-indolyl)-butyric acid) (Maybaker (S.A.) (Pty) Ltd., Port Elizabeth) and they were then placed under intermittent mist with basal heating at 25°C constant. Sample size consisted of 50 leaves for each treatment analysed.

Results

Most of the measured components of the growth flushes of juvenile-budded plants exceeded those of adult-budded plants. The range of variability of many parameters was very high. The coefficient of variation was as high as 33 percent in the determination of individual leaf areas, for example, but most coefficients of variation were in the vicinity of 10 percent.

Table 3.1 reveals that the greater vegetative vigour of juvenile scions results from greater stem and leaf growth. Significant differences were found in the number of leaves per plant and per individual flush; leaf area per plant and per leaf; stem length per plant and per internode; stem diameter and in dry mass/fresh mass percentage of both stems and leaves. In no instance did components of adult flush growth show a significant excess above that of juvenile shoots. Leaf fresh mass per shoot and per leaf and stem fresh mass per shoot and per individual stem were not significantly different.

Table 3.1 Parameters of three individual shoot flushes on ten plants each of adult- and juvenile-budded *Citrus sinensis* cv. Pickstone Valencia orange. Figures represent the mean determination for material collected from ten plants each for the two phases of growth. Figures in parentheses represent the standard error of the mean.

| PARAMETER MEASURED | JUVENILE | ADULT |
|------------------------------|-------------|------------|
| Leaf numbers | | |
| Total per plant | 24,0 (1,1) | 22,3 (2,1) |
| Mean per flush | 8,0 (0,4) | 7,4 (0,7) |
| Leaf area (cm ²) | | |
| Total per plant | 470 (33) | 330 (36) |
| Mean per leaf | 19,6 (6,6) | 14,7 (2,2) |
| Leaf fresh mass (mg) | | |
| Total per plant | 9407 (1247) | 9213 (916) |
| Mean per leaf | 391 (91) | 407 (32) |
| Stem length (mm) | | |
| Total per plant | 262 (7) | 202 (34) |
| Mean per internode | 11,1 (0,5) | 8,7 (0,8) |
| Stem diameter (mm) | 8,2 (0,8) | 7,7 (0,8) |
| Stem fresh mass (mg) | | |
| Total per plant | 977 (184) | 873 (62) |
| Mean per stem | 325 (61) | 291 (21) |
| Dry mass/Fresh mass (%) | | |
| Stem material | 38 (1) | 33 (2) |
| Leaf material | 38 (2) | 34 (3) |

The distribution of leaves along the length of an individual stem flush is presented in Figure 3.1. The most obvious difference between adult and juvenile flushes in this respect is that the distribution on juvenile stems is fairly even over the entire length with two peaks situated near the basal end of the flush. Adult stems, on the other hand, have the greatest density of leaves at the distal end of the flush. Fewer leaves are found at the basal end of the stem. Little difference was found in leaf distribution between adult and juvenile shoots near the midpoint of the flush stem. The distribution of leaves is obviously related to internode lengths. This phenomenon will be discussed shortly.

The results of a comparison of leaf area, internode length and leaf fresh and dry mass variation along the length of a flush stem are presented in Figure 3.2. Leaves situated near to the basal and distal ends of a flush were larger in surface area in juvenile shoots than in adult shoots while no significant difference was found for leaves situated between 40 and 80 percent of the length of the shoots (Figure 3.2 A). The greater average area of leaves from juvenile scions (Table 3.1) was a result of the preponderance of larger leaves situated at the basal and terminal ends of the flush.

The difference in the trends of variation of internode lengths was significant only in the more distal section of the flush length (Figure 3.2 B). Only at one point did the internode lengths of adult stems exceed those of juvenile stems (30 percent of stem length). The adult stems exhibited a greater decline of internode lengths nearer the distal end of the flush.

The variation of leaf fresh mass along the length of the flush was very similar in juvenile and adult flush growth (Figure 3.2 C). At one position only (20 percent of flush length) did the fresh mass of leaves from adult plants exceed that of juvenile plants. Variation of dry mass over the flush length showed considerable variation. This variation and the significant differences found were, however, of little value when analysed as such. The

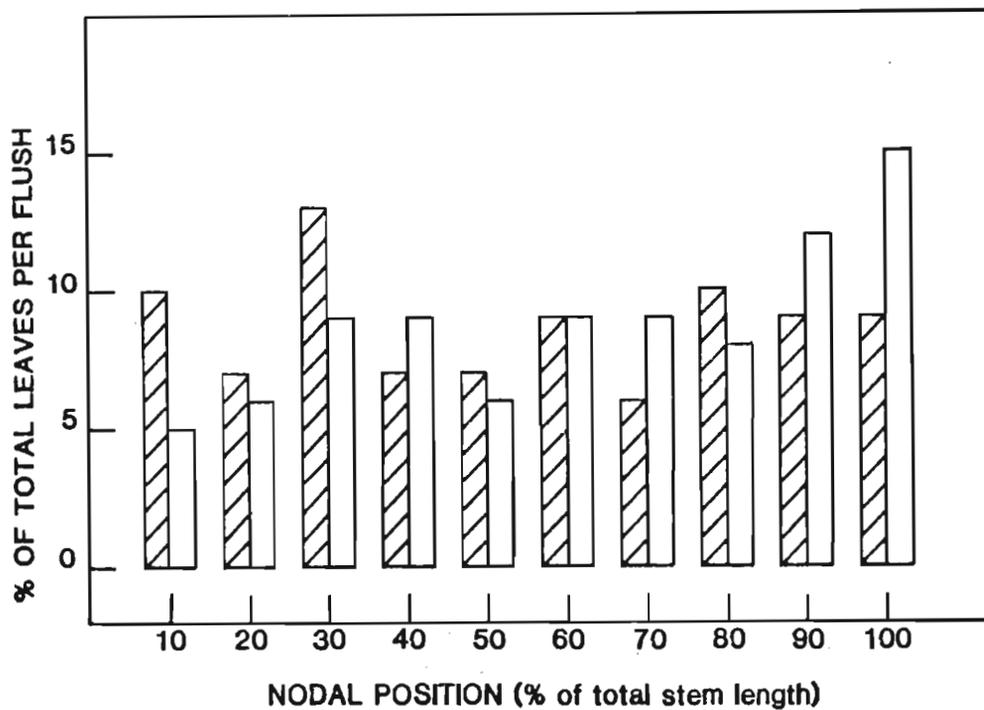


Figure 3.1 The distribution of leaves along the length of individual growth flushes of juvenile (cross hatched) and adult (clear) scions of *Citrus sinensis* cv. Pickstone Valencia orange. Results are presented as the frequency of leaves occurring at intervals over the stem segment produced in a single flush of growth.

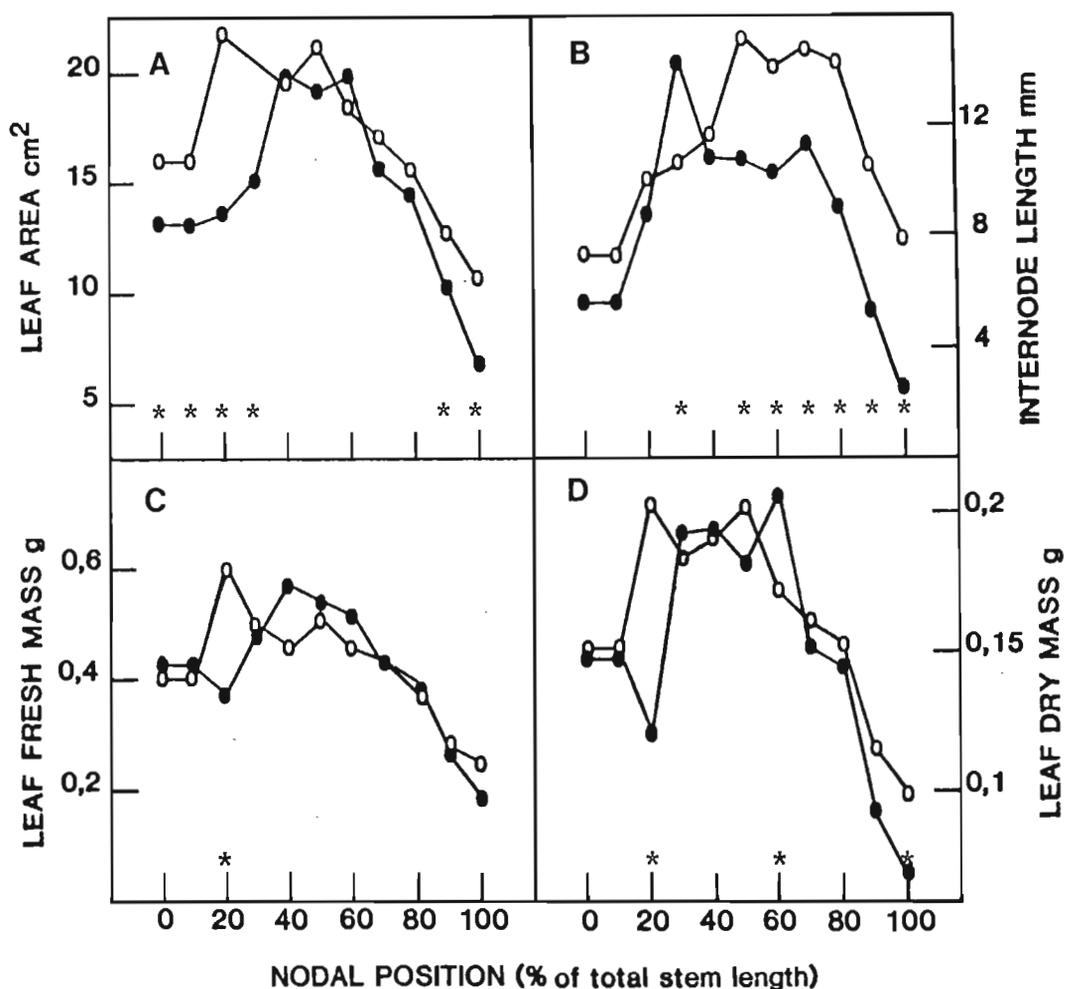


Figure 3.2 The variation of unit leaf area (A); internode lengths (B); unit leaf fresh mass (C) and unit leaf dry mass (D), over the length of an individual flush of growth from scion stems of juvenile (o) and adult (●) *Citrus sinensis* cv. Pickstone Valencia orange. Significant differences based on the overlap of standard errors of the means, are indicated by asterisks at respective nodal positions.

results of a related but more useful derived statistic, percentage of leaf dry mass, will be presented below.

Two more parameters of leaf growth which may be affected or regulated by hormonal influences were monitored along the length of a shoot flush. These were the width:length ratio of leaves and the dry mass percentage of fresh mass in leaves of juvenile and adult scions (Figure 3.3). The leaf width:length ratio of adult plants, expressed as a percentage, demonstrated a consistent trend of decreasing values from close to the base (30 percent of flush length) of the stem (Figure 3.3 A). The leaves from a juvenile flush varied little in this parameter along the length of the flush, from 57 to 61 percent, and no clear trend was apparent, as in the data for adult leaves. Despite the fact that no consistent differences between both fresh and dry mass of juvenile and adult leaves was apparent (Figure 3.2 C; D), the proportion of dry mass accumulation in juvenile leaves was found to be consistently higher in juvenile leaves along the entire shoot flush (Figure 3.3 B). There was much variation in this derived statistic and only near the terminal end of the flush was a significant difference found between juvenile and adult leaves.

A possible reason for the higher proportion of dry mass in leaves of juvenile scions may be found in the higher starch content of these leaves (Table 3.2). The starch content of leaves from adult scions was found to be less than half that of the juvenile leaves in quiescent plants. Relative chlorophyll levels, as determined by measuring absorbance at wavelengths of peak absorbance for chlorophyll (660 and 440 nm), indicated that there seemed to be a higher level of chlorophyll in juvenile leaves, although such differences were not statistically significant (Table 3.2).

The stomatal density on the abaxial surface of leaves from adult scions was exceeded by the density on leaves from juvenile scions (Table 3.2). This characteristic appeared to be very consistent for both plant types; the

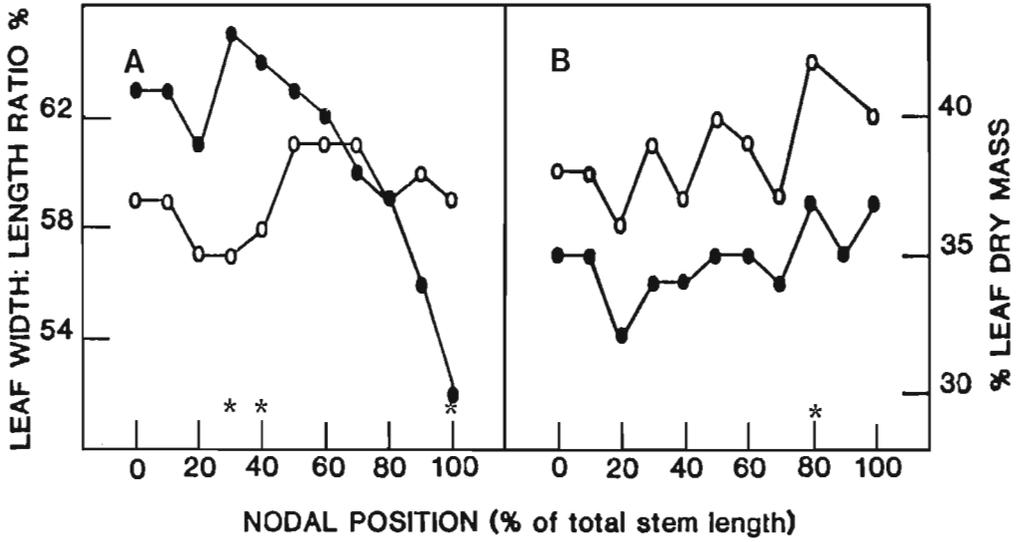


Figure 3.3 The variation in the unit leaf width:length ratio (expressed as a percentage) (A) and the percentage leaf dry mass (B) over the length of an individual growth flush from scion stems of juvenile (o) and adult (●) *Citrus sinensis* cv. Pickstone Valencia orange. Significant differences based on the overlap of standard errors of the means, are indicated by asterisks at respective nodal positions.

Table 3.2 A comparison of specific physiological characters in leaves of juvenile and adult scions of *Citrus sinensis* cv. Pickstone Valencia orange. Figures in parentheses represent the standard error of the mean of each determination.

| PARAMETER MEASURED | JUVENILE | ADULT |
|---|---------------|---------------|
| Starch content (percent of leaf dry mass) | 4,9 (2,1) | 2,0 (1,3) |
| Relative chlorophyll levels (absorbance) | | |
| 660 nm | 0,951 (0,091) | 0,906 (0,067) |
| 440 nm | 1,746 (0,151) | 1,673 (0,124) |
| Stomatal density (mm^{-2}) | 535 (18) | 459 (32) |
| Rooting ability of leaf cuttings (%) | | |
| Without buds | 63 | 0 |
| With buds | 76 | 22 |

coefficients of variation being three percent and seven percent for juvenile and adult leaves, respectively.

It was found that the comparative ease of rooting of juvenile tissues pertained even when the influence of an apical meristem (in the form of an axillary bud) was not a contributory factor. It is clear from the results presented in Table 3.2 that rooting in adult leaf cuttings was prevented in the absence of a bud. The presence of a bud on such cuttings seemed to enhance rooting in both adult and juvenile leaves but a marked difference in the final rooting percentage was still evident.

Experiment 2 Upper and lower shoot growth on single stems

The lower portions of a seedling tree remain, perhaps indefinitely, in the juvenile phase (FROST, 1952; CAMERON and FROST, 1968). Budwood and watershoots, which develop from near the crown of a seedling tree, produce thorny and vegetative shoots only. Shoots and buds collected from the distal region of Valencia orange trees, which are more than seven years old, will generally give rise to shoots which are thornless and which are sensitive to flowering induction. The results of the previous experiment indicated that under similar cultural conditions the growth of juvenile stem flushes is, with regard to certain features, in excess of that measured in adult stem flushes. Many factors may influence the growth of lateral shoots which arise along a single stem of a plant. One of these factors is the ontogenetical age differences which may exist, while proximity to roots or the shoot apical meristem may also be important. Only after the differences in growth of upper and lower shoot flushes have been examined can the possible causes of such differences, if any, be delved into.

Experimental procedure

Approximately three years prior to the performance of this experiment, buds were collected from a juvenile four-year-old nucellar seedling tree of

Outline of generalised experimental procedure for analysis of upper and lower shoot growth on single stems

Budding of Rough lemon rootstock with buds from four-year-old nucellar seedling of Pickstone Valencia orange

|

Three-year interval
(Plants produce stems ca. two metres tall with limited number of side branches)

|

Total defoliation of plants eight weeks prior to sampling

|

Parameters of growth of new shoots from upper and lower halves (potentially adult and juvenile, respectively) of scion stem analysed

Citrus sinensis cultivar Pickstone Valencia orange. They were budded onto two Troyer citrange rootstocks which were growing in 20 l black plastic bags in the soil medium described in Chapter II. A limited number of side branches were allowed to grow on the upright main stems, which had attained a height of about two metres when the experiment was carried out. Terminal buds had attained the adult phase as was evident from the flowers which emerged only from the terminal buds after the plants were subjected to a winter cold induction period. Growth flushes which emerged simultaneously from more basal buds were vegetative only.

Eight weeks prior to sampling, the plants were totally defoliated and all lateral branches were severed close to the main stem. Within the eight week period, buds had sprouted along the entire stem and about 40 shoot flushes developed per plant. In many cases more than one bud sprouted from each nodal position.

The individual leaf area, fresh mass and width:length ratio were recorded for each flush, as well as stem length and fresh mass. Variations of leaf parameters along the stem flush length were also calculated as outlined in the previous experiment.

Results

As was found in the previous experiment, variability of parameters was extremely high. Despite the fact that a fairly large number (40 each) of flushes were collectively analysed for lower shoot flush and upper shoot flush growth, coefficients of variation for some parameters exceeded 100 percent (Table 3.3). This high variability was partly due to the nature of the shoots which developed from sprouted buds. In those cases where more than one bud sprouted from a single leaf axil, only one flush developed normally. The remaining flushes were limited usually to about two leaves and a very short stem.

Despite these limitations in making comparisons of shoot growth parameters, it was apparent that the extent of growth from lower shoot flushes exceeded

Table 3.3 Parameters of shoot flushes from the lower, ontogenetically juvenile and the upper, ontogenetically adult halves of scion stems of *Citrus sinensis* cv. Pickstone Valencia orange. Figures represent the mean for each determination while the standard error of the mean is given in parentheses.

| PARAMETER MEASURED | LOWER STEM | UPPER STEM |
|------------------------------|-------------|------------|
| Leaf area (cm ²) | | |
| Total per flush | 78 (91) | 35 (29) |
| Mean per leaf | 12 (6) | 8 (5) |
| Leaf fresh mass (mg) | | |
| Total per flush | 1330 (1240) | 960 (760) |
| Mean per leaf | 280 (150) | 210 (140) |
| Stem length (mm) | | |
| Total per flush | 74 (78) | 23 (14) |
| Mean per internode | 8,4 (4,8) | 4,1 (2,3) |
| Stem fresh mass (mg) | | |
| Total per flush | 500 (730) | 180 (100) |

that of upper shoot flushes. No statistically significant differences were, however, detected in leaf or stem parameters (Table 3.3). The cumulative total leaf area produced per flush by individual shoots on the lower stem was more than double that of the upper shoots. Unit leaf areas were 50 percent greater in the lower shoots. Both the total leaf fresh mass per flush and the unit leaf fresh mass were about 38 percent greater in lower shoots. By far the greatest difference was located in the fresh mass of stems, where the unit mass per flush was 500 mg in lower flushes and 180 mg in upper flushes. Stem lengths and internode lengths were also greater in lower shoots.

Figure 3.4 reveals the changes in certain leaf parameters along the length of upper and lower stem flushes. The peak in leaf area of the upper and lower shoots coincided at about 30 percent of the total flush length (Figure 3.4 A). Unit leaf areas were consistently greater in lower flushes. The trend of changing leaf size was very similar in the upper and lower flushes. Leaf fresh mass of lower flushes reached a peak in leaves situated at about 20 percent of total shoot length and declined thereafter (Figure 3.4 B). The significant differences in leaf fresh mass were apparent in the basal part of flushes, but these differences diminished at 40 and 60 percent of stem length when the upper shoots produced heavier leaves.

Although there were initial differences in the leaf width:length ratio between upper and lower flushes, these differences disappeared fairly close to the basal portion of the stems (Figure 3.4 C). There was a trend of steady decline in this ratio in leaves of the lower flushes after the peak at 20 percent of flush length. Although upper leaves did follow the same trend of declining ratios, a much higher degree of variability was evident.

Experiment 3 Ultrastructure and anatomy of juvenile and adult buds

The growth emanating from terminal buds of juvenile and adult *Citrus* scion shoots has been shown to be different in many respects (FROST, 1952).

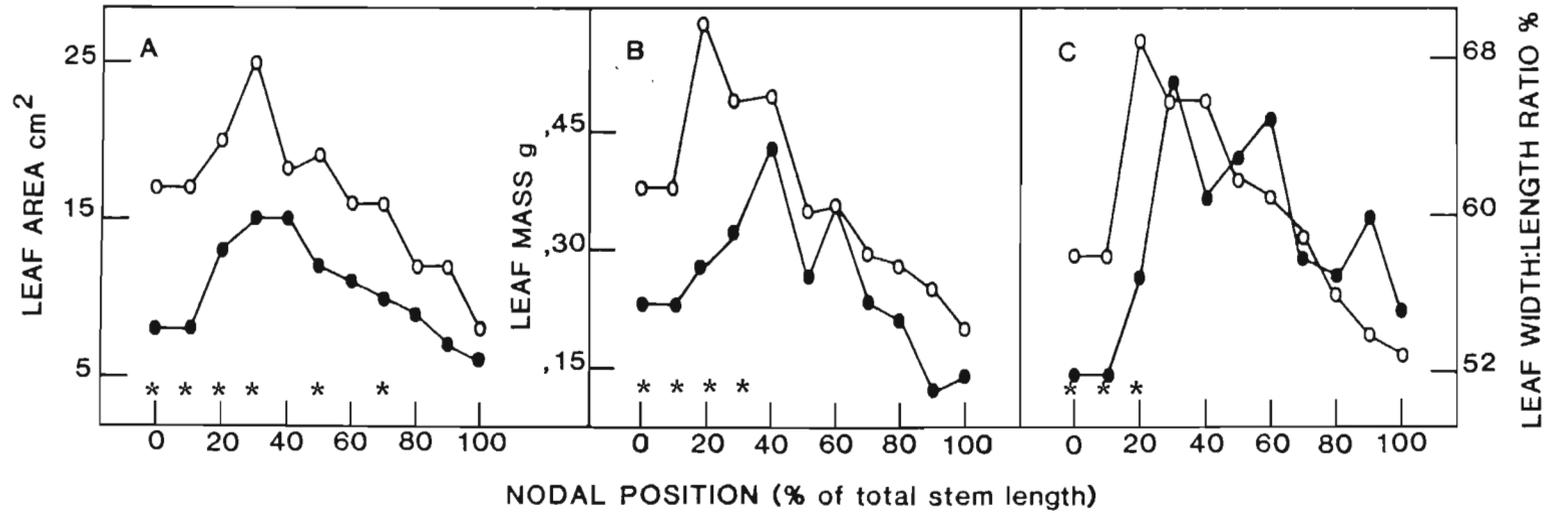


Figure 3.4 The variation of unit leaf area (A); unit leaf fresh mass (B) and unit leaf width:length ratios (expressed as a percentage) (C), over the length of an individual flush of growth. Parameters were measured for flushes from lower (o) and upper (●) stems of *Citrus sinensis* cv. Pickstone Valencia orange plants. Significant differences, based on the overlap of standard errors of the means, are indicated by asterisks at the respective nodal positions.

WILLIAMS (1975) showed that differences in the leaves of juvenile and adult *Eucalyptus bicostata* are evident at a very early stage of differentiation of the apex. Because no dimorphism is apparent in the leaves of juvenile and adult *Citrus sinensis* it would not be expected that anatomical differences would be apparent in the gross features of apical buds. However, since biochemical differences have been found in these tissues (HENDRY, VAN STADEN and ALLAN, 1982 b), an investigation of the ultrastructure of components of juvenile and adult apical buds was undertaken. Many researchers have found that plastids, for example, may be the locus of much of the metabolism of plant hormones (LAETSCH and BOASSON, 1972; RAILTON and REID, 1974; COOKE, SAUNDER and KENDRICK, 1975; FEIERABEND and DE BOER, 1978; NAITO, TSUJI, HATAKEYAMA and UEDA, 1979; PARTHIER, 1979). Observations for differences in organelles were made.

Experimental procedure

The terminal buds were collected from juvenile- and adult-budded Pickstone Valencia orange plants which were either dormant, or where bud sprouting was taking place. Dormant buds were obtained from plants which had been subjected to simulated winter conditions in a glasshouse for three months. Just prior to the onset of warmer conditions apical buds were dissected from the stem to provide an undamaged terminal segment of about two millimetres. About two weeks after the onset of warmer temperature conditions, sprouting buds with small primordial leaves visible were collected in a similar fashion.

All bud samples were immediately subjected to the fixation and embedding process described in Chapter II. During the trimming of specimen blocks, monitor sections were stained and viewed frequently under the light microscope. In this way it was possible to judge fairly precisely when the centre of the apical dome had been reached. At this point monitor sections were cut, stained and prepared for permanent mounting with D.P.X. mountant (B.D.H. Chemicals Ltd., Poole, England). These were viewed and photographed

using a Zeiss photomicroscope. Ultra-thin sections were then cut, stained, viewed and photographed on a transmission electron microscope.

Phyllotaxy of adult and juvenile shoots was determined in grafted plants.

Observations

Dormant shoot apices were well protected by a number of bud scales having cells with much wall thickening as shown by dark staining in the monitor sections (Plate 1 A; B). No differences were apparent between juvenile and adult dormant apices as far as the structure and dimensions of bud components were concerned (Plate 1 A and 1 B, respectively). Procambial strands were fairly well developed in adult and juvenile buds and extended into the bud scales.

The early development of the procambium in sprouting buds is also evident in the monitor sections of juvenile and adult buds (Plate 1 C and 1 D, respectively). *Citrus sinensis* has a single layered tunica (SCHNEIDER, 1968) and this is demonstrated especially clearly in the juvenile apex (Plate 1 C). Sprouting buds were collected within 14 days of the inception of warmer temperatures which are conducive to shoot growth. Cells of the subapical region of both juvenile and adult tissues appeared to have recently undergone many transverse divisions at this stage. No distinct differences were discernible between the subapical regions of adult and juvenile tissues. The cells of the tunica and corpus were relatively small. The cytoplasm of these cells appeared to be more dense than the cells in adjacent regions (Plate 1 C; D). Some dramatic changes were evident in cells from the peripheral zone of the corpus of juvenile and adult apices in the transformation from the dormant to the sprouting condition (Plates 2 A; B and 3 A; B, respectively). Chloroplasts of the peripheral corpus cells of the dormant juvenile and adult apices (Plates 2 A and 3 A, respectively) contained large quantities of starch while starch was not apparent in these organelles in similar cells of sprouting

Plate 1 Longitudinal sections of terminal apices of *Citrus sinensis*
cv. Pickstone Valencia orange.

- A; B Juvenile and adult dormant buds, respectively, showing a number of protective bud scales (Bs) covering the apical dome. Procambial strands (P) may be seen to reach as far as the bud scales.
- C; D Juvenile and adult sprouting buds, respectively, showing the tissues which were observed at greater magnification. Cells from the basal regions of developing leaflets, from the peripheral zone (Pz) and from the subapical region (Sr) of the shoot apex were examined in greater detail.

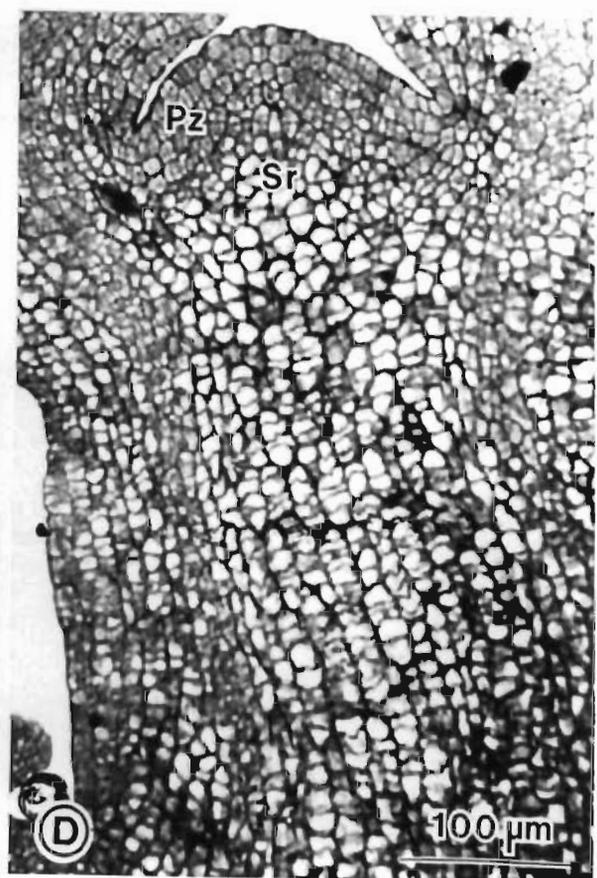
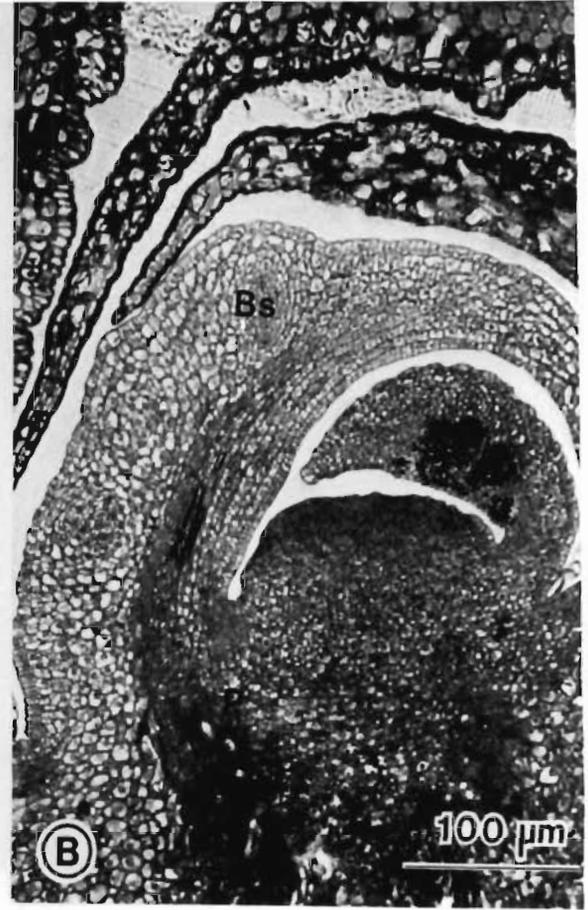
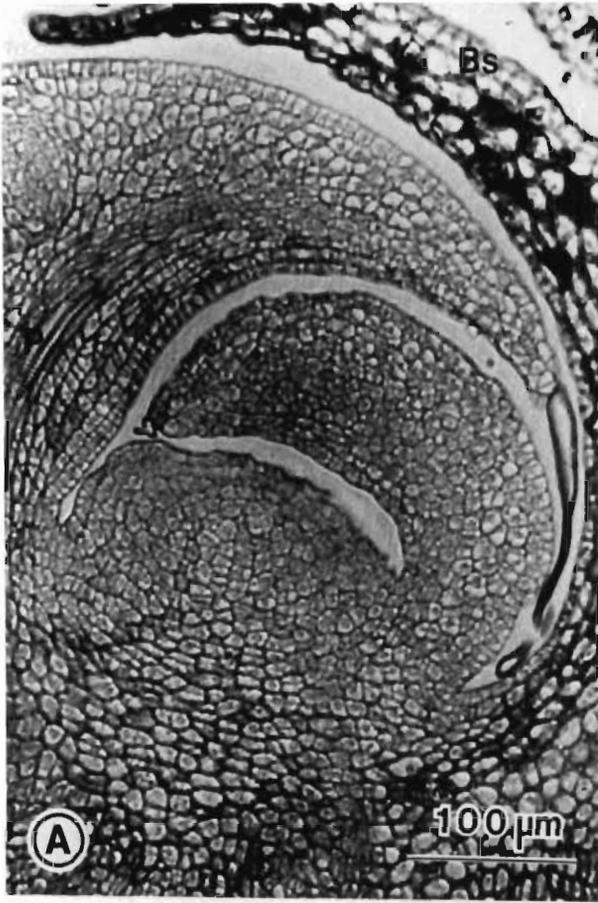


Plate 2 Cells of the peripheral zone of apices from juvenile *Citrus sinensis* cv. Pickstone Valencia orange.

A Cells from dormant apices showing the presence of many chloroplasts (Cp) with abundant starch (S).

B Cells of sprouting apices showed signs of recent mitotic activity. A nucleolus (Nu) with a central nucleolar vacuole was apparent within the nucleus (N) of some cells. Vacuoles (V) were present, as well as plastids (P) showing some signs of developing lamellae. Plasmodesmata (Pd) were also apparent.

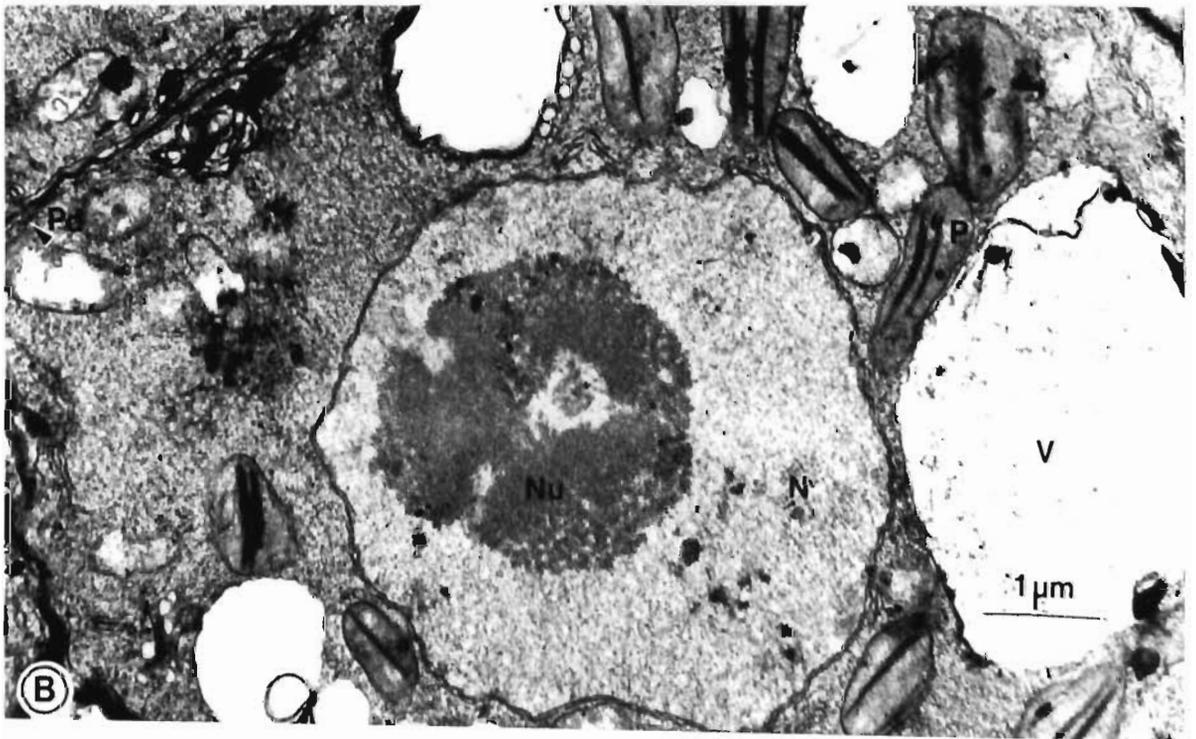
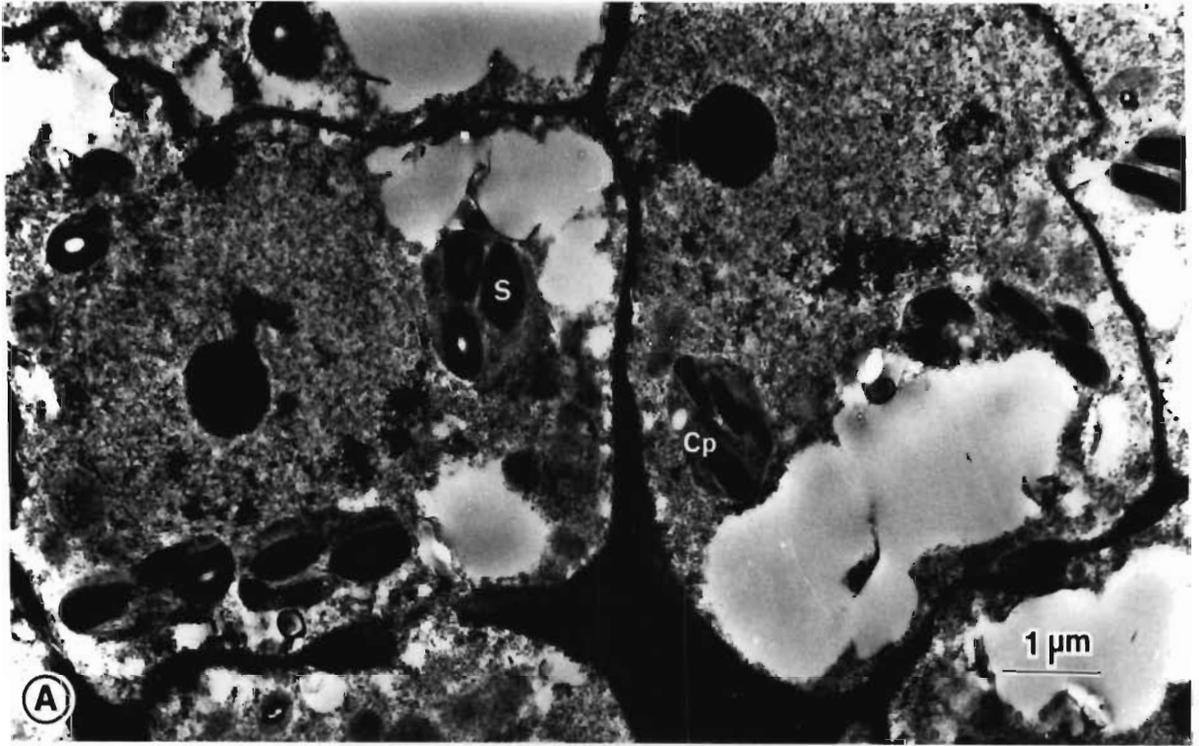


Plate 3 Cells of the peripheral zone of apices from adult *Citrus sinensis* cv. Pickstone Valencia orange.

A Cells from dormant apices showing that abundant starch (S) was present in the plastids. Plasmodesmata (Pd) were apparent in the thick cell walls. Mitochondria (M) were present in these cells as well as vacuoles (V).

B Cells of sprouting apices contained nuclei (N) with nucleoli (Nu). Vacuoles (V), plasmodesmata (Pd) and mitochondria (M) were evident in these cells.

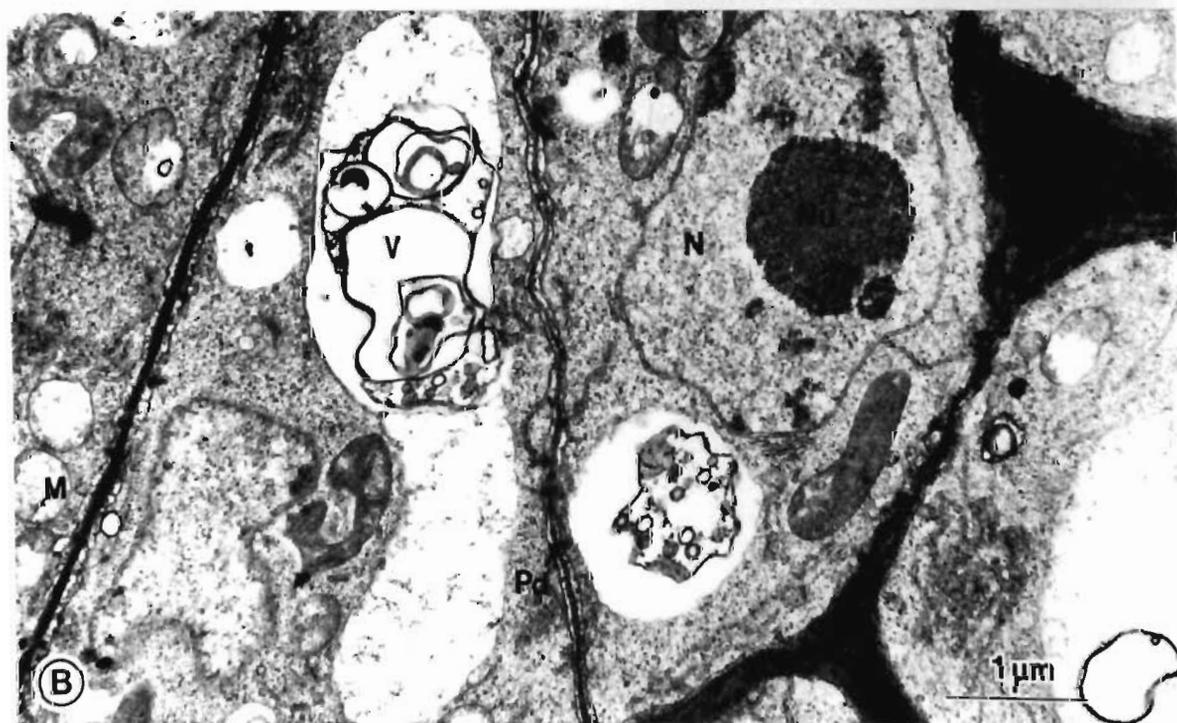
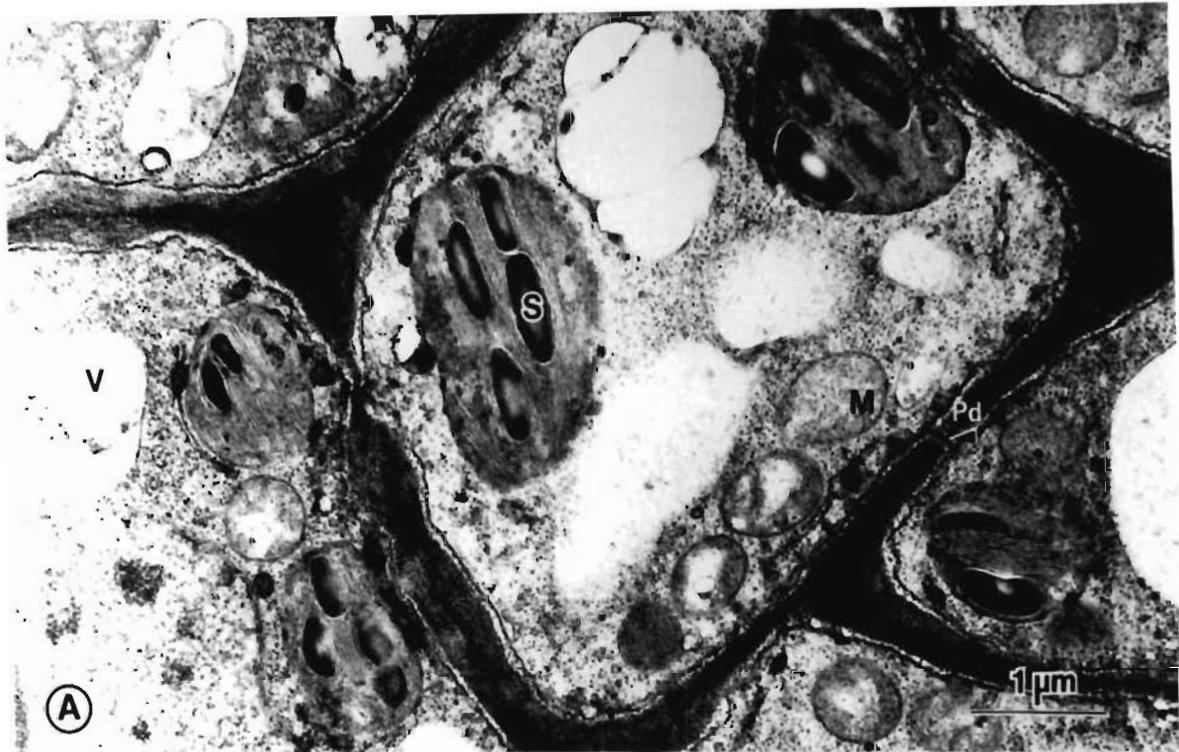


Plate 4 Cells of the subapical region of sprouting buds from adult and juvenile *Citrus sinensis* cv. Pickstone Valencia orange.

- A View of the cells from the subapical region of apices from juvenile plants showing the presence of developing plastids (P), nucleoli (Nu) and vacuoles (V).

- B The cells of the subapical region of apices from adult plants contained many developing plastids (P), vacuoles (V) and distinct nuclei (N) containing nucleoli (Nu).

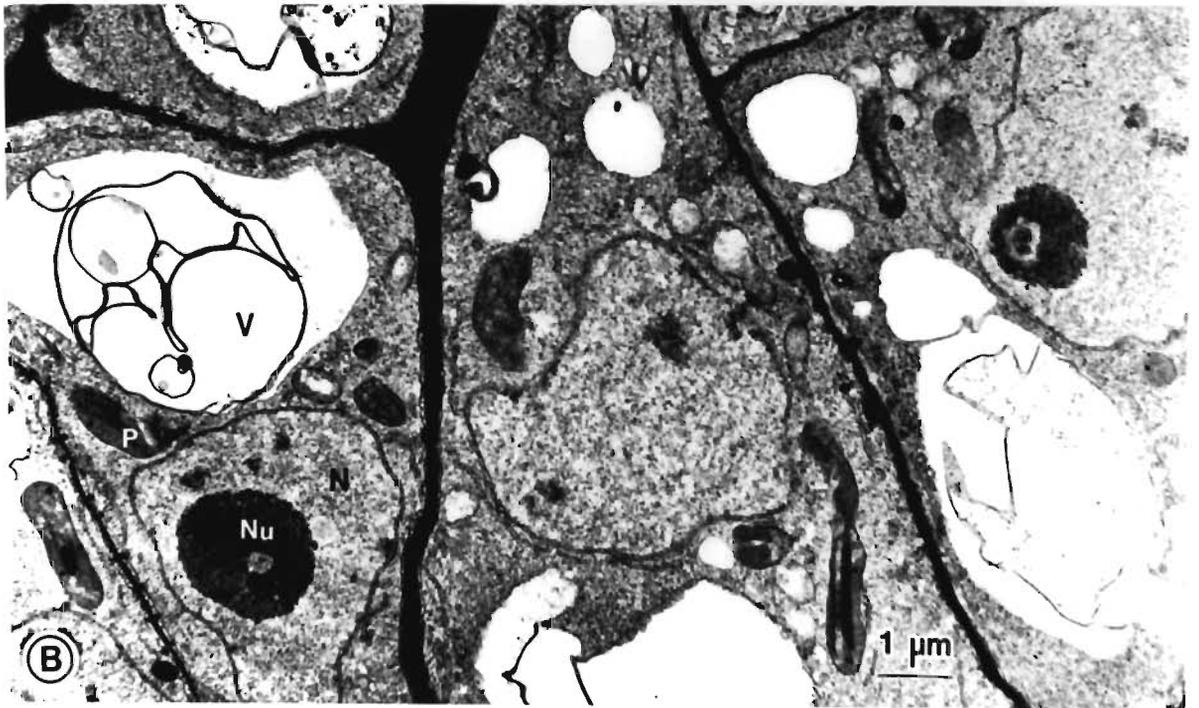
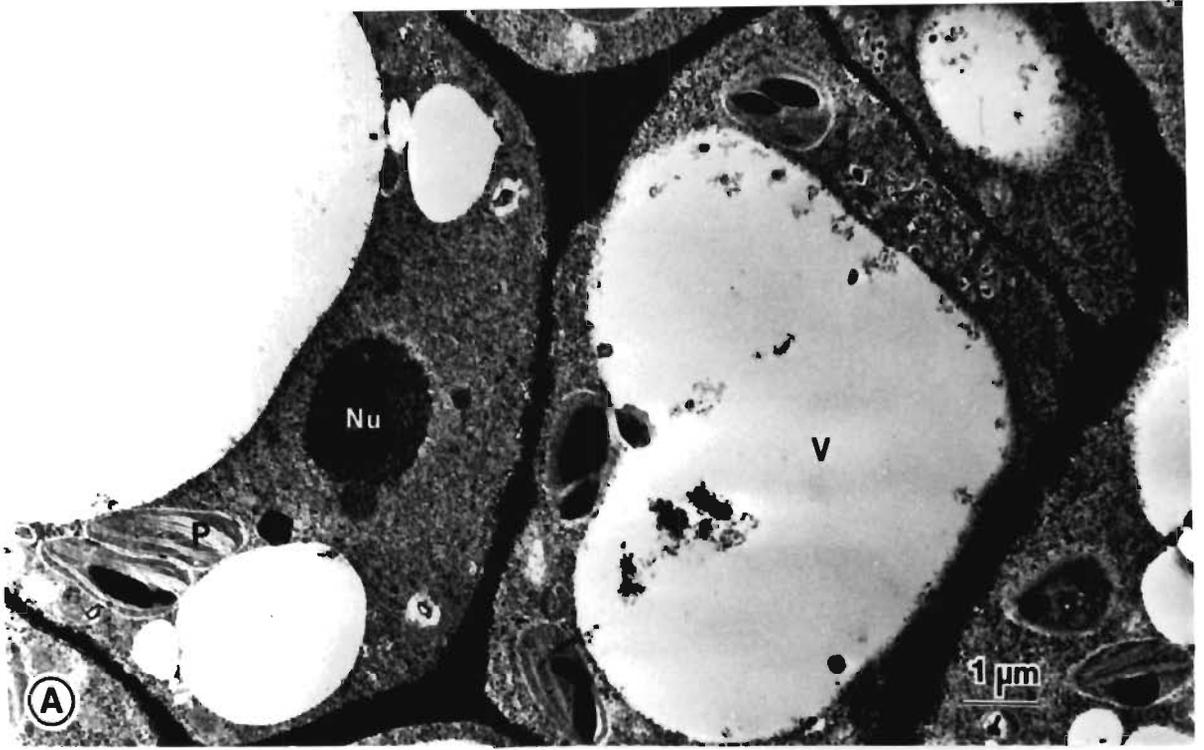
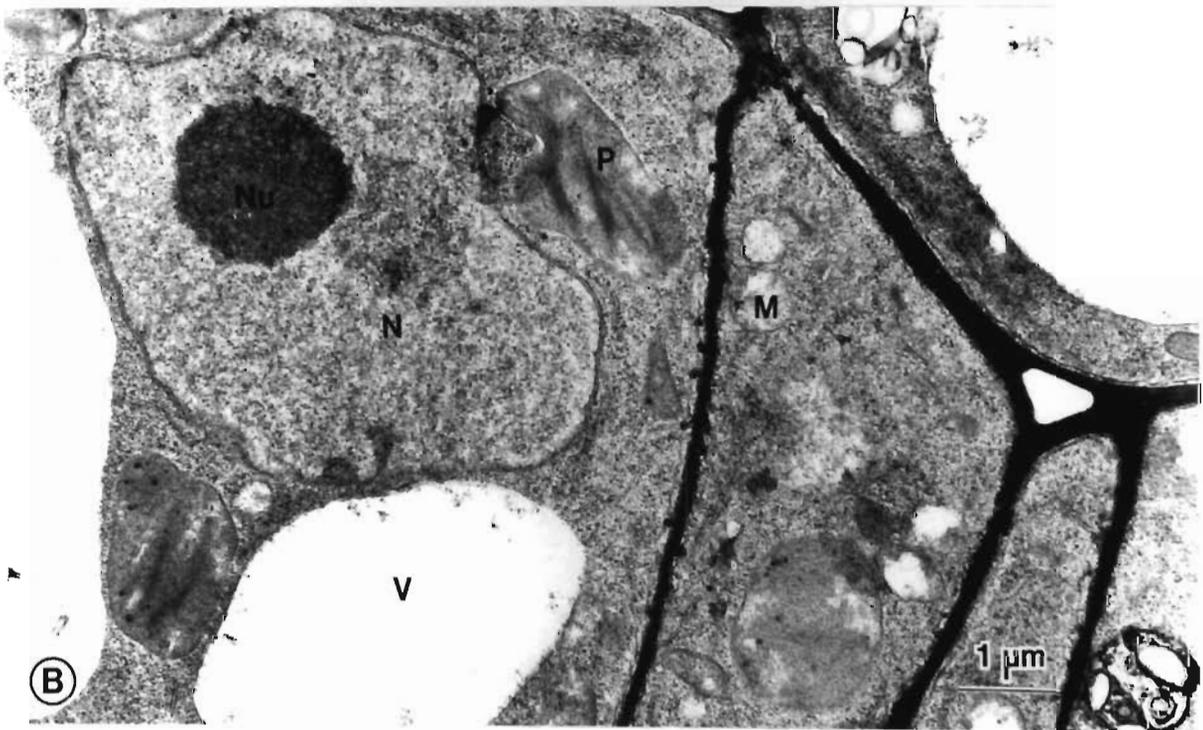
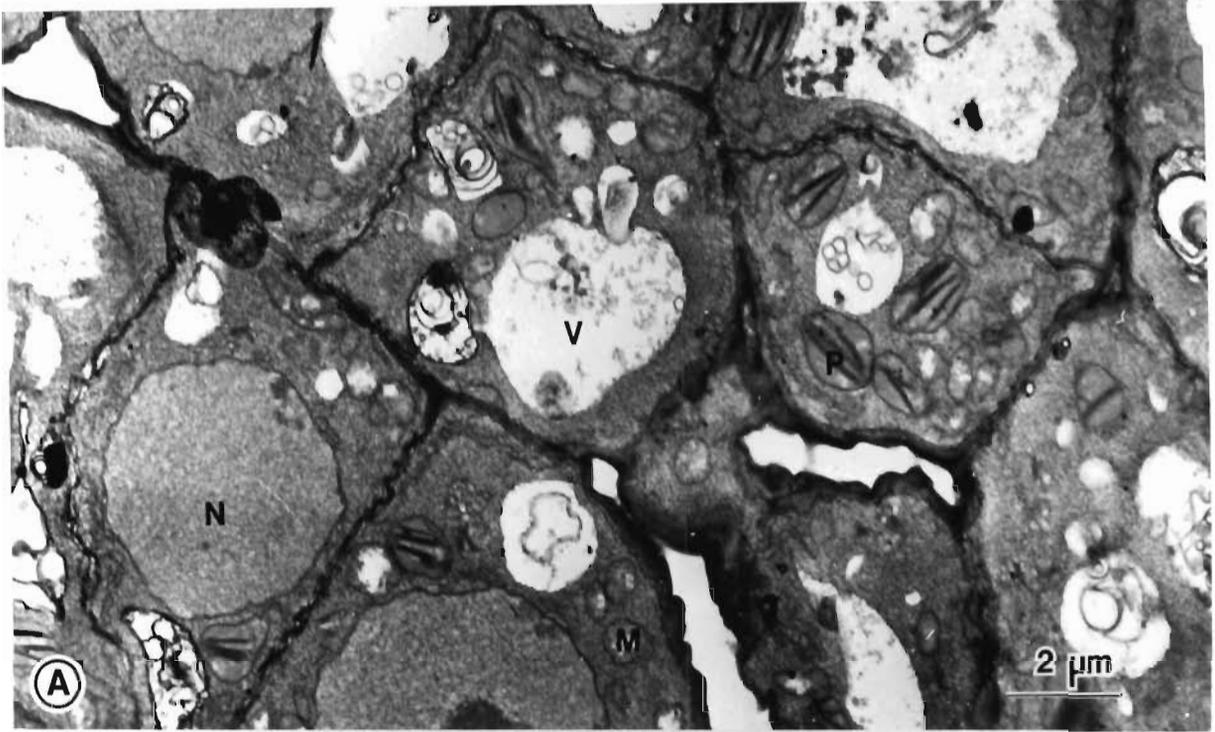


Plate 5 Cells of the basal regions of primordial leaflets from juvenile and adult *Citrus sinensis* cv. Pickstone Valencia orange.

- A The cells of developing leaflets of juvenile buds were characterised by the presence of many developing plastids (P). Mitochondria (M), vacuoles (V) and nuclei (N) were also evident.
- B Granular material was abundant in developing leaflets of adult buds. Nuclei (N) and nucleoli (Nu) were obvious, as well as mitochondria (M). Developing lamellae were shown clearly in plastids (P).



buds (Plates 2 B and 3 B, respectively). The walls of the peripheral corpus cells appeared to be thicker in the dormant apices. Large vacuoles were present in all corpus cells, but these vacuoles were not as large as in some of the subapical cells (Plate 1 C; D).

The nucleolus of peripheral corpus cells from juvenile, dormant apices was dense and contained fibrillar material characteristic of quiescent nucleoli (Plate 2 A). Although no nucleoli are shown in Plate 3 A, the same conditions of the nucleoli were evident in adult, dormant peripheral corpus cells.

In the peripheral corpus cells of sprouting juvenile and adult buds (Plates 2 and 3 B, respectively) the nucleolar organiser appeared to be functional; the nucleolar material was extended and the granular particles were dispersed. A nucleolar vacuole was present in some cells of the juvenile peripheral corpus region (Plate 2 B). These observations serve to indicate that the region was meristematically active. In addition the high density of polysomes and ribosomes typical of meristematic regions was also evident in peripheral corpus cells of both juvenile and adult active apices.

Cells of the subapical region in sprouting buds did not appear to differ markedly (Plate 4 A and B, respectively). The cells contained very large vacuoles and plastids which appeared to be developing into chloroplasts. Plastids of the cells from juvenile tissues are shown to contain starch (Plate 4 A). The apparent absence of starch from plastids of cells from adult subapical tissues (Plate 4 B) was by no means universal and the starch content of subapical cells in both juvenile and adult tissues was highly variable. Nuclei in these tissues, as in the peripheral corpus cells, present evidence of recent cell division.

Tissues of juvenile and adult developing leaflets close to the apex had abundant intercellular spaces, but did not differ in any major respects (Plate 5 A and B, respectively). Cells had thin walls, abundant mitochondria and plastids and large vacuoles and nuclei. No plastids were fully differentiated into

chloroplasts, but lamellae alignment and development was clear in the plastids of juvenile and adult leaflets. At the time when sprouting buds were sampled, these leaflets were yellow/pale green in colour.

Phyllotaxy of both juvenile and adult stems was found to be 2/5 and the direction of spirality varied between left and right.

Discussion

The purpose of the above experiments was to establish, on a quantitative basis where possible, the nature and extent of differences in shoots and shoot growth of juvenile and adult scion flushes. From the results of Experiments 1 and 2, above, it is clear that in many respects the shoot growth produced by juvenile-budded plants exceeds that of adult-budded plants. There seems to be an inherent property in juvenile shoots which allows for larger leaves and longer stems to be produced. It appears that this differential ability is fixed or controlled in shoot apices as is shown in grafting experiments where the particular phase of the parent apex is inherited somatically by the ensuing shoot growth. The differential ability has also been demonstrated in Experiment 2, above, where more and less vigorous shoots were produced by buds on single plants, which were juvenile and adult, respectively.

Certain parameters of the enhanced vigour of juvenile shoots might, in turn, contribute to the progressive acceleration of growth relative to that of adult plants. Amongst these characteristics may be included the greater leaf area and the higher level of deposited starch. Other significant differences, such as the larger stems and the greater stem diameters, may be part of the enhanced vegetative growth in juvenile stems but they play a small role, if any, in any further excessive growth relative to that of adult shoots.

Many of the features which characterize juvenile plants may be better understood after consideration of the position which such plants would naturally occupy. Seedling trees usually develop under conditions of low light intensit

under the canopy of the parent trees and perhaps also in competition with herbs and shrubs in the lower tiers (SCHWABE, 1976). Juvenile scion growth in *Citrus* shows a natural tendency to be lanky and thorny. The overall length and mean internode lengths of juvenile scion flushes are greater than in adult plants. A competitive advantage over adjacent plants is gained in the interception of light as a result of greater stem length. Thorns serve as a deterrent to browsing animals. Specific leaf characteristics should also be considered in the above context. Although differences were not statistically significant in the above experiments, it appears that juvenile leaves may contain more chlorophyll per unit area than adult leaves. Attention should be given to the findings of two researchers before an hypothetical reason may be offered for such a discrepancy. MONSELISE (1951 b) found that Sweet lime seedlings under reduced radiation grew better and became larger than sun seedlings. Light saturation was found to be at a higher level in adult *Hedera* leaves than in juvenile leaves (BAUER and BAUER, 1980). It may, therefore, be postulated that juvenile leaves develop and are adapted for existence in conditions of reduced radiant flux density such as in substrata of arboreal canopies. Additional evidence for such a postulation comes from the discrepancy in stomatal density. Adult leaves have a lower stomatal density. It seems that juvenile leaves are adapted, in this respect, for growth in a more humid and generally a less hostile environment. In considering the above and other dimorphic leaf characteristics found in juvenile and adult plants, it appears that changes in adult leaves tend to be in the direction of greater xeromorphy (BARTHOLOMEW and REED, 1943; BORCHERT, 1976).

Further development of the concept of adaptations to specific environments being inherent in the buds giving rise to the shoots, led to an examination of the conformation of juvenile and adult shoot flushes. In the first place, phyllotaxy of shoots was determined, since leaf arrangement obviously affects light interception and penetration to the lower leaves on a particular stem. Juvenile and adult scions of *Citrus sinensis* used in this experiment both

appeared to have a phyllotaxis of 2/5, which is identical to that of *Citrus grandis* and *Citrus paradisi* (BANERJI, 1952; SCHNEIDER, 1968). The finding of SCHROEDER (1953), that the direction of spirality is reversed with each growth flush, was corroborated in this study.

The arrangement of leaves on stems (phyllotaxy) of juvenile and adult plants was consistent, but their distribution was found to be different. The greatest density of leaves was found near the terminal end of adult shoot flushes while a more even spreading over the stem length was recorded in juvenile shoots. This was evident from both the actual measurement of leaf distribution (Figure 3.1) and the measurement of the variation in internode lengths along the length of the flush stem (Figure 3.2 B). The observed differences probably do affect light interception and penetration into the basal parts of the stem to some extent.

Investigations of the trends along the stem, of other shoot growth components showed that specific differences were evident in juvenile and adult stem conformation. Trends of variation in unit leaf area and unit leaf mass were fairly similar, though of different magnitude in both juvenile/adult and lower/upper shoot flushes. In only a very few instances did the magnitude of the above parameters of adult or upper shoots exceed those of the juvenile or lower shoots, respectively. Individual variation was fairly high, but peaks in these parameters were discernible in shoots from all four sources. Inconclusive evidence of the deposition of storage compounds in leaves along the length of a well matured flush of growth, was provided by the dry mass/fresh mass percentage data. A sharp increase in the dry mass per unit leaf area has been found in *Citrus* leaves just before the emergence of a growth flush (HENDRY and ALLAN, 1980). In addition, JONES and STEINACKER (1951) reported that starch accumulates in *Citrus* leaves up to the time of a new flush of growth. Measurement of the dry mass/fresh mass percentage showed that leaves situated near the terminal end of both juvenile and adult stem

flushes had accumulated higher proportions of dry mass than more basal leaves. Levels of dry mass/fresh mass percentage in juvenile leaves were always higher. If this parameter is indicative of relative starch deposition, the above findings provide scant evidence of a higher starch accumulation in terminal leaves of both scion types and an overall higher accumulation in juvenile leaves. However, dry matter increase in *Citrus* leaves continues up to a leaf age of about 18 months (ERICKSON, 1968). The measurement of the dry mass/fresh mass percentage may reflect developments other than starch deposition. Further direct measurement of storage compounds in leaves needs to be made before the rates of assimilation in leaves along the stem can be speculated upon.

The study of shoot conformation in juvenile and adult scions has provided some evidence that differences were not due simply to a consistent discrepancy of a resource or of a regulator. For although there were similar basic trends in leaf area and mass variation along a flush of growth, these trends were not always parallel and the curves of some parameters were distinctly divergent. The most obvious of these non-parallel trends was that of leaf shape, as reflected by the leaf width:length ratio. Adult leaves became relatively narrow towards the terminal end of a flush, while juvenile leaves did not follow a specific trend in this characteristic. Differences in internode lengths of juvenile and adult growth flushes became more pronounced towards the terminal end, while the only significant difference in the leaf dry mass percentage was recorded close to the tip of the flush. The possible causes or effects of these developments will be discussed later.

In seeking to understand the relationships involved in variations of shoot structure, some general allometric correlations were observed. In those parameters measured and plotted directly it is clear that leaf area, leaf mass and internode lengths were initially small, they increased and then tailed off towards the tip of the shoot flush. The peaks of these data did not always coincide and in some cases there was overlap of the curves from juvenile and adult parameters. The significance of these observations will be discussed later.

Shoot apices, both dormant and sprouting, were of interest because they give rise to the variety of structures composing a flush of shoot growth. Dormant apices have a similar cellular organization to those of non-dormant apices (LYNCH and RIVERA, 1981), yet they are probably in a more stable condition and provided material suitable for close comparison of juvenile and adult differences. As with the *Rhododendron* used by LYNCH and RIVERA (1981), the overwintering apex of *Citrus* is subtended by large photosynthetic leaves which remain on the plant during winter. The apex had access to water and nutrients which were obtained by the plant, in addition to the current photosynthetic products. Thus the everpresent plasmodesmata and the accumulated starch in most cells of dormant apices may be better understood for this reason. No translocation elements are available in apices and it is only through the plasmodesmata that water and reserves may reach the otherwise unconnected cells (SUSSEX, 1963).

It was the specific apical cells which give rise to the formative tissues of the shoot which were of interest (ESAU, 1977). Cells of the pith rib meristem, of the flank meristem or peripheral corpus zone and of basal parts of young leaflets were therefore examined. The zonation and terminology used were obtained from the review of shoot apices by GIFFORD and CORSON (1971). There was no evidence that leaves or stems were derived differently from juvenile and adult apices. Many proplastids were evident in most cells from all regions examined and their role in hormone synthesis or metabolism can only be speculated upon. It seems that differences in juvenile and adult shoot growth arise as a result of biochemical, but not ultrastructural differences during organogenesis and/or as a result of biochemical differences during the rapid development of specific organs. Although either hormones or reserve compounds may be the most important factors involved, it is suggested that both are high on the scale of determinant factors.

As has been stated above, it is commonly believed that the locus of differential behaviour or growth of adult and juvenile plants is the shoot apical meristem. From the tests for rooting ability, it does appear though that there is a retention of certain characteristics in somatic tissues generated from either juvenile or adult apices. In juvenile leaf petiole tissues there was an inherent greater ability to dedifferentiate tissues (DAVIES, LAZARTE and JOINER, 1982) and to produce root initials. It now seems that some somatic vegetative tissues have the ability, similar to meristematic apices, to exhibit differences in biochemical control mechanisms which are reflective of their ontogenetical age. If this possibility is acknowledged, it becomes more difficult to isolate specific regions or organs of adult or juvenile plants where the growth regulatory processes assume major importance in expressions of ontogenetical age.

It is important to realise that all of the above comparisons were conducted with adult and juvenile tissues which would normally be situated at the terminal reaches of exposed branches and at low tiers in the protective environment under a tree canopy, respectively. The measurement of shoot growth does, therefore, reflect the difference in response of juvenile and adult tissues to a specific environment. It is not a demonstration of the growth of juvenile and adult shoots to be found under natural conditions. What is shown here is that in juvenile and adult meristems, genes are differentially activated. There is a difference between the inherent differences of juvenile and adult shoot growth and the sometimes indistinguishable responses to the environment. Finally, two issues remain to be discussed. In the first place, all of the analyses of growth point to the presence of an inconsistent regulation of the magnitude of growth responses. In the sequential organogenesis and development of leaves, nodes and internodes, a regulatory mechanism is operative. This concept is part of an unexplored and little understood field and it can only be speculated that hormones may be involved. DORMER (1972) suggested

that a profitable approach to the study of growth correlations can be founded upon the realization that the growth of shoots involves a complex network of relations. Perhaps this network is regulated and directed by endogenous plant hormones.

The second issue to be considered is that of time measurement in buds, for that is where such a mechanism must be operative (ROBINSON and WAREING, 1969; SUSSEX, 1976). It seems that under most conditions which will permit growth to occur, there is a difference in the quality and quantity of shoot material produced by juvenile and adult meristems. ROBINSON and WAREING (1969) proposed that a system capable of measuring the number of cell divisions could be operative in ontogenetically ageing tissues. The mechanics of such a system, though obviously linked to gene expression, must remain as an enigma for the present. The effects of the system may operate through hormone balances and activities. This possibility is explored in the experiments outlined in the next two chapters.

CHAPTER IV

ENDOGENOUS HORMONES OF JUVENILE AND ADULT TISSUES

Introduction

A hormone has been defined as an organic substance synthesized in one part of an organism and translocated to another part, where it has a controlling or regulating effect (SALISBURY and ROSS, 1969). Hormones are regarded as co-ordinators of the growth and development of the plant (LETHAM, HIGGINS, GOODWIN and JACOBSEN, 1978). However one may define hormones, there is extensive evidence in support of a basic and widely accepted tenet of hormonal theory: differences in endogenous hormones are correlated with variations in growth patterns (WAREING and PHILLIPS, 1978).

In order to establish whether some relationship exists between endogenous hormones and the observed differences in shoot growth and differentiation of adult and juvenile plants, it would be required to examine the levels of hormones in specific tissues at critical times. Although these specific tissues and critical times may be the subject of some debate, they may generally be accepted as those meristematic regions giving rise to new shoots and those times immediately preceding and following the commencement of active growth, respectively.

Two sets of experiments were conducted with the Pickstone Valencia orange plants, which were propagated as described in Chapter II. In the first, levels of endogenous gibberellin-like, cytokinin-like and inhibitory substances were determined in the apical buds of the juvenile- and adult-budded plants at various stages of growth. These compounds were investigated in various other parts of the plants in the second experiment. Both experiments were conducted to determine whether these hormones may play a role in the greater vigour of vegetative shoot growth of the juvenile Pickstone Valencia orange as compared with the adult plants.

Experiment 1 Hormones in buds

Shoot growth of *Citrus* in subtropical regions occurs in distinct flushes. The first and major flush emerges in spring after dormancy has been broken with the onset of warmer weather and higher soil moisture levels. Two or three more flushes may emerge in a cyclic fashion during the ensuing summer growing season (GILFILLAN, 1977). The question arose whether the hormone levels or balances were most important, with regard to the extent of subsequent shoot growth, in apical buds either at the commencement of growth before bud burst, or during the actual growth of the flush.

The hormonal physiology of the stem apex remains largely unknown, but the response of isolated and *in situ* apices to known hormones indicates that they play some role in the coordination of shoot growth (GOODWIN, 1978). In this experiment differences in extractable gibberellin-like, cytokinin-like and inhibitory substances were determined for adult and juvenile buds in various conditions of growth.

Experimental procedure

Three sets of analyses were performed with bud samples. Initially, gibberellins and inhibitors were analysed in dormant buds. This was followed by comparisons of quiescent and sprouting buds from juvenile and adult scions in terms of gibberellin-like, cytokinin-like and inhibitor levels. Various methods of gibberellin extraction were also tested. Finally, ontogenetically related hormone differences on the same plant were compared.

Seasonal gibberellin variation at midwinter and just before the onset of growth in spring was determined when plants were subjected to simulated winter temperatures in a glasshouse (Table 4.1) from February to the end of July. No alteration to the natural daylength of Pietermaritzburg was made and the duration of day and night temperatures was extended or shortened so as to coincide with the natural daylength. For the analysis of buds from glasshouse-grown dormant plants the apical and the first

Table 4.1 Monthly temperature settings ($^{\circ}\text{C}$) maintained in the glasshouse in which the *Citrus sinensis* cv. Pickstone Valencia orange plants were grown

| Month | Feb | Mar | Apr | May | Jun | Jul |
|-------|-----|-----|-----|-----|-----|-----|
| Day | 27 | 26 | 24 | 22 | 19 | 20 |
| Night | 22 | 20 | 18 | 15 | 12 | 12 |

Subapical buds were collected from every branch of each of 20 adult- and 20 juvenile-budded plants on 30th June and 20th July, respectively. The total collective fresh mass of the adult and juvenile bud samples was 300 mg each. Samples were stored at -20°C . The tissues were macerated and extracted for 24 h in 80 percent ethanol and purified according to the method of REEVE and CROZIER (1978) as described in Chapter II. Purified extracts were separated on Whatmans No. 1 chromatography paper using the *iso*-propanol: 25% NH_4OH : H_2O (10:1:1 v/v) solvent and assayed using the dwarf rice micro-drop bioassay. In an attempt to make use of one extract for analysing both gibberellin-like substances and inhibitory substances, the aqueous phase residues were reduced to dryness after partitioning with ethyl acetate and *n*-butanol. The residue was taken up in diethyl ether and applied to sheets of Whatmans No. 1 paper for chromatographic separation. Chromatograms were divided into 10 R_f strips and these were analysed for growth inhibitor activity using the wheat coleoptile bioassay.

The use of one extract for the analysis of two hormone types in this way proved to be unsatisfactory. Consequently, plants were prepared for separate determinations of gibberellin-like and inhibitor activity. Three methods of extraction for gibberellins were employed. In the first instance the non-ionic detergent TRITON X-100 (BDH Chemicals Ltd., Poole, England) (BROWNING and SAUNDERS, 1977) was used to dissolve membranes to which gibberellins may have been adhering. Sprouting buds were collected (1400 mg) from adult and juvenile scions and these were macerated and extracted in 80 percent ethanol for 12 h at 10°C . The extract was then reduced to the aqueous phase *in vacuo* at 30°C without filtering. The extract volume was then made up to 100 ml with phosphate buffer (pH 8.0) and detergent so that the final concentration of TRITON X-100 in the extract was two percent (v/v). The extract was then incubated at 10°C for a further 12 h. It was then filtered *in vacuo*. The filtrate was partitioned against

petroleum ether, slurried with PVP, adjusted to pH 2,5 and then partitioned against ethyl acetate and *n*-butanol. The ethyl acetate and *n*-butanol soluble gibberellins were separated on Whatmans No. 1 chromatography paper with *iso*-propanol: 25% NH₄OH:H₂O (10:1:1 v/v) solvent and assayed using the dwarf rice microdrop bioassay.

In the second instance, samples of sprouting buds (2000 mg) were placed on moist filter paper and exposed to red light for 10 minutes before storage at -20°C. The standard extraction and purification procedure of REEVE and CROZIER (1978) was then followed before separation and bioassay as for the TRITON X-100 extracted samples. The third extraction procedure was employed after noting that the dwarf rice microdrop bioassay is very specific for gibberellin response (GRAEBE and ROPERS, 1978) and that the bioassay is not greatly affected by other growth promoting or inhibiting compounds. In this instance, both dormant and sprouting bud samples (1000 mg) were collected and stored at -20°C until extracted. Samples were macerated and extracted in 80 percent ethanol for 24 h at 10°C. The extract was filtered *in vacuo*, reduced to the aqueous phase *in vacuo* at 30°C and diluted to 100 ml with phosphate buffer (pH 8,0). The diluted extract was then slurried with PVP. After filtration the extract was reduced to dryness and separated on Whatmans No. 1 chromatography paper and bioassayed as for the TRITON X-100 extracted sample above.

In all three of the above methods synchronous flushing was induced in the plants by placing 10 adult and 10 juvenile plants for each sample collected into a controlled environment cabinet and subjecting them to the dormancy induction conditions described in Chapter II. Sampling for dormant buds took place when plants had been in the controlled environment cabinets for four weeks and sampling for sprouting buds took place about 10 days after removal from controlled environment cabinets and placement in a glasshouse held at a 30°/20°C diurnal temperature regime under natural daylight, which

was supplemented with five 300 W Hg-vapour lamps to provide daylengths of 16 h.

Analysis of cytokinin-like components was carried out with samples treated for the induction of synchronous flushing, as described above. The extraction and purification of cytokinins from dormant and sprouting buds was performed as described in Chapter II. Samples were then separated into the various cytokinin-like components on a Sephadex LH-20 column eluted with 10 percent methanol. Dormant and sprouting buds were bioassayed separately using the soybean callus bioassay. For the purpose of undertaking a sequential analysis of cytokinins in bursting buds, plants were first reduced to the dormant state in order to synchronise the commencement of flushing. Sixty adult- and sixty juvenile-budded plants were incubated in a glasshouse held at a 20⁰/12⁰C diurnal temperature regime for eight weeks. Sunlight provided illumination of the plants during the eight-week period from June to July. At the end of this eight-week period the temperature in the glasshouse was changed to a 30⁰/20⁰C diurnal regime and the daylength was increased to 16 h with supplemental lighting provided by five 300 W Hg-vapour lamps suspended over the plants. Twenty plants each, of the juvenile and adult types, were sampled five days after the return to the higher temperatures. Three and nine days later the sampling was repeated with a further 20 plants on each occasion. By day 14 (third sample) small leaves had begun to appear from the buds. Samples (300 mg) were extracted and purified for cytokinin analysis as specified in Chapter II and detection was by means of the soybean callus bioassay, after separation on Whatmans No. 1 chromatography paper.

Inhibitor activity was assessed in dormant and sprouting buds also. Plants were incubated in controlled environment cabinets as described above. In the first instance, samples (500 mg) were extracted in 80 percent ethanol, reduced to the aqueous phase, diluted with phosphate buffer (pH 7.4) and

slurried with PVP. After filtration, extracts were reduced to dryness, separated on Whatmans No. 1 chromatography paper with *iso*-propanol: 25% NH₄OH: H₂O (10:1:1 v/v) solvent and assayed using the wheat coleoptile bioassay. The analysis of hormones in such unpurified extracts was found to be unsatisfactory when using this bioassay system. The inferiority of these detection methods probably arose from interference of compounds retained in the extract after partial purification.

Buds were therefore specifically extracted for abscisic acid and additional steps were incorporated in the purification procedure for abscisic acid recovery. The method of SAUNDERS (1978a) for extraction and purification of abscisic acid was adopted. Accordingly, a batch of 20 juvenile and 20 adult plants was incubated in the controlled environment cabinets under dormancy inducing conditions. Buds were collected as for the above determinations and the purification procedures of SAUNDERS (1978 a) were instituted, followed by separation of the residue on silica gel TLC plates using toluene: ethyl acetate: acetic acid (40:5:2 v/v) as the solvent. Plates were developed to 150 mm in three successive runs before division into 10 R_f strips and elution of each strip with methanol. Methanolic eluates were placed directly in the repli dish compartments and the methanol was evaporated off under a stream of air before the wheat coleoptile bioassay was performed.

Gibberellin-like, cytokinin-like and inhibitory substances were analysed from sprouting buds situated on the upper, ontogenetically more adult part and from the lower, ontogenetically more juvenile part of two large Pickstone Valencia orange trees. The trees were growing in 20 l plastic bags and had been budded with juvenile scions three years prior to sampling. All branches were removed from the two-metre-tall trees and they were totally defoliated to stimulate bud sprouting along the stem (DE LANGE, 1980). Although the basal part of the stem retained large thorns, indicative of the juvenile condition, the terminal bud had attained the adult condition as was evident from the appearance of flowers after a cold induction

period. Plants were grown in a glasshouse at a 30⁰/20⁰C diurnal temperature regime and sprouting buds were collected from the lower and upper one metre of stem, respectively. After buds had been removed for gibberellin analysis, adventitious buds sprouted and these were collected 14 days later for cytokinin analysis. Fourteen days later more buds had sprouted and these were collected for abscisic acid analysis. Bud samples averaged one gramme in mass and about 70 buds in number. Buds to be analysed for gibberellins were extracted and purified according to the method of REEVE and CROZIER (1978), while the buds to be analysed for cytokinins were extracted using the procedure outlined in Chapter II. In both cases purified extracts were separated on Whatmans No. 1 chromatography paper using *iso*-propanol: 25% NH₄OH: H₂O (10:1:1 v/v) as the solvent. Chromatograms were divided into 10 equal R_f strips and assayed for gibberellin- and cytokinin-like activity using the dwarf rice microdrop and soybean callus bioassays, respectively. Buds to be analysed for abscisic acid were extracted and purified according to the method of SAUNDERS (1978 a) and separated on silica gel TLC plates using toluene: ethyl acetate: acetic acid (40:15:4 v/v). The increase in the ethyl acetate and acetic acid proportions was suggested by DUTTON (1982) as a possible means of separating abscisic acid from coumaric acid, as they normally have very similar chromatographic properties. The plates were developed to 150 mm in two successive runs before division into 10 equal R_f strips and assay using the wheat coleoptile bioassay.

In all analyses the equivalent fresh mass of bud material was extracted and bioassayed simultaneously for the various comparisons made. Estimated gibberellin A₃ and kinetin equivalents were calculated from linear curves of response for the standards included in each bioassay.

Results

The analysis of dormant buds for gibberellin-like activity revealed that in both juvenile and adult buds there were very low levels of the acidic ethyl acetate soluble and acidic *n*-butanol soluble forms (Figure 4.1).

The bioassay of these fractions revealed that although no statistically significant levels of gibberellin-like substances were detected, there may, nevertheless, be different forms present as is evident from the small peaks manifest at different R_f zones. By extrapolating from the standards included with the bioassay it appears that recoverable gibberellin-like activity did not exceed 10 ng g^{-1} fresh mass of buds, at any time in any of the dormant buds. When the aqueous phase residue from the above gibberellin extraction was analysed for inhibitor activity using the wheat coleoptile bioassay, very little difference was found between juvenile and adult responses on either date of sampling (Figure 4.2). The only zone showing the presence of inhibitors in all four analyses was below R_f 0.4. It is known that wheat coleoptiles do respond to growth promoters (MILBORROW, 1978) and indeed, a region of growth promotion was found (R_f 0,4 - 1,0) in all extracts. Such unpurified extracts were obviously of no value in the determination of inhibitors, as the growth promoters probably had an overriding effect.

Figure 4.3 illustrates the change in extractable gibberellins in relatively unpurified extracts, obtained from dormant and sprouting buds. No detectable amounts of gibberellin-like substances were found in either juvenile or adult dormant buds. In the sprouting buds, however, a marked difference existed in the measured response. Juvenile buds yielded a polar (R_f 0,1 - 0,2 and non-polar (R_f 0,7 - 1,0) zone of activity which was notably greater than the single, polar (R_f 0,2) peak of activity detected in adult buds.

GOODWIN (1978) stated that reported endogenous gibberellin levels may have to be re-evaluated, following the findings that plant material extracted in buffered TRITON X-100 detergent yields higher gibberellin levels than normal

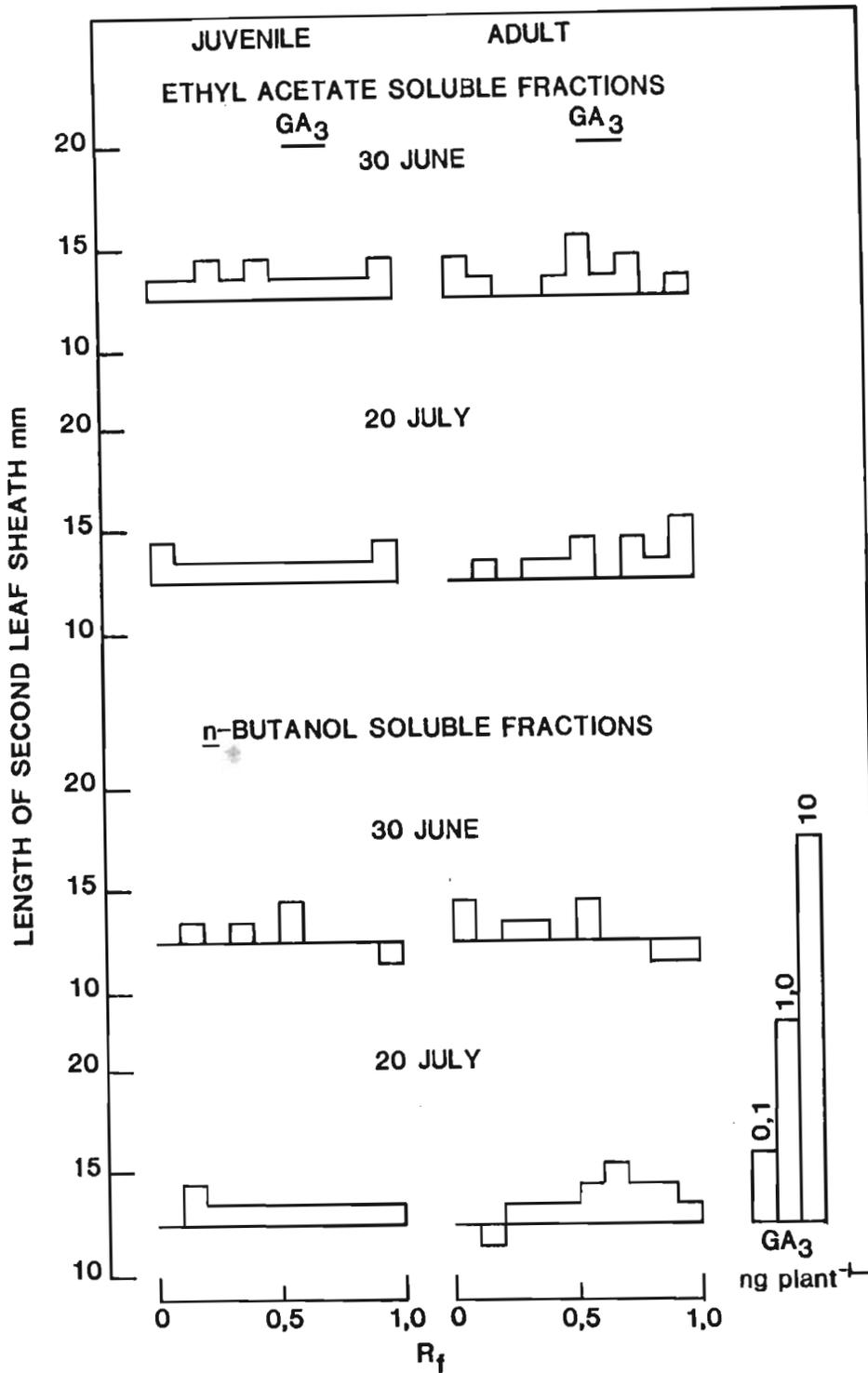


Figure 4.1 Gibberellin activity in extracts of buds obtained from dormant adult and juvenile *Citrus sinensis* cv. Pickstone Valencia orange, which were collected in mid- (30 June) and late (20 July) winter, respectively. Acidic ethyl acetate and acidic *n*-butanol soluble fractions were obtained by means of solvent partitioning and these were separated on Whatmans No. 1 chromatography paper with *iso*-propanol: 25% NH₄OH: H₂O (10:1:1 v/v) as the solvent. Activity was detected using the dwarf rice microdrop bioassay. GA₃ = gibberellin A₃.

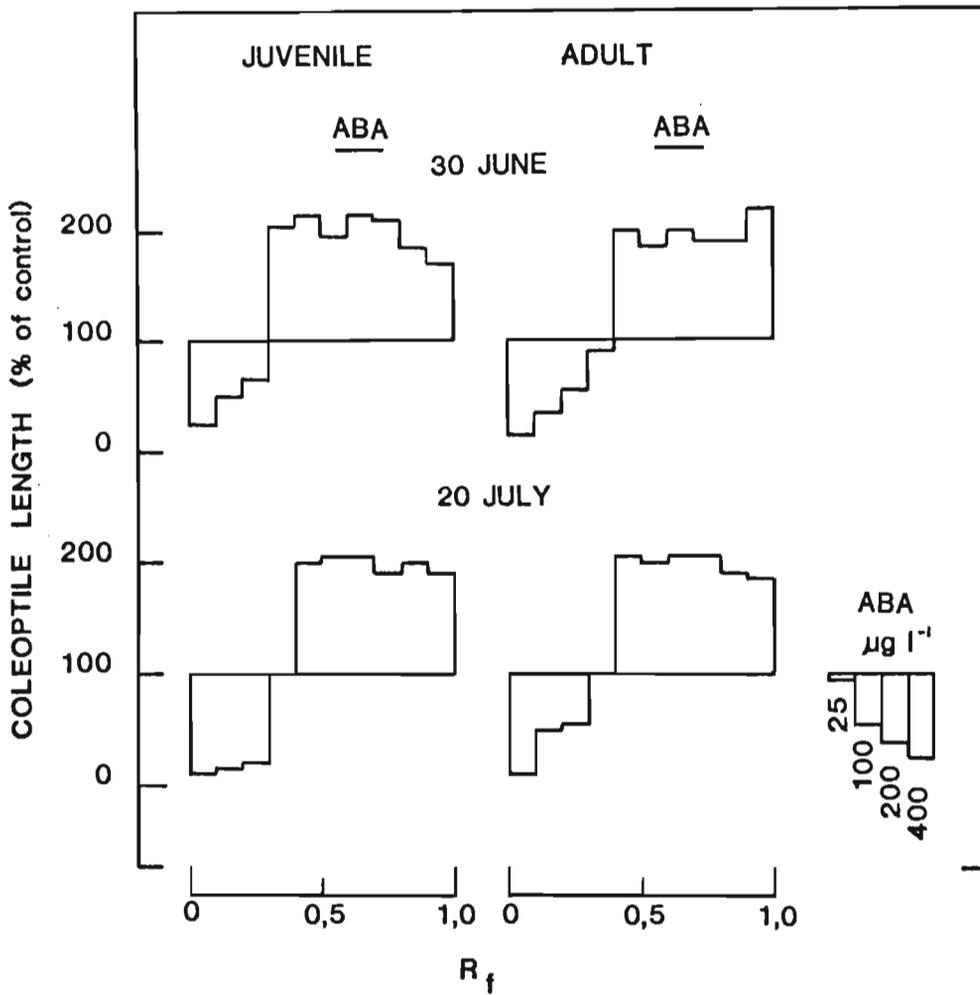


Figure 4.2 Bioassay for inhibitor activity in the aqueous phase residue after partitioning extracts of dormant buds, collected in mid- (30 June) and late (20 July) winter, against ethyl acetate and *n*-butanol. The residue was separated on Whatmans No. 1 chromatography paper using *iso*-propanol: 25% NH_4OH : H_2O (10:1:1 v/v) as the solvent. Activity was detected using the wheat coleoptile bioassay. ABA = abscisic acid.

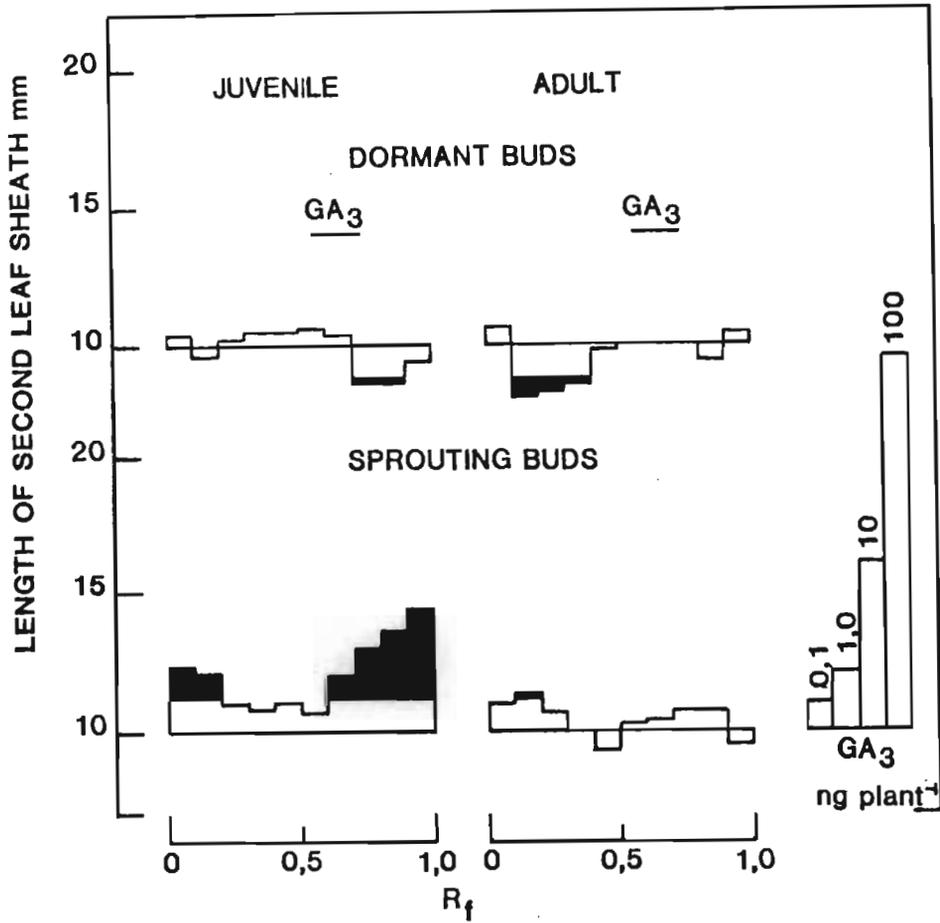


Figure 4.3 Gibberellin activity in extracts of dormant and sprouting buds obtained from *Citrus sinensis* cv. Pickstone Valencia orange. Extracts were purified by slurring with PVP before being separated on Whatmans No. 1 chromatography paper with *iso*-propanol: 25% NH₄OH: H₂O (10:1:1 v/v) as the solvent. Activity was detected using the dwarf rice microdrop bioassay. Shaded areas represent regions significantly different from the controls at the level $P = 0,05$. GA₃ = gibberellin A₃.

methanolic/ethanolic extracts. Membrane-bound gibberellins are thought to be released following the membrane-dissolving action of the detergent, thus resulting in detection of that part of the gibberellin pool which is normally discarded with the residue of particulate matter after extract filtration. Figure 4.4 shows that in all likelihood the membrane-dissolving properties of the detergent also result in the release of inhibitors which interfere with the gibberellin response in the bioassay. These inhibitory compounds partitioned into the ethyl acetate fraction and spread over a wide band on paper chromatograms (R_f 0,4 - 1,0), probably as a result of overloading the chromatograms. The *n*-butanol soluble fractions yielded small peaks of activity which differed in polarity between adult and juvenile extracts, but were not very different as far as total response was concerned. Prior treatment of bud samples with red light before extraction for gibberellins, using the method of REEVE and CROZIER (1978), did not markedly change the gibberellin recovery compared with that recovered from unpurified extracts in terms of total estimated gibberellin A_3 equivalents measured (Table 4.2). Nevertheless, very much higher levels of response were observed in the polar regions of chromatographic separation of the ethyl acetate soluble fraction in juvenile bud extracts than in adult bud extracts (Figure 4.5). Very low levels of acidic *n*-butanol soluble gibberellins were recovered from both bud types.

A striking difference in the cytokinin content of sprouting juvenile and adult buds was noted when the dynamics of these compounds were monitored during emergence from the dormant state. Figure 4.6 shows that five days after the plants were removed from the cold conditions, the juvenile buds yielded very much lower levels of the cytokinin-like compounds than adult buds. In adult buds the active compounds appeared to be of a polar form (R_f 0,3 - 0,4). No apparent qualitative changes in components of this peak occurred during bud development into new shoots, although the concentration

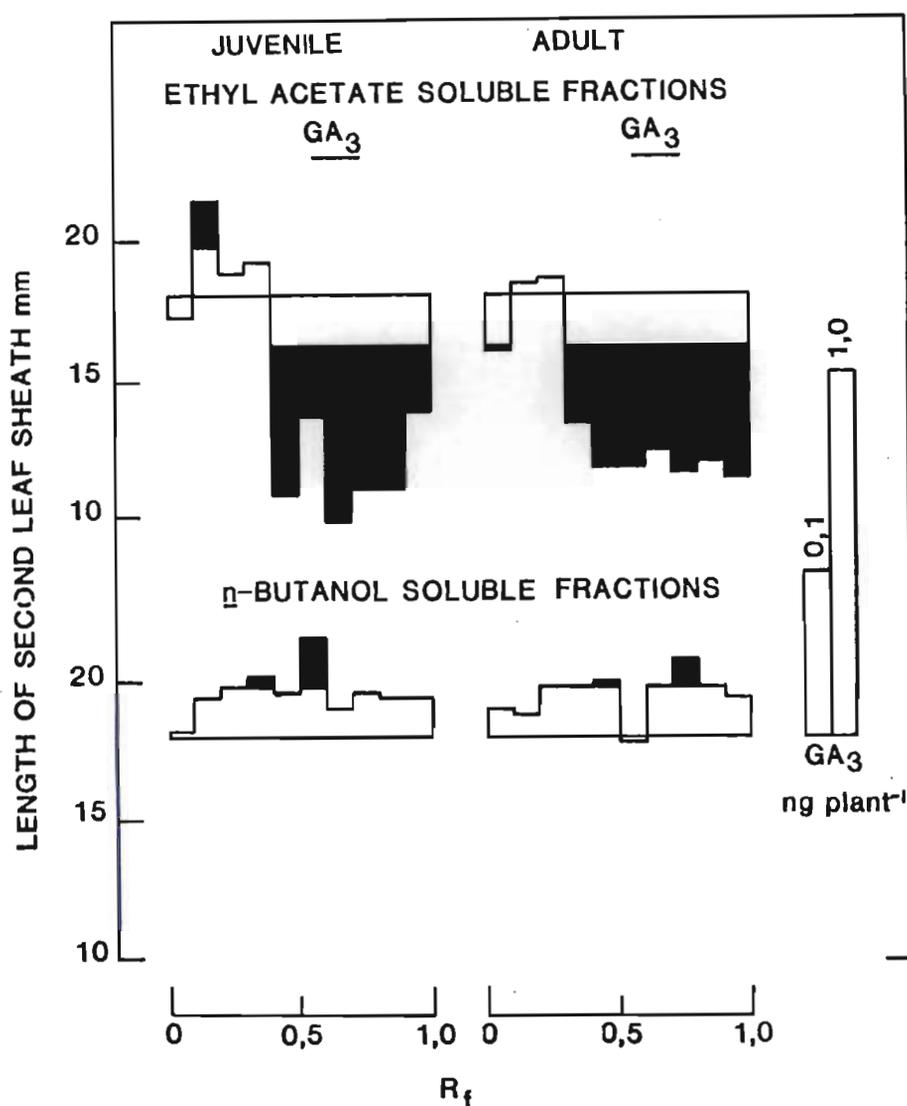


Figure 4.4 Gibberellin activity in extracts of sprouting buds collected from juvenile and adult *Citrus sinensis* cv. Pickstone Valencia orange. Acidic ethyl acetate and acidic *n*-butanol soluble fractions were obtained by solvent partitioning after samples were extracted for 12 h in 80 percent ethanol, followed by extraction for 12 h in buffered (pH 8,0) TRITON X-100 (2% v/v) detergent. Extracts were separated on Whatmans No. 1 chromatography paper using *iso*-propanol: 25% NH_4OH : H_2O (10:1:1 v/v) as the solvent. Activity was detected using the dwarf rice micro-drop bioassay. Shaded areas represent regions significantly different from the controls at the level $P = 0,05$. GA_3 = gibberellin A_3 .

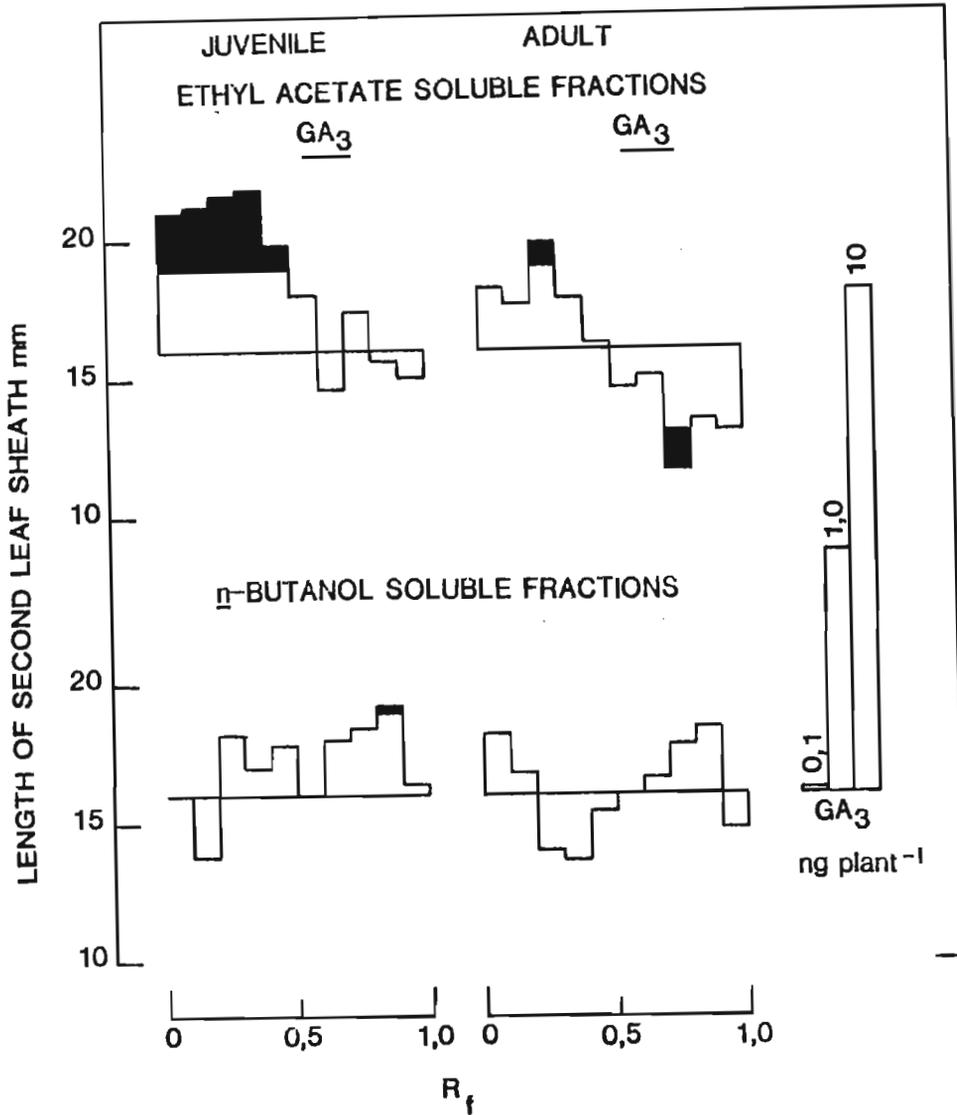


Figure 4.5 Gibberellin activity in extracts of sprouting buds collected from juvenile and adult *Citrus sinensis* cv. Pickstone Valencia orange. Acidic ethyl acetate and *n*-butanol soluble fractions were obtained by solvent partitioning. Bud samples were exposed to red light for ten minutes prior to storage at -20°C , before extraction. Extracts were separated on Whatmans No. 1 chromatography paper using *iso*-propanol: 25% NH_4OH : H_2O (10:1:1 v/v) as the solvent. Activity was detected in the dwarf rice microdrop bioassay. Shaded areas represent regions significantly different from the controls at the level $P = 0,05$. GA₃ = gibberellin A₃.

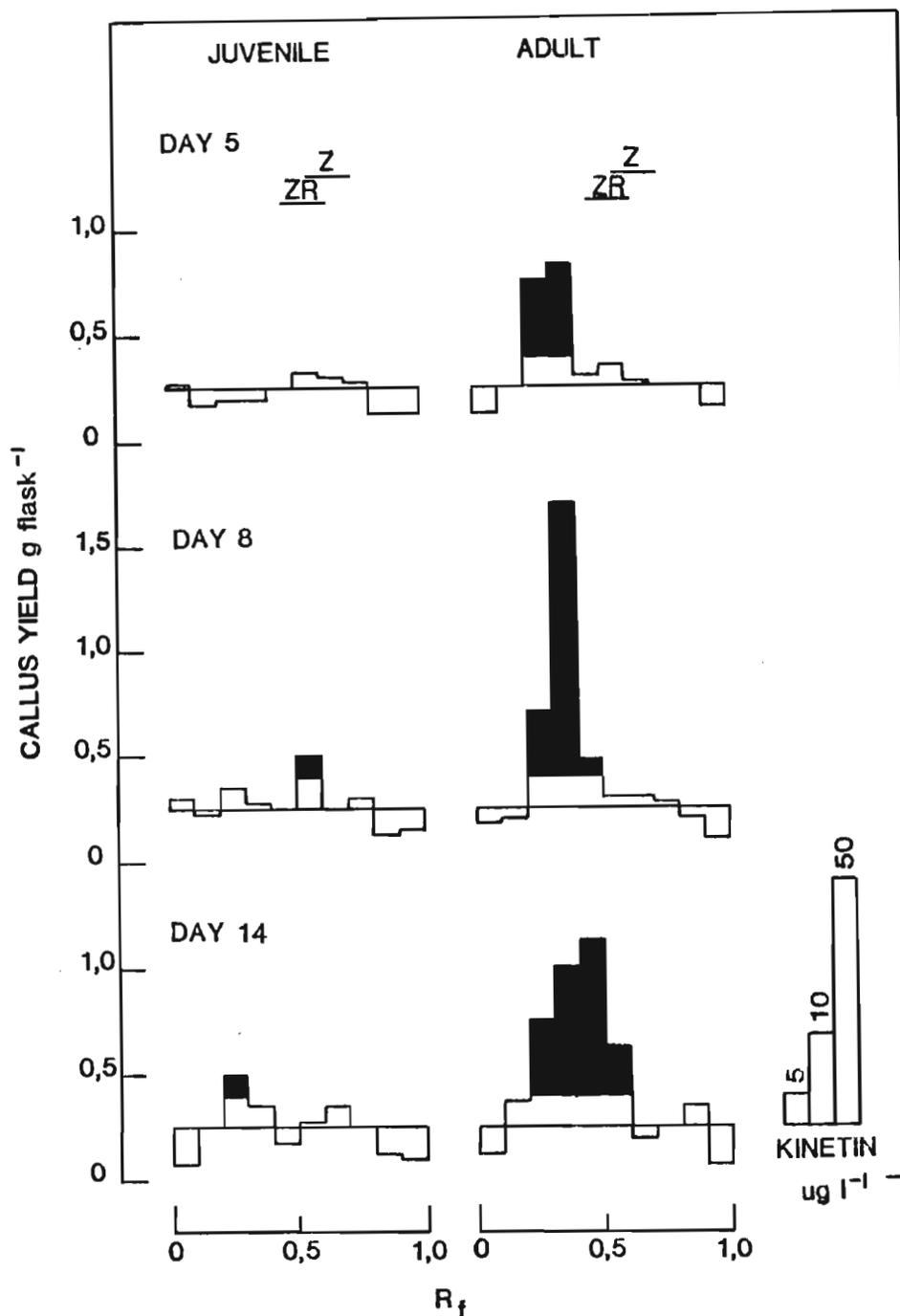


Figure 4.6 Cytokinin activity in sprouting buds of *Citrus sinensis* cv. Pickstone Valencia orange. Buds were collected on the fifth, eighth and fourteenth days after the induction of growth. Extracts were purified on Dowex 50 cation exchange resin and separated on Whatmans No. 1 chromatography paper using *iso*-propanol: 25% NH₄OH: H₂O (10:1:1 v/v) as the solvent. Activity was detected using the soybean callus bioassay. Shaded areas represent regions significantly different from the controls at the level P = 0,05. Z = zeatin; ZR = ribosyl zeatin.

did appear to increase up to day eight. By day 14 at least part of the peak of activity co-chromatographed with ribosylzeatin. In juvenile buds the only cytokinin-like compounds were a ribosylzeatin-like component on day eight and a more polar component on day 14 of sampling. Both peaks of activity indicated a low measurable cytokinin level in juvenile buds.

Cytokinin-like components from dormant and sprouting buds were bioassayed separately and the results are therefore not directly comparable. The total of recovered activity, measured in terms of kinetin equivalents shows, however, that overall activity in adult dormant buds appeared to be lower than in juvenile buds (Table 4.2). In sprouting buds (Figure 4.7), on the other hand, juvenile buds yielded much lower cytokinin-like activity than adult buds. The cytokinins appeared to be present in the free base forms in all cases, including some unidentified peaks, at elution volumes of 720-760 ml, 840-920 ml and 2300-2400 ml (Figures 4.7 and 4.8). The peak eluting at 360-400 ml in dormant juvenile buds (Figure 4.8) may have co-chromatographed with glucosylated ribosylzeatin (VAN STADEN, 1982). It is immediately apparent that a lower level of cytokinins was recovered in sprouting buds. While both adult and juvenile sprouting buds appear to contain at least one unidentified slow-eluting peak of activity (>1600 ml), the major obvious difference between these extracts (Figure 4.8) lies in the absence of detectable activity of the recognisable free base forms in juvenile buds. Adult buds yielded a significant amount of cytokinin-like activity which co-chromatographed with dihydrozeatin (1080-1160 ml). The early-eluting peak from juvenile buds coeluted with purinyl glycine, an oxidation product of zeatin (VAN STADEN, DREWES and HUTTON, 1982).

In attempting to analyse inhibitors in bud extracts it became obvious that use of the wheat coleoptile bioassay made it imperative to assay only purified samples. Figure 4.9 reveals that in extracts purified only by slurring with PVP the growth promoters and inhibitors beyond R_f 0,1 may have had

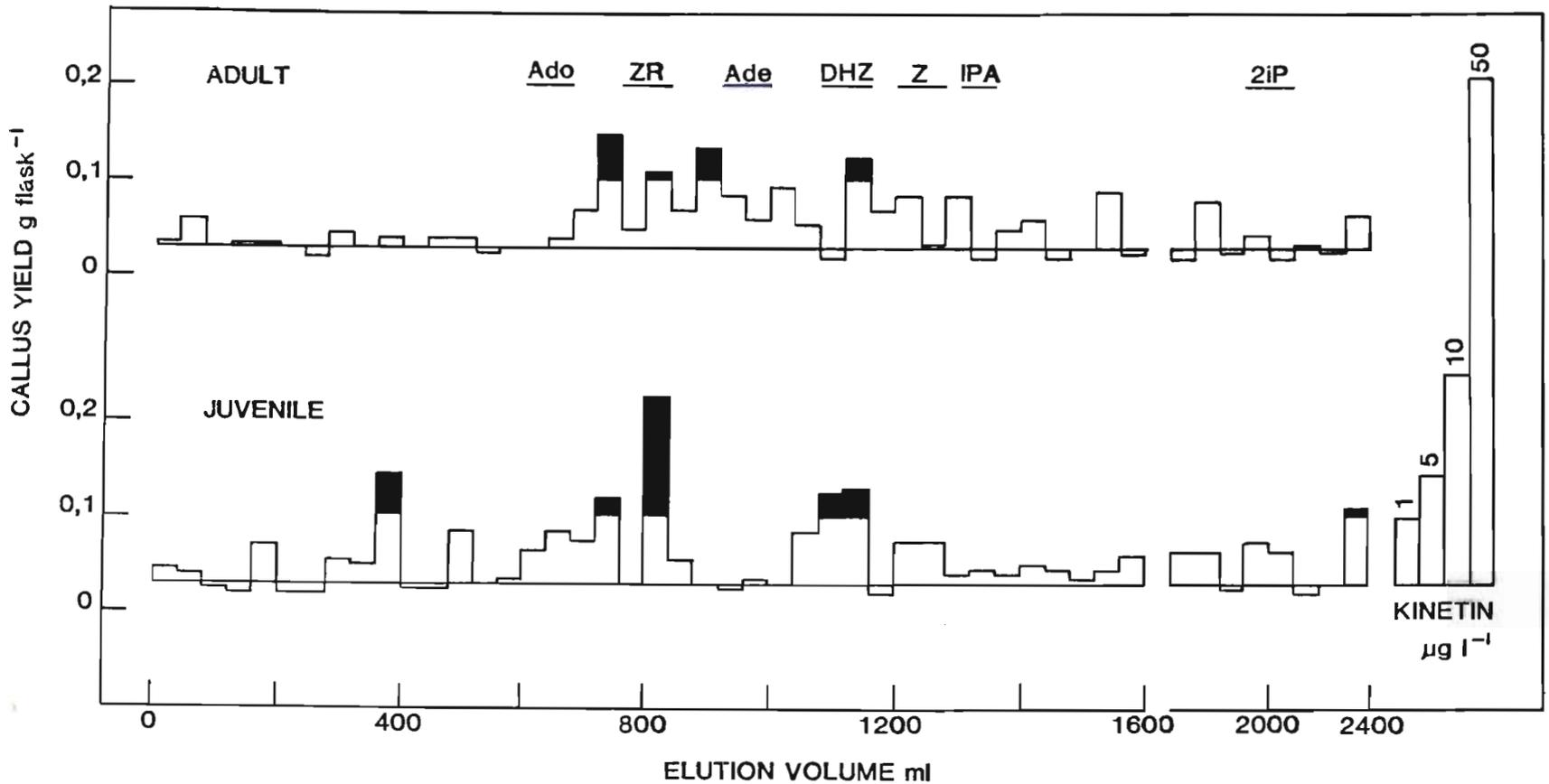


Figure 4.7 Cytokinin activity in dormant buds collected from juvenile and adult *Citrus sinensis* cv. Pickstone Valencia orange. Dowex 50 purified extracts were fractionated on Sephadex LH-20 columns eluted with 10 percent methanol. Activity was detected using the soybean callus bioassay. Shaded areas represent regions significantly different from the controls at the level $P=0,05$. Ado = adenosine; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = *iso*-pentenyl adenosine and 2iP = *iso*-pentenyl adenine.

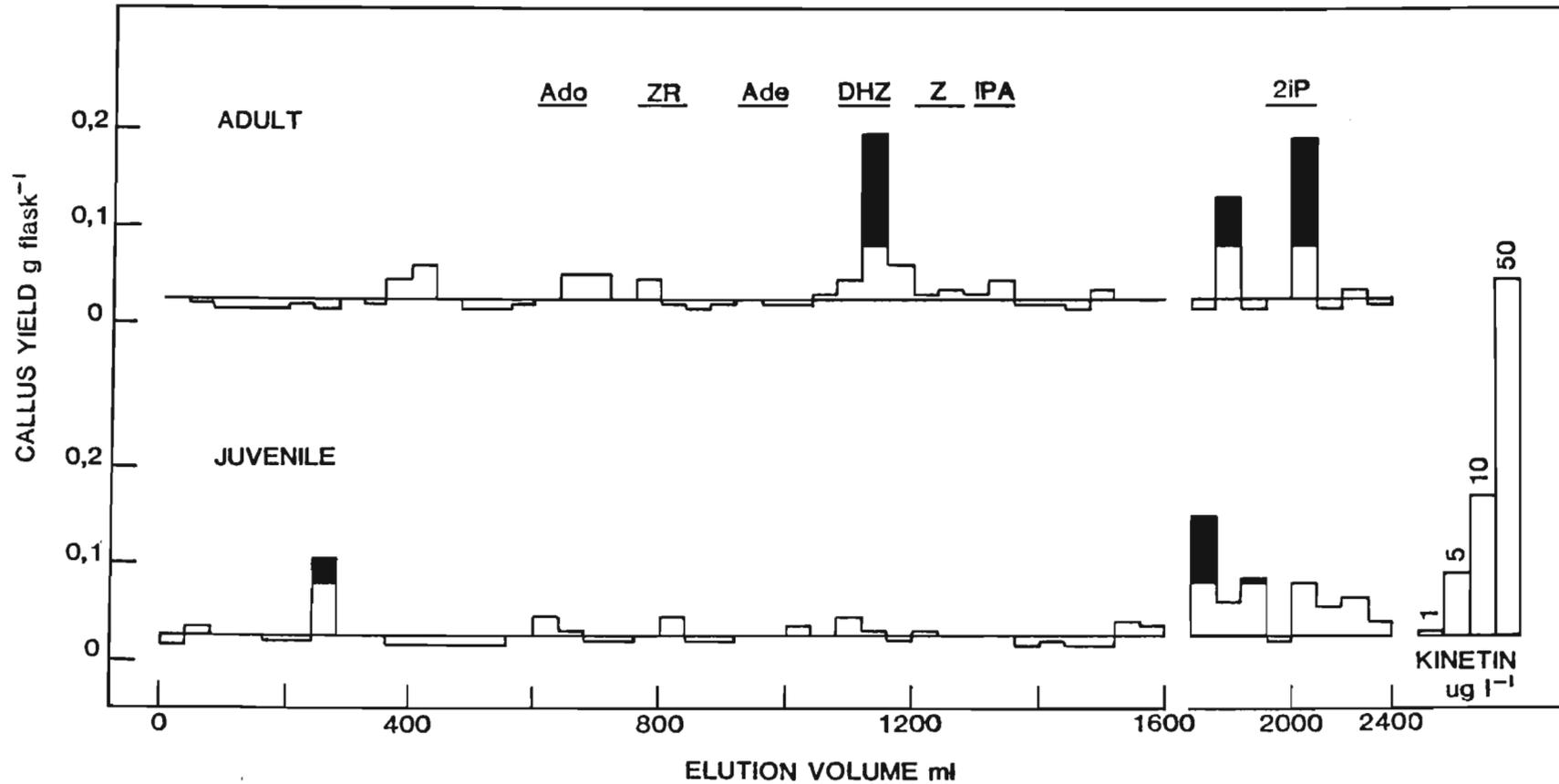


Figure 4.8 Cytokinin activity in sprouting buds collected from juvenile and adult *Citrus sinensis* cv. Pickstone Valencia orange. Dowex 50 purified extracts were fractionated on Sephadex LH-20 columns eluted with 10 percent methanol. Activity was detected using the soybean callus bioassay. Shaded areas represent regions significantly different from the controls at the level $P = 0,05$. Ado = adenosine; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = *iso*-pentenyl adenosine and 2iP = *iso*-pentenyl adenine.

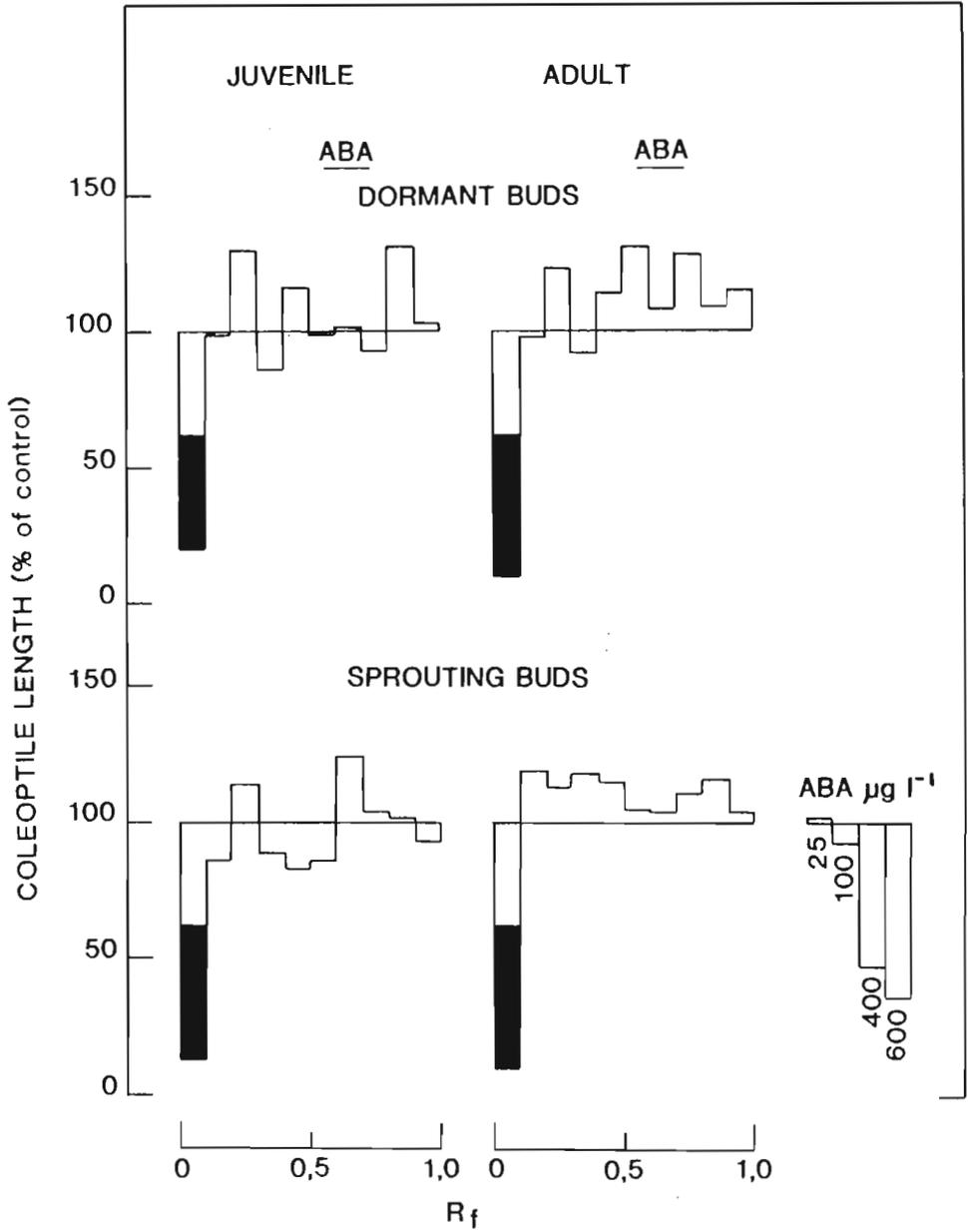


Figure 4.9 Inhibitor activity in dormant and sprouting buds collected from juvenile and adult *Citrus sinensis* cv. Pickstone Valencia orange. Extracts were purified by slurring with PVP before being separated on Whatmans No. 1 chromatography paper with *iso*-propanol: 25% NH₄OH: H₂O (10:1:1 v/v) as the solvent. Activity was detected using the wheat coleoptile bioassay. Shaded areas represent regions significantly different from the controls at the level $P = 0,05$. ABA = abscisic acid.

counteractive effects and thus provided results which showed only small differences from the controls. Such an argument was verified to some extent by the results presented in Figure 4.10 for extracts additionally purified by solvent partitioning. Significant depression of growth occurred in zones co-chromatographing with abscisic acid in juvenile dormant and sprouting buds and in adult sprouting buds. Differences in concentration were small as evinced by this bioassay result. Other regions of growth inhibition were also evident but were due to other sources of inhibition.

Results of the analyses of sprouting buds from the basal (juvenile) and distal (adult) portions of tall grafted plants are presented in Figure 4.11 for gibberellin-like substances, Figure 4.12 for cytokinin-like substances and in Figure 4.13 for abscisic acid-like components. In the gibberellin bioassay, the lower sprouting buds yielded a comparatively large peak of activity at R_f 0,5 - 0,6 in the acidic ethyl acetate soluble fraction and no detectable gibberellin-like activity in the acidic *n*-butanol soluble fraction. The detectable gibberellin-like substances of the acidic ethyl acetate fraction from upper buds appeared to consist of a polar (R_f 0,1) and a non-polar fraction (R_f 0,7 - 1,0), while the acidic *n*-butanol soluble fraction contained two regions of activity, at R_f 0,7 - 0,8 and at R_f 1,0. Total gibberellin-like activity, as estimated by the collective gibberellin A_3 equivalents for the significant peaks from the extracts respectively, was greater in the lower buds (Table 4.2).

In sprouting buds on the main stem, little difference in extractable cytokinins was detected in respect of both quantity and quality of cytokinin-like activity (Figure 4.12). The cytokinin-like compounds from both bud sources co-chromatographed with zeatin and ribosylzeatin (R_f 0,6 - 0,8).

As with the gibberellin analysis, the detectable abscisic acid-like content in upper sprouting buds was also lower than in the more basal buds (Figure 4.13), but the difference was less marked. Although the buds were

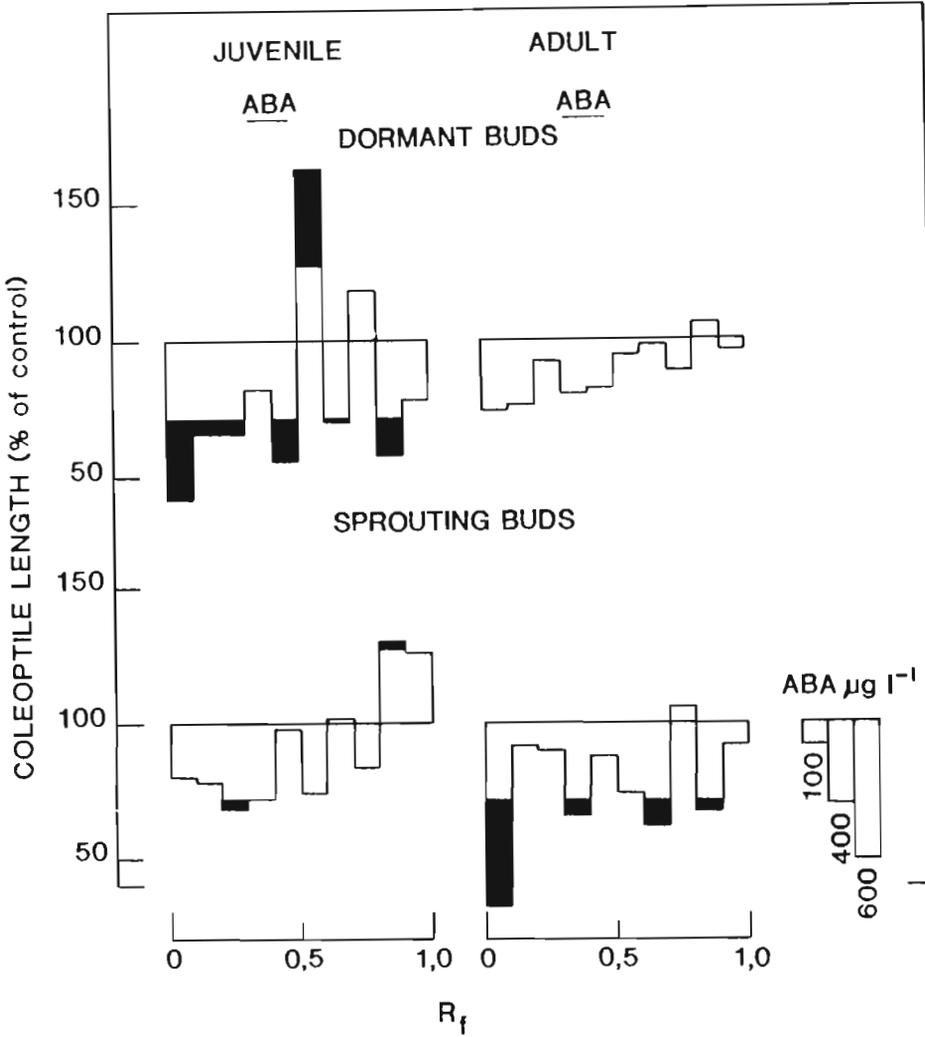


Figure 4.10 Inhibitor activity in dormant and sprouting buds collected from juvenile and adult *Citrus sinensis* cv. Pickstone Valencia orange. Diethyl ether soluble fractions were obtained after solvent partitioning with sodium hydrogen carbonate and these were separated on silica gel TLC plates using toluene: ethyl acetate: acetic acid (40:5:2 v/v) as the solvent. Activity was detected using the wheat coleoptile bioassay. Shaded areas represent regions significantly different from the controls at the level $P = 0,05$. ABA = abscisic acid.

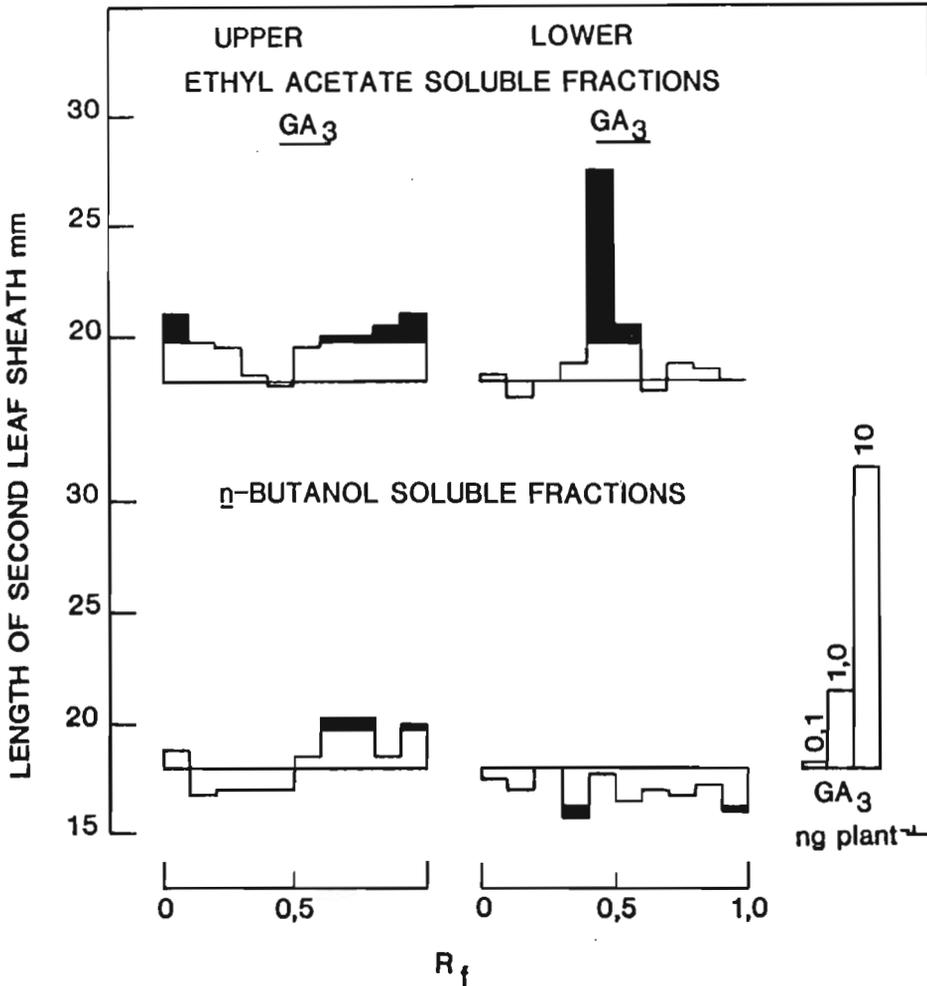


Figure 4.11 Gibberellin-like activity in sprouting buds collected from the lower (basal) and upper (distal) halves of two metres tall stems of *Citrus sinensis* cv. Pickstone Valencia orange. Acidic ethyl acetate and acidic *n*-butanol soluble fractions were obtained by solvent partitioning and these were separated on Whatmans No. 1 chromatography paper using *iso*-propanol: 25% NH_4OH : H_2O (10:1:1 v/v) as the solvent. Activity was detected using the dwarf rice microdrop bioassay. Shaded areas represent regions significantly different from the controls at the level $P = 0,05$. GA_3 = gibberellin A_3 .

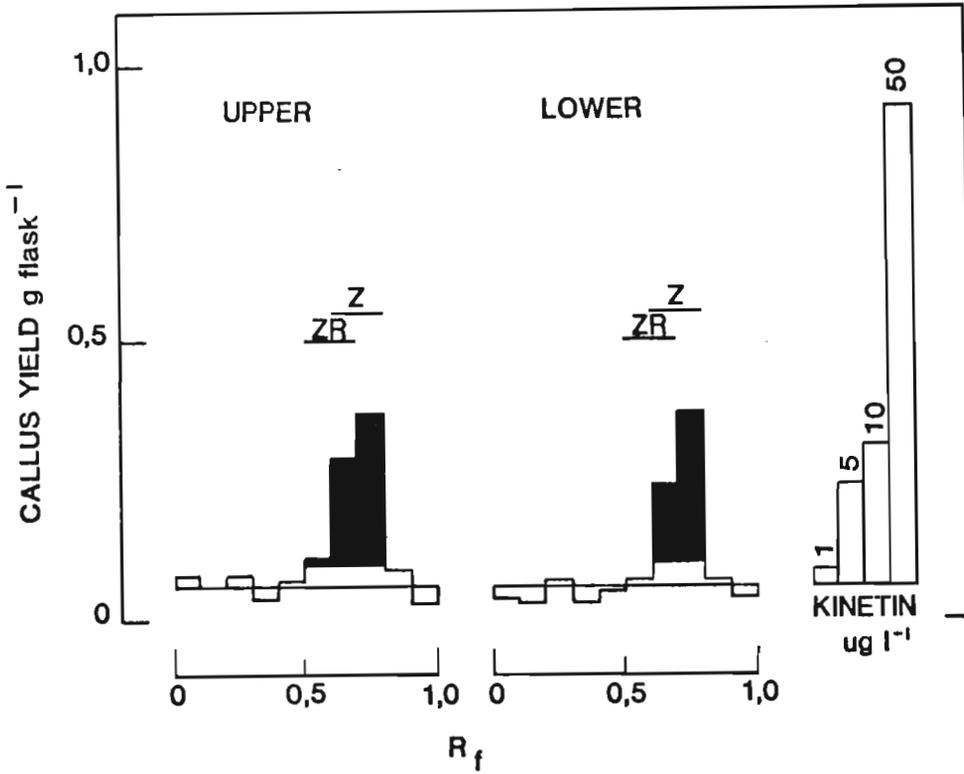


Figure 4.12 Cytokinin activity in sprouting buds collected from the lower(basal) and upper (distal) halves of two metres tall stems of *Citrus sinensis* cv. Pickstone Valencia orange. Extracts were purified on Dowex 50 cation exchange resin and separated on Whatmans No. 1 chromatography paper using *iso*-propanol: 25% NH₄OH: H₂O (10:1:1 v/v) as the solvent. Activity was detected using the soybean callus bioassay. Shaded areas represent regions significantly different from the controls at the level P = 0,05. Z = zeatin; ZR = ribosylzeatin.

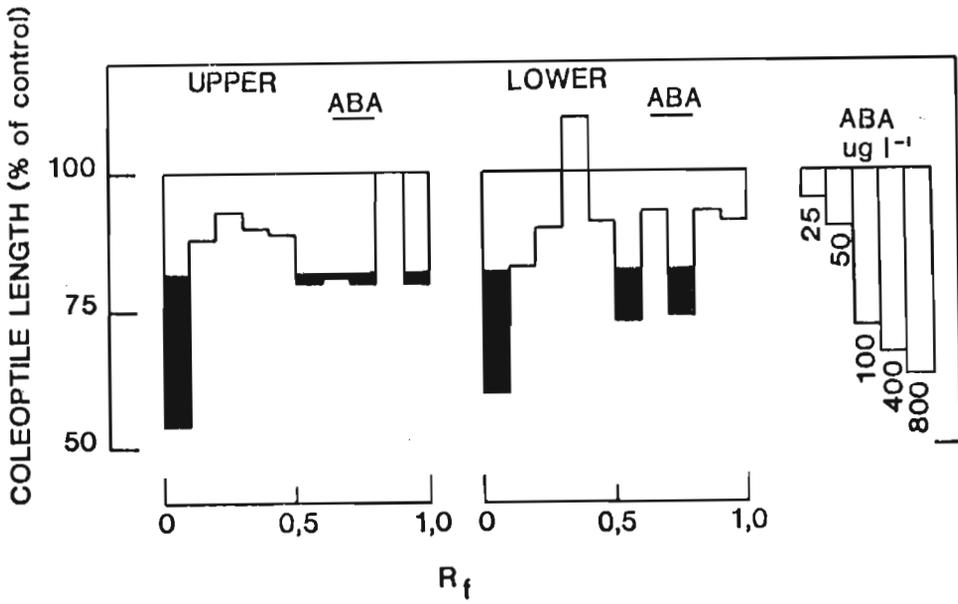


Figure 4.13 Inhibitor activity in sprouting buds collected from the lower (basal) and upper (distal) halves of two metres tall stems of *Citrus sinensis* cv. Pickstone Valencia orange. Diethyl ether soluble extracts were obtained after solvent partitioning with sodium hydrogen carbonate and these were separated on silica gel TLC plates with toluene: ethyl acetate: acetic acid (40:15:4) as the solvent. Activity was detected in the wheat coleoptile bioassay. Shaded areas represent regions significantly different from the controls at the level $P = 0,05$. ABA = abscisic acid.

Table 4.2 The total combined levels of detectable gibberellin- and cytokinin-like hormones in buds of *Citrus sinensis* cv. Pickstone Valencia, as estimated from regression lines calculated from bioassay data

| Bud source/ condition | Gibberellin-like compounds ng g ⁻¹ GA ₃ equivalents | | Cytokinin-like compounds ng g ⁻¹ Kinetin equivalents | | Data source |
|--------------------------|---|---------|---|-------|----------------------|
| | Juv. | Adult | Juv. | Adult | |
| Dormant buds (winter) | <10 | <10 | 672 | 492 | Fig. 4.1 Fig. 4.6 |
| Sprouting buds i* | 98 | 47 | | | Fig. 4.3 |
| ii. | <10 | <10 | | | Fig. 4.4 |
| iii. | 105 | 30 | 70 | 329 | Fig. 4.5 Fig. 4.7 |
| Developing buds | | **Day 5 | <15 | 374 | |
| | | Day 8 | 104 | 1656 | |
| | | Day 14 | 107 | 1006 | Fig. 4.10 |
| Lower buds | 1348 | | 312 | | Fig. 4.11 |
| Upper buds | | 616 | | 400 | Fig. 4.12 |

* extraction method: i. partial purification
ii. Triton X-100
iii. red light pretreatment

** time from growth stimulation - see text

specifically extracted and purified for the abscisic acid-like component, other zones of inhibition, as detected with the wheat coleoptile bioassay, were also evident. Some of these zones of inhibitor activity were common to both extracts, viz. at R_f 0,1 and R_f 0,6 - 0,7; while only the upper buds appeared to contain inhibitor-like activity at R_f 1,0.

The concentration gradient of standards included with each bioassay was used to estimate the fresh mass concentration of active hormones, based on interpolating statistically significant bioassay responses in linear regression curves of concentration and bioassay response. These values are presented in Table 4.2. In all bud samples analysed, the juvenile tissues contained equal or greater concentrations of gibberellin-like substances. The situation varied for cytokinins and the implications of the detectable differences will be discussed later.

Experiment 2

Although it is apparent from grafting experiments that the locus of juvenile or adult growth control lies in the shoot apex (CAMERON and SOOST, 1952; HENDRY and ALLAN, 1980), it is not immediately apparent how other parts of the plant may respond to such directed behaviour. There are a number of reasons justifying an examination of hormone levels in plant parts distant from the shoot apex. By definition, hormones are produced at one site and are active elsewhere. Even when investigating only a particular organ, the synthesis, transport, storage and metabolism of hormones needs to be considered in relation to adjacent organs or plant parts. It would be simplistic to search for correlations between the growth rate of plant tissues and endogenous hormone levels. Nevertheless, there is a system of "spatial coordination" (WAREING and PHILLIPS, 1978) operative in plants and this system appears to depend upon the movement of regulatory substances between cells and tissues. The differences in many measurable aspects of growth in shoots, stems and roots between juvenile and adult *Citrus* plants (HENDRY and ALLAN, 1980) may be related to hormone differences.

In this experiment the differences in gibberellin- and cytokinin-like substances and inhibitory substances were investigated in vital plant parts with the aim of elucidating their role in regulating the greater overall vigour of juvenile- as opposed to adult-budded *Citrus* plants.

Experimental procedure

Material used in these experiments was collected from plants which were grown either in a controlled environment cabinet under dormancy induction conditions, as described in Chapter II, or in a glasshouse under conditions suitable for active growth. For the purpose of analysing gibberellin-like activity in expanding shoots, plants in which the lowest leaf in the new flush had reached approximately maximum leaf area were selected. The whole shoot, including the leaves and stem, was harvested from such plants to supply a total fresh mass of 10 g each from juvenile and adult plants. The gibberellin-like substances were recovered, after extraction and purification as described in Chapter II. These extracts were assayed using the dwarf rice microdrop bioassay. The ethyl acetate soluble and *n*-butanol soluble fractions were bioassayed separately.

Extracts were obtained from the fibrous roots of dormant and flushing plants. These were purified for gibberellin detection and separated as described in Chapter II and the ethyl acetate soluble and *n*-butanol soluble fractions were assayed separately using the dwarf rice microdrop bioassay. Similar samples of fibrous roots from dormant plants were extracted, purified and separated for the recovery of cytokinin-like compounds. Ten grammes of material was used for each gibberellin and cytokinin extraction. Cytokinin-like activity was determined using the soybean callus bioassay after purification and separation on a Sephadex LH-20 column eluted with 10 percent aqueous methanol, as described in Chapter II.

The investigation of transportation of hormones relies in part on detection of these compounds in plant parts or structures differentiated for the

purpose of providing translocation passages. Xylem exudate from decapitated plants is commonly analysed for root derived substances. However, *Citrus* plants do not yield any significant amount of xylem exudate when decapitated. An alternative method of employing centrifugal force for the extraction of stem- and root-localized substances (GOLDSCHMIDT and MONSELISE, 1968; MORENO and GARCIA-MARTINEZ, 1980) was therefore employed. Scion stems, rootstocks and tap roots from flushing and quiescent plants were cut into 100 mm segments and placed in glass centrifuge tubes with the ends of the segments dipping into two millilitres of methanol. Each sample consisted of three plants. Centrifugation provided a force of 3000 g for 30 minutes on the segments. The methanol extracts were combined for each specific tissue extracted and strip-loaded onto half sheets of Whatmans No. 1 chromatography paper and separated with the *iso*-propanol: 25% NH₄OH: H₂O (10:1:1 v/v) solvent system. The chromatograms were divided into 10 equal R_f strips and these were bioassayed for gibberellin-like activity using the dwarf rice microdrop bioassay. The extracts from the flushing and quiescent plants were analysed in separate bioassays. This technique was refined further in the analysis of cytokinin-like compounds in rootstock stem bark and wood. Three adult and three juvenile flushing plants were randomly selected and the bark was stripped from the rootstock stem. The total adjusted fresh mass of bark analysed was 22 g and of wood was 62 g for both juvenile and adult tissues. The bark was deep-frozen, freeze-dried, ground to a fine powder and extracted, purified and separated on a Sephadex LH-20 column as described in Chapter II. The wood was cut into 100 mm segments and subjected to centrifugation as described above. The methanol fractions were combined for the juvenile and adult centrifugates respectively, taken to dryness *in vacuo*, redissolved in one millilitre of 10 percent methanol and applied to a Sephadex LH-20 column which was eluted with 10 percent methanol. Fractions were collected and bioassayed as described in Chapter II. Bark extracts and wood centrifugates of juvenile and adult plants were bioassayed separately.

Inhibitor activity in the scion stems of dormant adult and juvenile plants was tested by centrifuging the stems of three juvenile and three adult plants which had been incubated at 13⁰/9⁰C; 8 hour daylength for 28 days. The centrifugate extraction procedure followed was as outlined above. The total fresh mass of stems was 75 grammes. Methanolic centrifugates were strip-loaded onto Whatmans No. 1 chromatography paper and separated with the *iso*-propanol: 25% NH₄OH: H₂O (10:1:1 v/v) solvent system. The 10 R_f strips were bioassayed using the wheat coleoptile bioassay as described above.

Results

The analysis of gibberellin-like substances in expanding, but fairly well developed stem flush material revealed that juvenile flushes contained higher levels of relatively polar gibberellin-like substances in comparison with adult flushes (Figure 4.14 A). The detectable gibberellin-like substances appeared to be of a polar nature as they migrated to zones below R_f 0,5 in both adult and juvenile extracts. Significant inhibition of growth was observed in part of the less polar region of the chromatograms (R_f 0,7 - 0,9). In the acidic *n*-butanol soluble fractions the reverse situation held. Figure 4.14 B reveals that the adult tissues appear to contain higher levels of the highly polar or conjugated gibberellin-like substances and that little or no inhibitors which are active in the dwarf rice bioassay are present in these fractions. The zones of significant activity in the extract of adult tissues were R_f 0,2; 0,5 and 1,0.

There appears to be a higher level of gibberellin-like substances in the fibrous roots of both dormant and flushing adult-budded plants than in similar juvenile-budded plants. Although all levels of response were low, the slightly higher levels of gibberellin-like substances in fibrous roots of adult-budded plants were evident in both the acidic ethyl acetate soluble fraction and the acidic *n*-butanol soluble fraction (Figure 4.15 A,B). Conversely, cytokinin-like substances in the fibrous roots of juvenile-budded

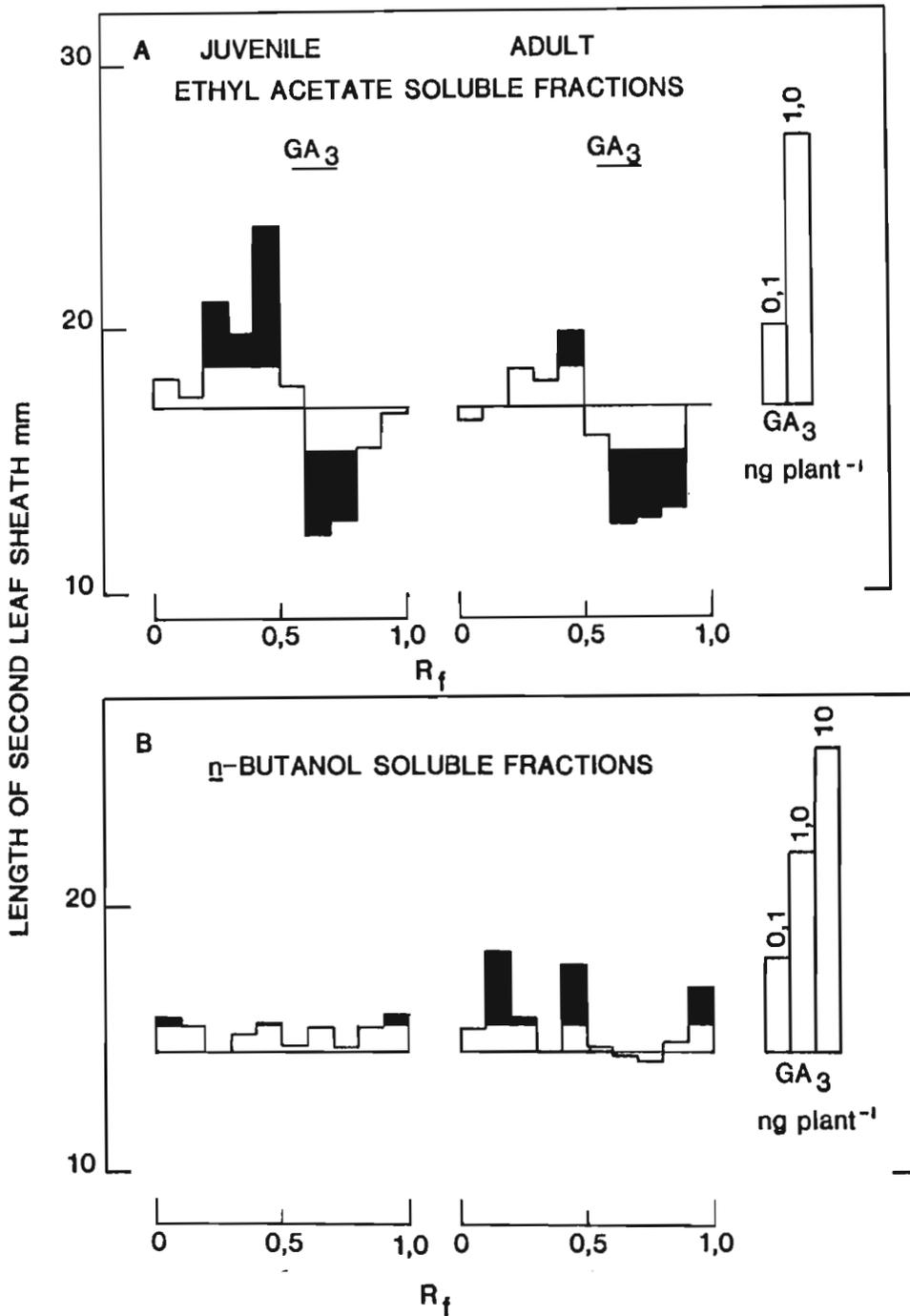


Figure 4.14 Gibberellin activity in developing shoot flushes of juvenile and adult *Citrus sinensis* cv. Pickstone Valencia orange. Acidic ethyl acetate soluble (A) and acidic *n*-butanol soluble (B) fractions were obtained by solvent partitioning and these were separated on Whatmans No. 1 chromatography paper with *iso*-propanol: 25% NH_4OH : H_2O (10:1:1 v/v) as the solvent. Activity was detected in the dwarf rice microdrop bioassay. Shaded areas represent regions significantly different from the controls at the level $P = 0,05$. GA_3 = gibberellin A_3 .

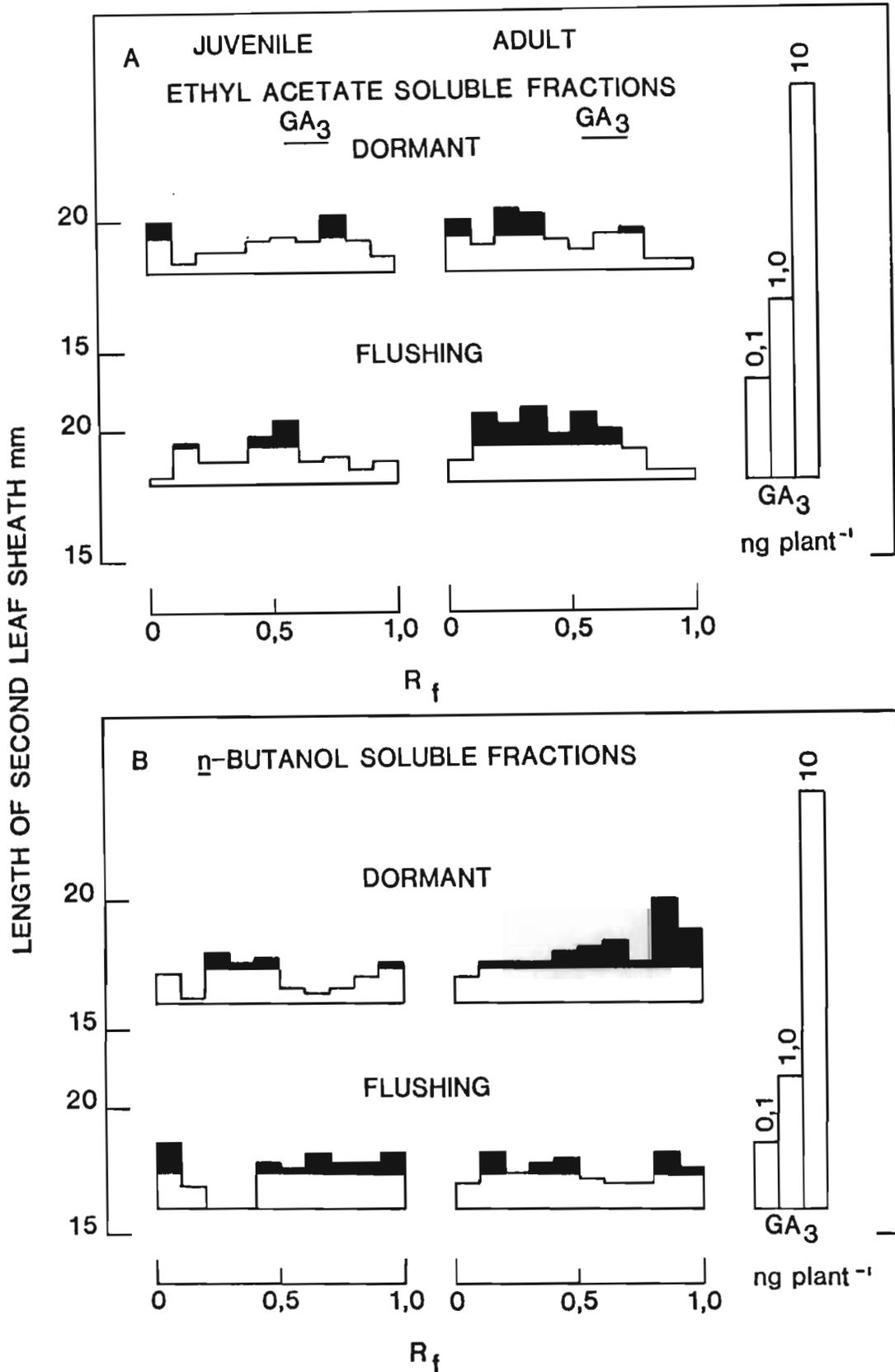


Figure 4.15 Gibberellin activity in the fibrous roots of *Citrus limon* cv. Rough lemon, which had been budded with either juvenile or adult *Citrus sinensis* cv. Pickstone Valencia orange. Root samples were collected from both dormant and flushing plants. Acidic ethyl acetate (A) and acidic *n*-butanol (B) soluble fractions were obtained by solvent partitioning and these were separated on Whatmans No. 1 chromatography paper with *iso*-propanol: 25% NH₄OH: H₂O (10:1:1 v/v) as the solvent. Activity was detected in the dwarf rice microdrop bioassay.

plants were present at a higher level than in adult-budded plants (Figure 4.16). A trace amount of a substance coeluting with zeatin was detected in juvenile-budded plant extracts, while the remainder of the activity appeared in the peak coeluting with ribosylzeatin. The adult-budded plants yielded only a ribosylzeatin-like peak of activity.

Figure 4.17 depicts the gibberellin-specific response of the centrifugate obtained from the scion stems, rootstock stems and tap roots of flushing adult- and juvenile-budded Pickstone Valencia plants. Little difference was apparent in the level of response from scion and rootstock stem centrifugates. The scion stem fractions of both adult and juvenile tissues yielded a polar component (R_f 0,1 - 0,2) which contributed most of the gibberellin-like activity detected. Tap roots of adult-budded plants yielded greater gibberellin-like activity than the tap roots of juvenile-budded plants. The analysis of the gibberellin-specific response from centrifugates of both adult- and juvenile-budded plants revealed that the organs analysed contained much higher levels of gibberellin-like substances when the plants were in the quiescent state (Figure 4.18). These centrifugates yielded fairly well defined peaks of activity compared with extracts obtained from macerated tissues. Tissues from adult-budded plants showed a response only in the zone corresponding with GA_3 migration (R_f 0,6), while stem and root segments from juvenile-budded plants yielded centrifugates showing a response at three specific zones: R_f 0,1; R_f 0,4 - 0,6 and R_f 0,8. In all cases levels of gibberellin-like substances in dormant juvenile-budded plant tissues appeared to exceed those in dormant adult-budded plant tissues. Levels of gibberellin-like compounds in the tap root centrifugate of adult-budded plants were very low. In comparing the levels of cytokinin-like compounds obtained by centrifugation from the rootstock wood with those obtained from macerated and extracted bark, it was clear that a marked difference exists in this respect

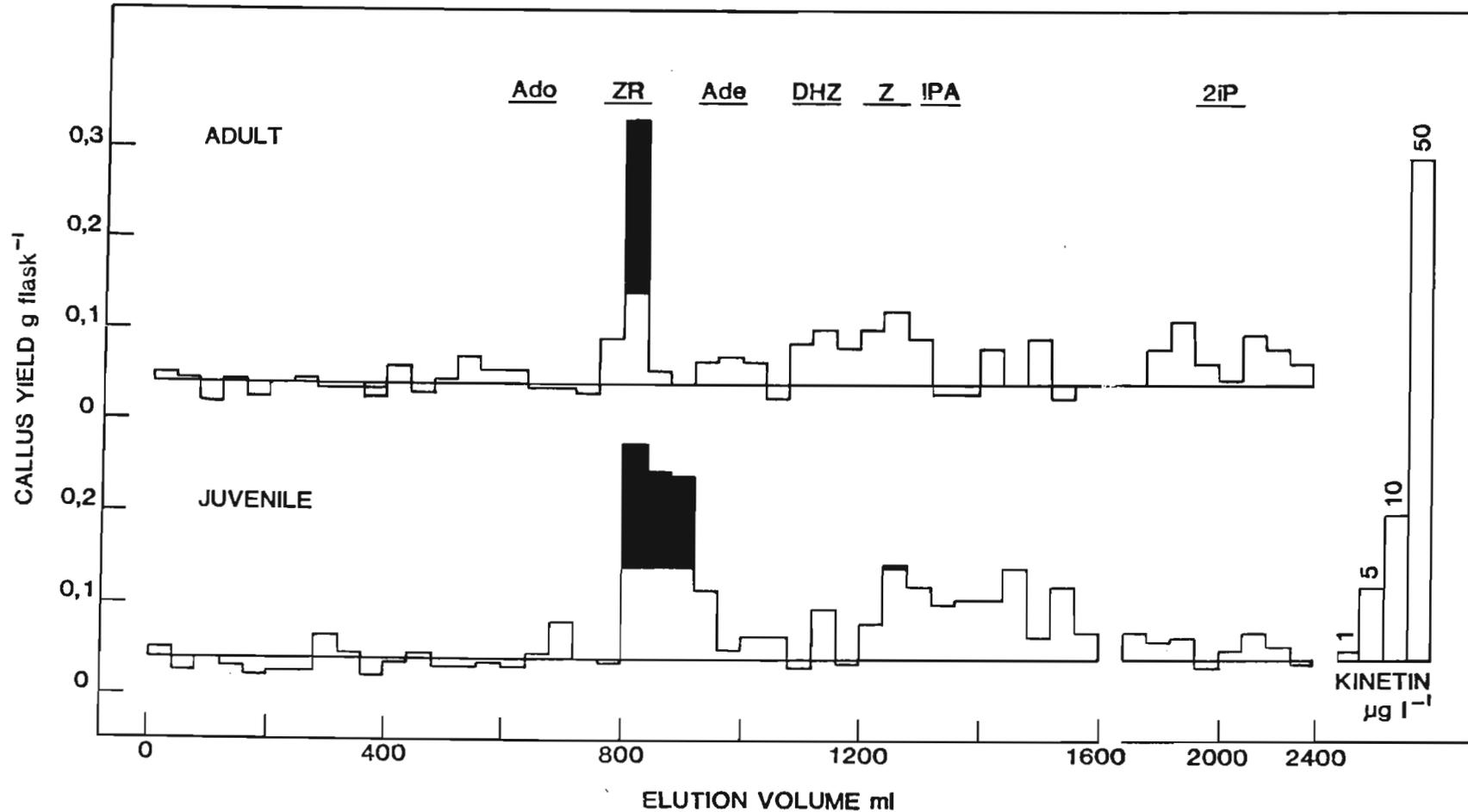


Figure 4.16 Cytokinin activity in the fibrous roots of *Citrus limon* cv. Rough lemon, which had been budded with either juvenile or adult *Citrus sinensis* cv. Pickstone Valencia orange. Plants were dormant when samples were collected. Extracts were purified with Dowex 50 cation exchange resin and fractionated on Sephadex LH-20 columns eluted with 10 percent methanol. Activity was detected using the soybean callus bioassay. Shaded areas represent regions significantly different from the controls at the level $P = 0,05$. Ado = adenosine; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = *iso*-pentenyl adenosine and 2iP = *iso*-pentenyl adenine.

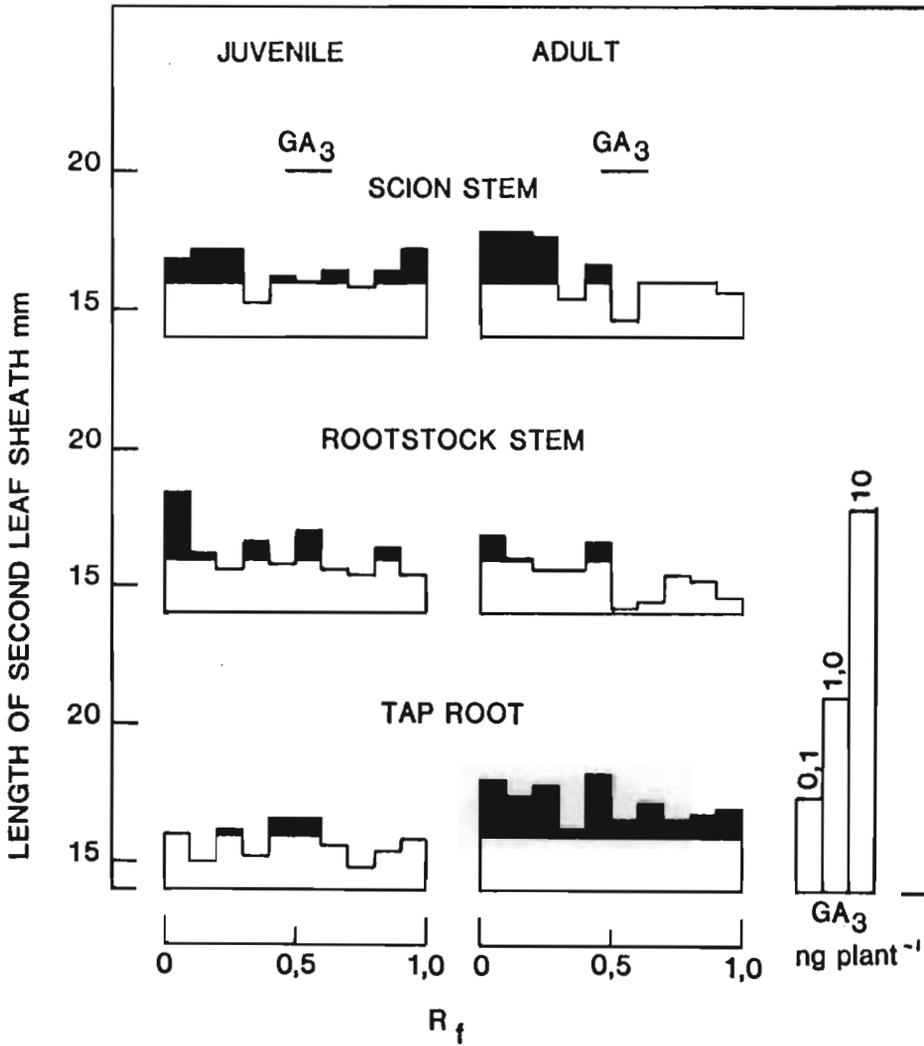


Figure 4.17 Gibberellin activity in the centrifugate obtained from the scion stem, rootstock stem and tap root of flushing plants of juvenile- and adult-budded *Citrus sinensis* cv. Pickstone Valencia orange. Centrifugates were separated on Whatmans No. 1 chromatography paper with *iso*-propanol: 25% NH₄OH: H₂O (10:1:1 v/v) as the solvent. Activity was detected using the dwarf rice microdrop bioassay. Shaded areas represent regions significantly different from the controls at the level P = 0,05. GA₃ = gibberellin A₃.

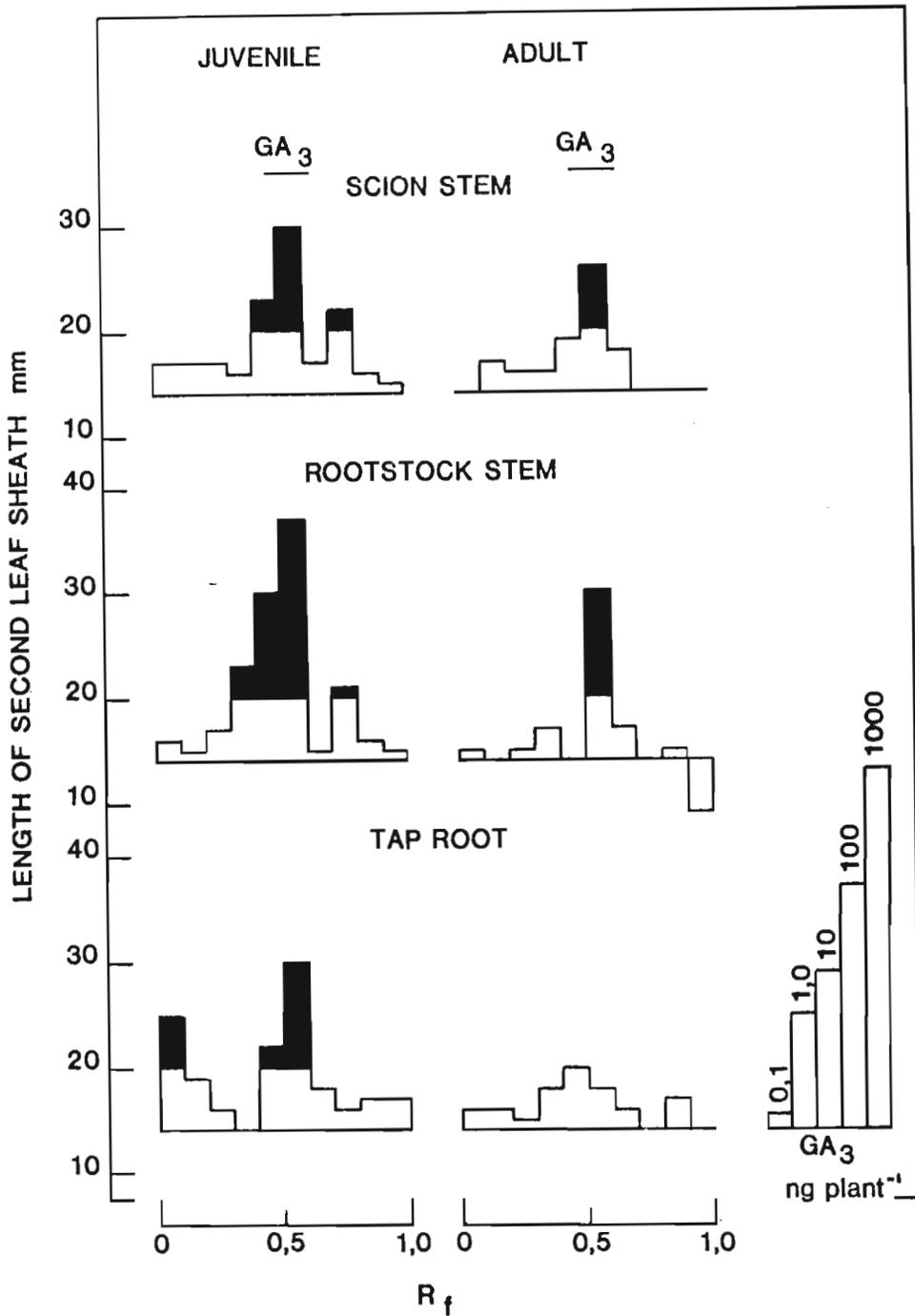


Figure 4.18 Gibberellin activity in the centrifugate obtained from the scion stem, rootstock stem and tap root of dormant plants of juvenile- and adult-budded *Citrus sinensis* cv. Pickstone Valencia orange. Centrifugates were separated on Whatmans No. 1 chromatography paper with *iso*-propanol: 25% NH_4OH : H_2O (10:1:1 v/v) as the solvent. Activity was detected using the dwarf rice micro-drop bioassay. Shaded areas represent regions significantly different from the controls at the level $P = 0,05$. GA_3 = gibberellin A_3 .

between the adult- and juvenile-budded plants (Figures 4.19 and 4.20). Not only were the levels in the juvenile-budded, dormant plants lower than in adult plants (Table 4.3), but the relative levels in wood and bark and the components showing activity also differed. The wood of juvenile plants (Figure 4.19) appears to contain high levels of a substance co-eluting with *iso*-pentenyl adenine and significant levels of compounds co-eluting with other cytokinins, as well as a rapidly eluting (320-360 millilitres) unidentified compound. The bark of juvenile-budded rootstocks contained low but significant levels of cytokinin-like substances with the major peak eluting at 1160-1200 millilitres. In contrast, the bark of adult-budded rootstocks yielded a large peak of activity with an elution volume of 320-640 ml and very little other activity except for an unidentified peak with an elution volume of 1600-1800 millilitres (Figure 4.20). The wood centrifugate of adult-budded plants yielded fast-eluting active compounds (280-620 ml elution volume) and a substantial quantity of a substance co-eluting with *iso*-pentenyl adenine.

The analysis of inhibitors in scion stem centrifugate of both adult- and juvenile-budded dormant Pickstone Valencia plants reveals the presence of high inhibitor activity in a wide range of R_f zones. In the classical "inhibitor β " zone (R_f 0,6 - 0,7) similar levels of inhibitor activity were detected in the juvenile and adult scions (Figure 4.21). The inhibition normally detected in unpurified extracts at R_f 0,1 is marginally greater in centrifugate obtained from juvenile stems.

In attempting to make direct comparisons of the levels of gibberellin-like and cytokinin-like substances from the different tissues analysed, regression analyses of the standards included in each bioassay were used to derive the data presented in Table 4.3. With the exception of the fibrous roots the levels of gibberellin-like substances appear to be higher in tissues of juvenile-budded plants. The situation pertaining to cytokinin-like substances is not as simple. Fibrous roots of juvenile-budded plants

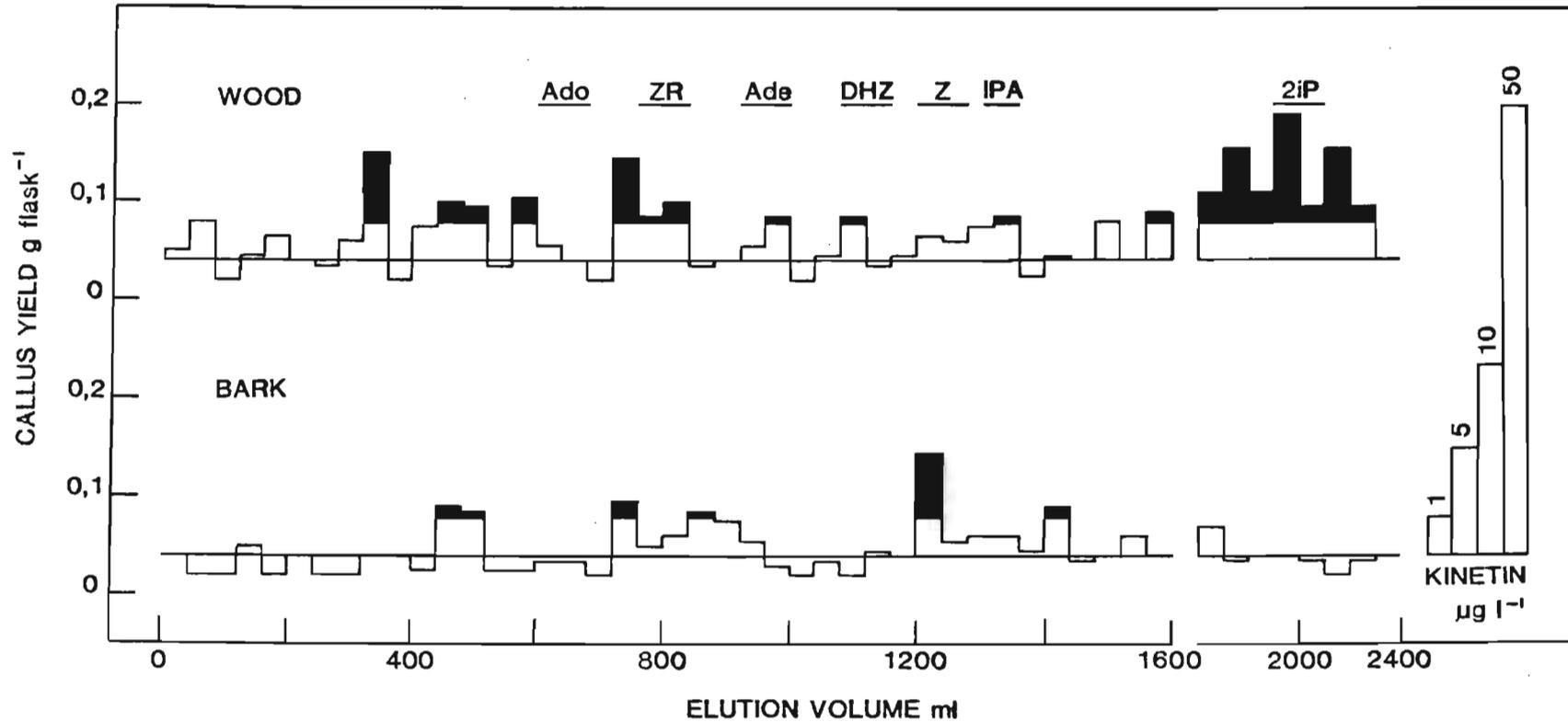


Figure 4.19 Cytokinin activity in the wood centrifugate and the bark extract of the rootstock stem of plants having scions of juvenile *Citrus sinensis* cv. Pickstone Valencia orange. Dowex 50 purified extracts were fractionated on Sephadex LH-20 columns eluted with 10 percent methanol. Activity was detected using the soybean callus bioassay. Shaded areas represent regions significantly different from the controls at the level $P = 0,05$. Ado = adenosine; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = *iso*-pentenyl adenosine; and 2iP = *iso*-pentenyl adenine.

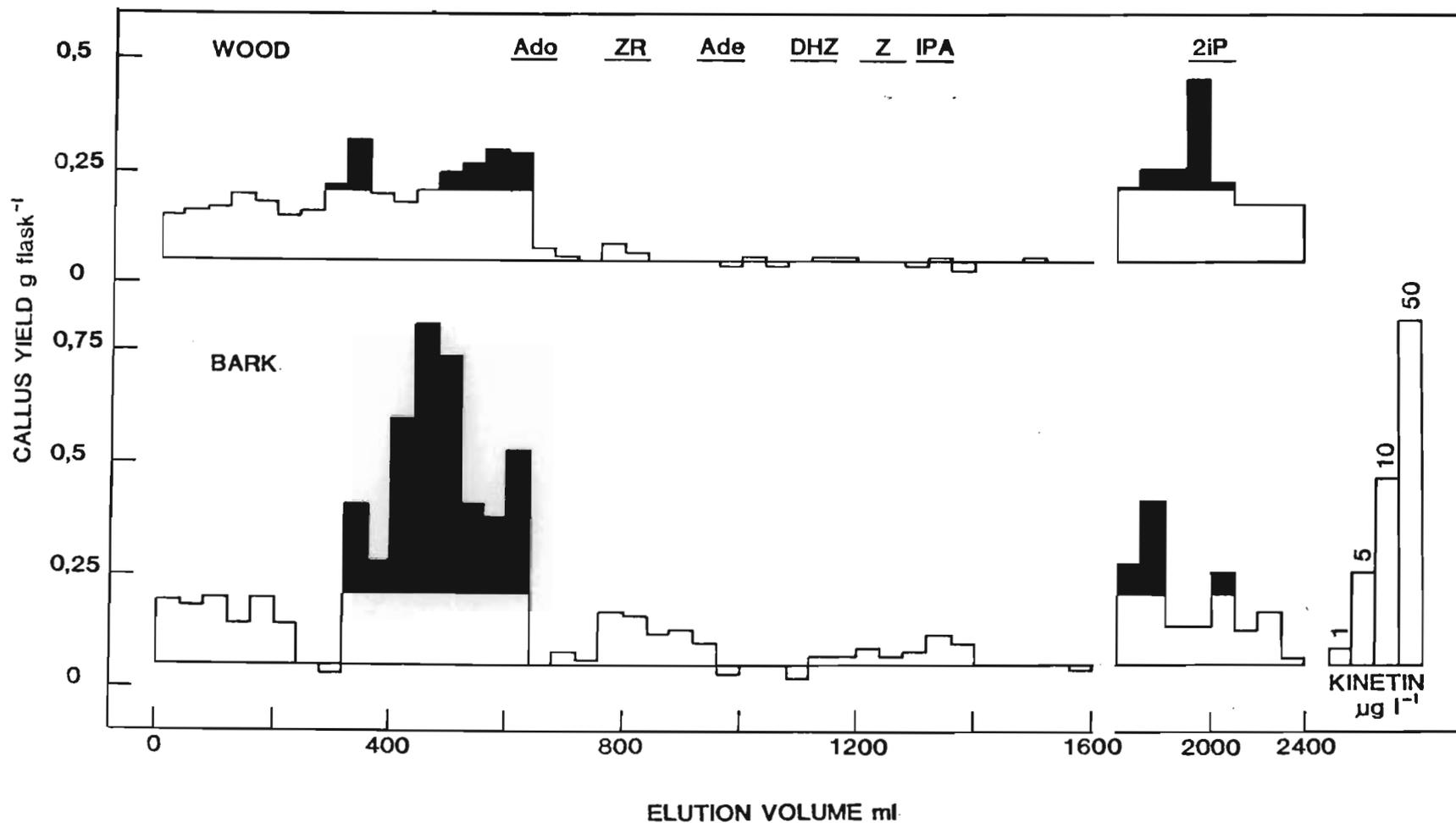


Figure 4.20 Cytokinin activity in the wood centrifugate and the bark extract of the rootstock stems of plants having scions of adult *Citrus sinensis* cv. Pickstone Valencia orange. Dowex 50 purified extracts were fractionated on Sephadex LH-20 columns eluted with 10 percent methanol. Activity was detected using the soybean callus bioassay. Shaded areas represent regions significantly different from the controls at the level $P = 0,05$. Ado = adenosine; ZR = ribosylzeatin; Ade = adenine; DHZ = dihydrozeatin; Z = zeatin; IPA = *iso*-pentenyl adenosine and 2iP = *iso*-pentenyl adenine.

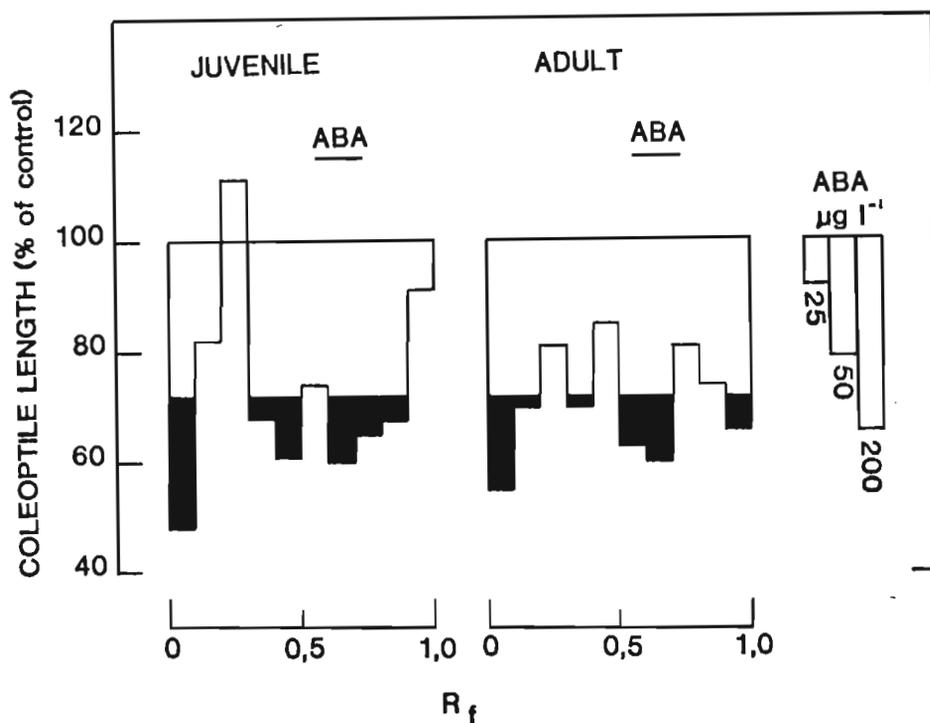


Figure 4.21 Inhibitor activity in scion stem centrifugate of dormant plants of juvenile and adult *Citrus sinensis* cv. Pickstone Valencia orange. Centrifugates were separated on Whatmans No. 1 chromatography paper. Activity was detected in the wheat coleoptile bio-assay. Shaded areas represent regions significantly different from the controls at the level $P = 0,05$. ABA = abscisic acid.

Table 4.3 The total combined levels of detectable gibberellin- and cytokinin-like hormones in various tissues of *Citrus sinensis* cv. Pickstone Valencia, as estimated from regression lines calculated from bioassay data

| Tissue source | Gibberellin-like compounds ng g^{-1} GA ₃ equivalents | | Cytokinin-like compounds ng g^{-1} Kinetin equivalents | | Data source |
|------------------|---|-------|---|-------|------------------------|
| | Juv. | Adult | Juv. | Adult | |
| Expanding shoots | 89 | 15 | | | Fig. 4.14 |
| Fibrous roots | F 28 | 86 | | | Fig. 4.15 |
| | D 60 | 70 | 160 | 100 | Fig. 4.15 Fig. 4.16 |
| Centrifugates: | | | | | |
| Scion stem | F 2 | 2 | | | Fig. 4.17 |
| | Q 62 | 17 | | | Fig. 4.18 |
| Rootstock stem | F 1 | <10 | | | Fig. 4.17 |
| | Q 267 | 26 | | | Fig. 4.18 |
| Tap root | F <10 | 3 | | | Fig. 4.17 |
| | Q 70 | 3 | | | Fig. 4.18 |
| Flushing plants | Wood centrifugate | | 12 | 15 | Fig. 4.19 |
| | Bark extract | | 9 | 123 | Fig. 4.20 |

F: flushing Q: quiescent D: dormant

appeared to contain 60 percent more cytokinin-like activity than adult-budded plants. In the stem tissues little difference was found in the wood centrifugate while the level of cytokinin-like compounds in the bark of adult-budded plants far exceeded that of juvenile-budded plants.

Discussion

The purpose of the experiments described in this chapter was to explore the extent of hormonal differences in organs and plant parts which play a role in the synthesis, transport, storage and metabolism of hormones in the final regulation of juvenile and adult vegetative shoot growth. Important differences in all organs vitally concerned with these processes were found. Though there were shortcomings in the work and in the literature referred to, it remains to present an objective interpretation or evaluation of the hormonal role in discrepancies of shoot vigour of juvenile- and adult-budded plants.

Before attributing importance to any particular findings it is worth considering some of the limitations which still exist regarding extrapolations of hormone data to growth responses. CROZIER, REID and REEVE (1973) advise that apparent differences in gibberellin levels of less than three to four orders of magnitude may be nothing more than a consequence of inaccurate estimation. On the other hand TREWAVAS (1982) argues that simple interaction of a small molecule with a single, specific receptor site should involve at the most two orders of magnitude variation in concentration for a change in effect. He develops the argument further by assigning major importance to the sensitivity of plants to growth substances. This is an important concept and will be discussed later. There is undoubtedly some significance to differences in hormone concentrations and this has been established beyond doubt in the case of the juvenility phenomenon where reversion from the adult to juvenile phase was caused by application of gibberellic acid

(ROBBINS, 1957 b, 1960; GOODIN and STOUTEMYER, 1961). Nevertheless, in the light of the above arguments and after noting that reactions to different compounds within a class of hormones show varying response curves in bioassays (range and gradient), with the result that bioassay data does not necessarily reflect true concentration or specific biological activity, the evaluation of the significance of hormone differences remains a fairly subjective operation.

Because ontogenetic age appears to be determined in shoot apices and because these organs give rise to new flushes, they were examined for hormone activity in the form of whole shoot tips. The results support the premise of JONES and PHILLIPS (1966, 1967) that gibberellins are produced in the young leaves of the apical bud. Dormant and quiescent buds have only non-developing leaf primordia and small leaflets. Analysis of the apical buds from flushing adult and juvenile scions for gibberellin-like activity revealed the presence of higher levels of total activity in the latter tissues. It may be reasonable to assume that gibberellins were not being produced in the dormant buds, nor was there a detectable level as a result of importation from other sites of synthesis or from residual levels from the previous growth flush.

The lower, ontogenetically juvenile and upper, adult sprouting buds on the same plant yielded levels of gibberellin-like activity in accord with those of adult and juvenile buds on separate plants. This leads to the conclusion that buds derived from ontogenetically younger shoots are capable of generating, within the structures of a sprouting bud, higher levels of gibberellins than comparable adult buds. Juvenile buds may, in addition or alternately, be capable of acting as stronger sinks for gibberellins produced elsewhere in the plant. Root tips may be a site of gibberellin synthesis (GRAEBE and ROPERS, 1978), which means that the proximity of buds

to roots may affect the gibberellin levels in buds at different heights along the stem. The high levels recorded in watershoots of *Citrus unshiu* (KAWARADA and SUMIKI, 1959) (1700 ng g^{-1}) support such an hypothesis.

Developing and expanding shoots of juvenile-budded plants also exceed shoots of adult-budded plants as far as levels of gibberellin-like substances are concerned. While the scion stem, rootstock stem and tap root centrifugates of flushing plants yield low and similar levels of gibberellin-like substances, the quiescent plants yield significantly higher levels. These organs of the central plant axis contain higher gibberellin levels in juvenile-budded plants.

The findings are consistent with those of other researchers who have shown that juvenile tissues contain higher gibberellin levels than adult tissues (FRYDMAN and WAREING, 1973 a, b; SCHWABE and ALDOORI, 1973). The question which arises from such data concerns the role these higher levels play in the enhanced vigour of juvenile shoot growth. In the light of the suggestion of TREWAVAS (1982) that it is sensitivity to growth substances, not their concentrations, which is the limiting factor in plant development, the question of WAREING and FRYDMAN (1976) must be repeated: Are differences in gibberellin a cause or effect of differences in ontogenetical age? The reversion caused by gibberellin applications in some cases (ROBBINS, 1957 b, 1960; GOODIN and STOUTEMYER, 1961) provides scant evidence that it may play a role in causing juvenile growth phenomena.

The collective sum of cytokinin-like activity appears to be higher in juvenile buds only when new flushes are not growing and, therefore, when active root growth may be taking place (MARLOTH, 1950; REUTHER, 1977).

The following facts provide evidence for an hypothesis that accumulated or stored cytokinins, which were produced in actively growing root tips, play an integral role in shoot vigour differences: 1. Cytokinin levels (probably

ribosylzeatin) in the fibrous roots of plants with quiescent stems were higher in juvenile plants; 2. A peak co-eluting with what is most probably glucosylated ribosylzeatin (VAN STADEN, 1982) was evident in dormant juvenile buds, but was not detectable in adult buds; 3. The bark of actively flushing adult plants contained more of what may be glucosylated cytokinins (VAN STADEN, 1982) and 4. Purinyl glycine, an oxidation product of zeatin (MILLER, 1965; LETHAM, SHANNON and McDONALD, 1967) was evident in sprouting juvenile buds and not in adult buds. Although these facts do not come near to providing concrete evidence, they may indicate that in the more vigorous juvenile tissues: 1. a greater demand or sink strength for cytokinins may stimulate greater production in actively growing roots; 2. there may be a greater relative ability in juvenile buds to sequester or hydrolyse storage forms of cytokinins (VAN STADEN and DIMALLA, 1981) from the bark and 3. there may be greater oxidative metabolism, resulting in the build-up of purinyl glycine in juvenile buds. Glucosylated cytokinins are thought to be storage or inactive forms (LETHAM, PARKER, DUKE, SUMMONS and McLEOD, 1976; VAN STADEN, 1976 a, 1977; HENSON, 1978). The greater accumulation of glucosylated cytokinins in the bark of adult-budded plants may indicate that the tissues are unable to metabolise the free base cytokinin forms, probably as a result of a lower metabolic and growth rate. Receptors for plant hormones are likely to participate in the plant growth responses to hormones (GRAEBE and ROPERS, 1978; HIGGINS and JACOBSEN, 1978) and are, therefore, worthy of discussion. TREWAVAS (1982) postulated that the interaction of a growth substance with its receptor site can probably be summarised in the simple equation:

$$\text{growth substance (GS) + receptor (R) } \rightarrow \text{(GS.R) } \rightarrow \text{biological response.}$$

However, HIGGINS and JACOBSEN (1978) believe that the great variety of processes which are affected by such a few compounds suggests that a number of

receptors, mediators or recognition factors must exist in order to "define priorities between the various processes competing for the one hormone". In the arguments developed here, where it is postulated that greater use is made of cytokinins and gibberellins for promoting the vigour of juvenile shoot growth, the analogous reasoning might therefore be followed, that more receptor sites for this purpose must be available in juvenile tissues. If this line of thought is to be followed, it may be argued that a lower sensitivity, or less receptor sites are available in adult-budded plant tissues for cytokinin utilisation in growth processes. GRAEBE and ROPERS (1978) argue conversely, that the typical increases in gibberellin-like activity in developing flowers and in germinating seeds point to an increased ability to produce gibberellin when needed at the site of utilization. In other words, hormone synthesis may ultimately regulate the biological response. The decline in gibberellin after these "spurts" probably indicates inactivation after the high levels of hormone are no longer needed. The appearance of a purinyl glycine-like substance in juvenile sprouting buds provides scant evidence of metabolic oxidation of zeatin (Figure 4.8) and therefore of greater utilization of cytokinins than in adult buds where a purinyl glycine-like substance was not apparent. There are no known means of detecting relative rates of gibberellin metabolism using biological assays. The end products of gibberellin metabolism are 2 β -hydroxylated gibberellins and their conjugates, both of which are inactive or have very low biological activity (GRAEBE and ROPERS, 1978). The ability of plants to maintain a specific level of gibberellin activity has been shown by incorporation experiments (VAN DEN ENDE and ZEEVAART, 1971) and also by the fact that gibberellin-like activity in shoots rapidly disappears when gibberellin biosynthesis is inhibited by AMO-1618 (ZEEVAART, 1971). The information gathered in these experiments is not adequate for discounting or verifying either of the previously postulated mechanisms for regulating hormone activity.

The analysis of inhibitors was undertaken following the suggestion by FORTANIER and JONKERS (1976) that the ratio between growth promoting and growth retarding substances may be involved in the ability of maturing plants to flower. It is now obvious that the search for such a growth retardant would be an extensive project. Although slight differences in an abscisic acid-like component were found, the relevance of these differences and the significance of balances or ratios with, for example, gibberellins and cytokinins, would be difficult to establish.

MARLOTH (1950) proposed that in the growth of *Citrus* plants there exists an alternating cycle of root and shoot growth. This pattern is not easily discernible in samples of plants which do not have synchronised cycles of flushing (HENDRY and ALLEN, 1980). The sharply contrasted high and low levels of gibberellin-like substances in centrifugates from artificially synchronised quiescent and flushing plants, respectively, may be related to this alternating pattern. During the period of quiescence, when the growth of shoot flushes ceases, the high levels of gibberellin-like substances in centrifugates may be accompanied by cambial growth in these tissues. It is interesting to note that the higher levels found in tissues from juvenile-budded plants extend to the seedling rootstock. It appears that a transmissible factor may render the rootstock as vigorous or as slow growing as the juvenile and adult scions, respectively, as indicated by HENDRY, VAN STADEN and ALLEN (1982 b). Such a process may be more widely operative in the regulation of root:shoot ratios in plants generally.

In conclusion it might be said that, in terms of the limited evidence available, a tenable explanation has been offered for the involvement of differing hormone levels found in juvenile- and adult-budded plants, in the unequal vigour of such plants. As far as the gibberellin-like compounds are concerned, the relationship appears to be fairly well established and to be consistent with other researchers' findings. What remains to be established, among other facts, is the actual involvement in particular

aspects of the disparity in growth between juvenile- and adult-budded plants; the sites or organs of action and the mechanism which regulates hormone synthesis. The relationship between cytokinins and shoot growth is not clear. There does appear to be a pattern of greater synthesis, storage and possibly of cytokinin utilization in juvenile-budded plants. More evidence is required, however, before such an hypothesis can be accepted. The involvement of inhibitors in the juvenility phenomenon remains largely an unknown quantity. This aspect of the work does seem to be more complex and requires an in-depth study incorporating physico-chemical detection methods coupled to a number of bioassay systems.

It appears that both gibberellin-like and cytokinin-like hormones are actively involved in the juvenility phenomenon. However, the findings of the above experiments still do not clarify where the effects of these hormones fit in the series of reactions constituting cause and expression of ontogenetical age.

CHAPTER V

THE EFFECT OF SYNTHETIC GROWTH REGULATORS ON SHOOT GROWTH

Introduction

Previous experiments, documented in Chapter III and Chapter IV, revealed that differences in the growth patterns and in the quality and quantity of endogenous hormones exist in juvenile- and adult-budded Pickstone Valencia orange plants. In an attempt to further characterize the role of specific classes of plant growth regulators in directing growth in components of shoot growth and as causal agents in differences of juvenile/adult shoots, the response to applications of such compounds was observed and recorded. It would be impossible to undertake to elucidate these cause and effect relationships without considering interactions of different growth regulators, relative concentrations of the active ingredient and the timing of application or effectiveness, amongst many other variables.

Nevertheless, with due consideration of the above and with the possibility of commercial application in mind, there is just cause to examine simple application and response effects between hormones and shoot growth, respectively. Two aspects of such a relationship merit investigation. In the first place, the metabolism in leaves; the relationship of sink to source and the mobilisation of reserves is deserving of more attention. A limited amount of knowledge has been gained in this field with respect to fruit crops, such as grapes (HOAD, LOVEYS and SKENE, 1977; NIIMI, 1979), tomatoes (VARGA and BRUINSMA, 1974), apples (RICHARDS, 1980), beans (PATRICK, JOHNSTONE and WAREING, 1979) and to a lesser extent with *Citrus* (MONSELISE, 1979). In the second place, studies of the relationship between conformation, structure or shape of shoots and shoot components with growth regulator levels and balances could provide useful information for improving pruning and tree shaping practices and light interception properties. In this respect growth retarding chemicals may have the greatest potential (MONSELISE, 1979). They have been

used extensively in modifying the shapes of potted plants (CATHEY, 1964; WITTWER, 1978) and some are used in changing tree branch architecture (WITTWER, 1978).

It appears from the experiments of Chapter III and Chapter IV that juvenile and adult plants are physiologically adapted to different rates of hormone metabolism and, perhaps consequentially, to differences in patterns of growth and differentiation. The aims of the experiments described below, were to determine how these patterns could be modified by the application of plant growth regulators.

Experiment 1 Applications to the apical bud during a growth flush

As has been discussed previously (Chapter I), the locus of control of juvenile and adult growth differences is thought to be in the shoot buds and the apical meristem. Analyses of endogenous hormones in these meristems revealed that sharp contrasts existed here. In order to test for the localized effect on growth produced by such apical meristems, it seemed logical to apply the growth regulators to buds which were in the process of differentiating new shoot material.

The effects of exogenous growth regulators are often short-lived (BEN-GAD, ALTMAN and MONSELISE, 1979) especially in situations where dynamic growth is occurring, with the result that most experiments of this nature rely on repeated applications (see for example FISHER, 1980; JUNTILLA, 1980; MOORE, 1980; WHEELER, WAMPLE and PHARIS, 1980). The resultant flush growth after the uninterrupted application of a gibberellin, a cytokinin and abscisic acid to the apical differentiating bud, was recorded and analysed for adult and juvenile scions.

Experimental procedure

Juvenile and adult plants used in this experiment were produced and cultured as described in Chapter II. Twenty-eight each of adult- and juvenile-budded

plants were incubated in a growth chamber maintained at a 20⁰/14⁰C temperature regime and an eight hour daylength, for 28 days. After removal to a glasshouse maintained at a 30⁰/20⁰C temperature regime and a daylength artificially extended to 16 h with five 300W Hg-vapour lamps, a flush of growth emerged and matured in about eight weeks. Plants were then decapitated above the lowest leaf on the scion stem. The leaf was removed and growth regulator treatments were applied to the axillary bud after bud burst had occurred. All other buds which sprouted on the stems were removed.

Gibberellin A₃, kinetin and abscisic acid solutions of one gramme per litre were applied as 0,02 ml drops in 0,05 percent aqueous TWEEN 20, control plants being treated with TWEEN 20 solution only. This meant that 0,02 mg of active ingredient was applied to each terminal bud with each daily treatment. The treatment was continued for as long as the terminal bud continued to differentiate new leaves. Seven adult- and seven juvenile-budded plants were used for each treatment and results presented represent the mean for seven plants. About eight weeks after bud burst the new shoot flush was considered to have matured. Various parameters of leaf and stem growth were measured and in some cases, derived statistics were calculated. The phyllotaxis of all shoots was recorded.

Results

Growth of the flushes from juvenile and adult control plants was very similar to those recorded in Chapter III. The deviations of basic leaf and stem parameters of growth regulator-treated plants are presented as a percentage of the respective juvenile and adult controls (Table 5.1). Of all leaf and stem characteristics determined in juvenile plants, none was found to be reduced by any of the regulators below that of the control. Many characteristics exceeded those of the controls. Leaf numbers were significantly increased by gibberellin treatments; unit leaf area was increased by abscisic acid treatments; total leaf mass per flush was increased by the abscisic acid treatment.

Table 5.1 Parameters of shoot growth in juvenile and adult scions of *Citrus sinensis* cv. Pickstone Valencia orange, which had been treated with gibberellin A₃ (GA₃), kinetin (KIN) and abscisic acid (ABA). The growth regulators were applied to the shoot apical bud as droplets for as long as the new stem was differentiating new growth. Results are presented as a percentage difference from the respective juvenile and adult control treatments. Those responses which were significantly different from the control treatments are indicated with an asterisk. (+ = greater than control; - = less than control)

| PARAMETER MEASURED | JUVENILE | | | ADULT | | |
|-------------------------|-----------------|------|------|-----------------|-----|------|
| | GA ₃ | KIN | ABA | GA ₃ | KIN | ABA |
| Leaf number | +34* | -8 | -5 | +23* | -6 | -13 |
| Leaf area | | | | | | |
| Total per stem | +15 | -1 | +8 | -13 | -6 | -16 |
| Mean per leaf | -13 | +8 | +16* | -30* | -6 | -3 |
| Leaf mass | | | | | | |
| Total per stem | +18 | +7 | +21* | -6 | +3 | -7 |
| Mean per leaf | -10 | +17* | +31* | -24* | +3 | +6 |
| Leaf mass per unit area | +3 | +9 | +13 | +8 | +9 | +10 |
| Stem | | | | | | |
| Total mass | +56* | +7 | +20 | +21* | +4 | -19* |
| Total length | +59* | -1 | -2 | +43* | 0 | -18* |
| Total shoot mass | +28* | +7 | +21 | +19 | +20 | +5 |
| Phyllotaxy | 2/5 | 2/5 | 2/5 | 2/5 | 2/5 | 2/5 |

while unit leaf mass was also increased by abscisic acid and by kinetin treatments. Stem mass, total stem length and the total mass of the new shoot flush was enhanced by the gibberellin treatments.

The situation in adult plants was similar, but not identical. Leaf numbers on the new flush were increased significantly by the gibberellin treatments; unit leaf area was reduced by gibberellin treatments, as was the unit leaf mass while total flush stem mass and length were both increased by the gibberellin treatments. These two parameters were both significantly reduced by the abscisic acid treatments.

In all cases phyllotaxis was unaltered, it being 2/5 in the left or right hand direction of spirality in all treatments of adult and juvenile scions.

In the earlier observations on patterns within the growth of a shoot flush (Chapter III), changes in leaf area, leaf mass and leaf shape seemed to follow a particular sequence of change. These leaf characters appeared to be differentially susceptible to the modifying effects of the growth regulators, depending on the position of the leaf along the length of the stem. The differential effect also depends on the type or class of hormone applied. Gibberellin A₃ treatments to the apical bud, for example, caused the basal leaves on a flush to expand to a greater extent than the same leaves on the control plant (Figure 5.1 A). The effect was reduced and eventually reversed in the more distal leaves on a flush, with the result that leaves became smaller and smaller near to the terminal end of the flush. Juvenile and adult flushes followed similar trends, though the change in leaves of juvenile scions was less extreme than the changes in leaves of adult scions. Kinetin applications had little effect on the pattern of leaf area change along the length of the adult flush stem (Figure 5.1 B). Leaves of juvenile scions showed peaks of response to kinetin near to 60 percent and 90 percent of the total stem length. Abscisic acid depressed the leaf area of leaves closer to the basal end of adult stem flushes, but thereafter no significant change from the control was

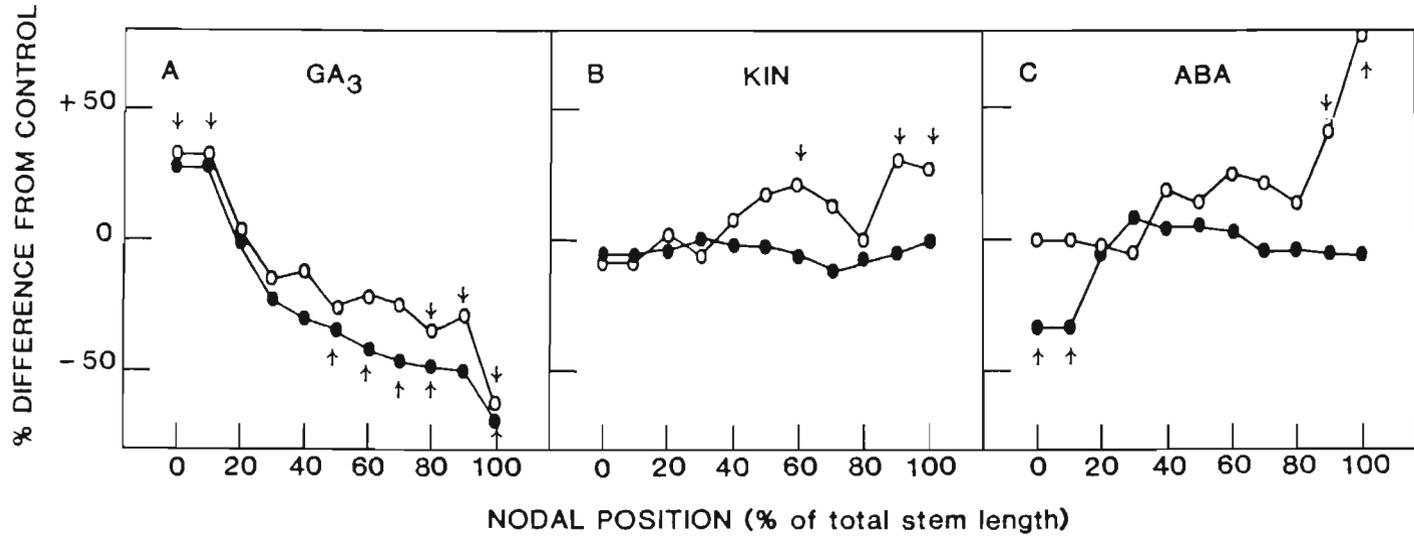


Figure 5.1 The effect of gibberellin A₃ (GA₃), kinetin (KIN) and abscisic acid (ABA) on the variation of unit leaf area along the length of a shoot flush. The compounds were applied daily throughout the development of a growth flush, to *Citrus sinensis* cv. Pickstone Valencia orange plants. Results are presented as a percentage difference from the respective juvenile (o) and adult (●) control treatments. Points significantly different from the respective control values are indicated by arrows.

observed (Figure 5.1 C). The basal leaves of juvenile stem flushes, on the other hand, were not significantly different from those of the control plants, while the leaves situated between 40 and 100 percent of the stem length were larger in area than those of the control treatments.

Figure 5.2 A shows that very similar effects to the above were found regarding leaf mass. Continuous gibberellin A_3 applications resulted in a steady decline in leaf area, relative to the controls, over the length of both juvenile and adult shoot flushes. Kinetin did not promote leaf mass accumulation in juvenile shoots as it did increase the leaf area (Figure 5.2 B and 5.1 B, respectively). Leaf mass of both juvenile and adult shoots was not found to vary significantly from the controls. Abscisic acid was only effective in producing disproportionately large leaves near the distal end of juvenile flushes and did not change leaf areas of adult flushes, relative to the controls, over the entire shoot length (Figure 5.2 C).

Gibberellin A_3 was the only treatment which was effective in bringing about a significant change in leaf shape (Figure 5.3). A consistent trend of leaf reduction (relative to the controls) was evident over the shoot flush lengths of juvenile and adult scions (Figure 5.3 A). A greater reduction in the leaf area of adult leaves was evident in the basal half of the stems. No significant change was effected in leaf shape by kinetin or abscisic acid applications to the terminal bud (Figure 5.3 B and C, respectively).

Experiment 2 Applications to the apical bud before the emergence of a growth flush

That hormonal changes accompany the loss of dormancy in buds is beyond doubt (SAUNDERS, 1978 b with references). It also seems that the extent of these changes may affect the nature of the growth emanating from buds, with respect to cytokinins (DOMANSKI and KOZLOWSKI, 1968; HENDRY, VAN STADEN and ALLAN, 1982 a, b) and gibberellins (COOPER, YOUNG and HENRY, 1969). In addition,

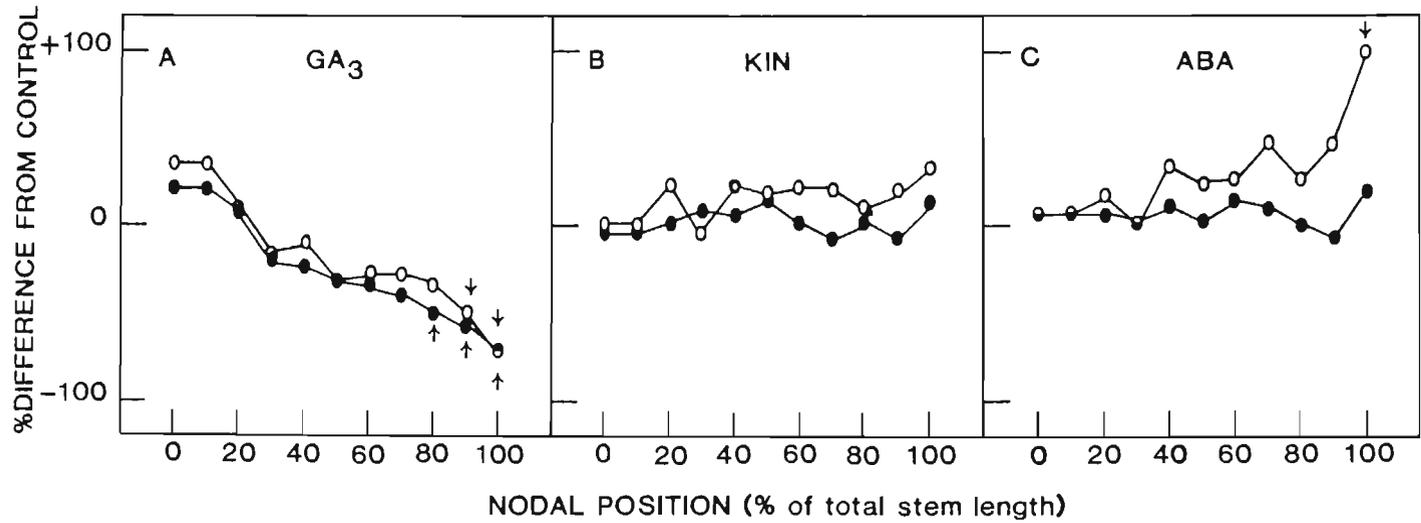


Figure 5.2 The effect of gibberellin A₃ (GA₃), kinetin (KIN) and abscisic acid (ABA) on the variation of unit leaf mass along the length of a shoot flush. The compounds were applied daily throughout the development of a growth flush, to *Citrus sinensis* cv. Pickstone Valencia orange plants. Results are presented as a percentage difference from the respective juvenile (o) and adult (●) control treatments. Points significantly different from the respective control values are indicated by arrows.

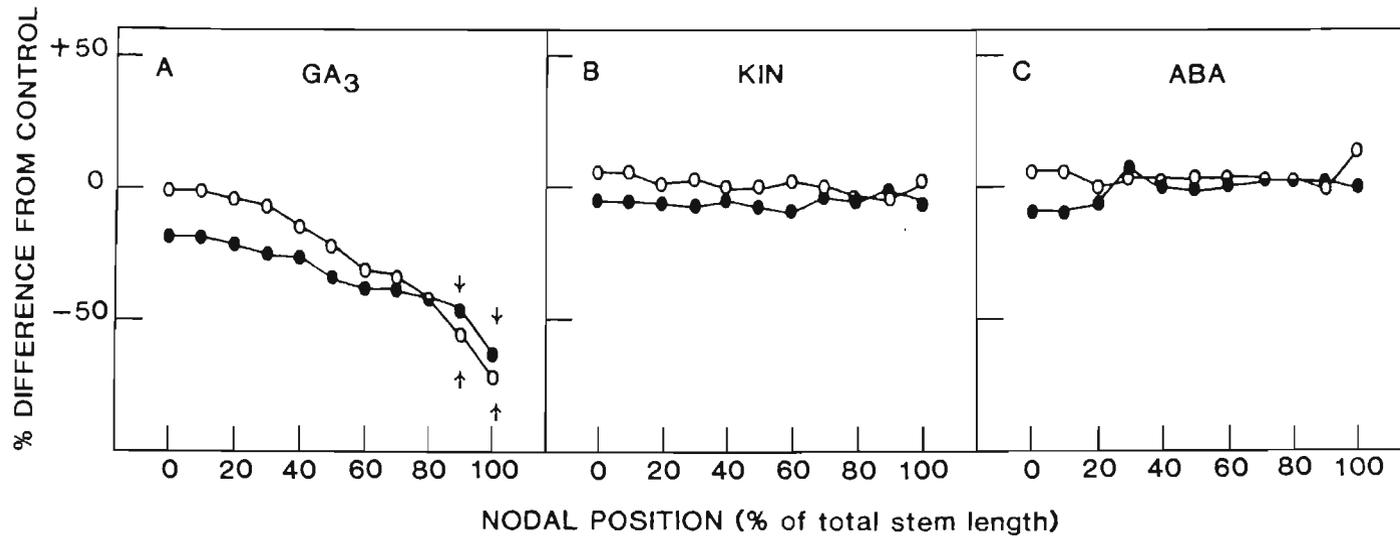


Figure 5.3 The effect of gibberellin A₃ (GA₃), kinetin (KIN) and abscisic acid (ABA) on the variation of the leaf width:length ratio (expressed as a percentage) along the length of a shoot flush. The compounds were applied daily throughout the development of a growth flush, to *Citrus sinensis* cv. Pickstone Valencia orange plants. Results are presented as a percentage difference from the respective juvenile (○) and adult (●) control treatments. Points significantly different from the respective control values are indicated by arrows.

the findings of Experiment 1, above, show that growth regulators applied to differentiating buds are effective in modifying growth from a very early stage of bud sprouting. From the point of view of potential practical applicability the quiescent bud presents an easily recognisable stage of development, between growth flushes in summer, for application of growth regulators.

The experiment described below was similar to Experiment 1, above, with the difference that growth regulator applications were made to the apical bud before bud sprouting occurred. As gibberellins appear to play a major role in shoot differentiation and assimilate distribution in *Citrus* (MARTH, AUDIA and MITCHELL, 1956; COOPER, YOUNG and HENRY, 1969; DE LANGE, 1974; BEN-GAD, ALTMAN and MONSELISE, 1979) and as CCC is known to inhibit endogenous gibberellin synthesis (JONES and PHILLIPS, 1967) and to affect the growth from buds of *Citrus* (MONSELISE, GOREN and HALEVY, 1966), CCC was included as an additional treatment in this experiment. The necessity for repeated applications, as outlined above, was relevant in this experiment, too.

Experimental procedure

Forty-two each of the juvenile- and adult-budded plants, produced and cultured as described in Chapter II, were induced to the dormant state as described in Experiment 1, above. After removal to the glasshouse, where conditions were more conducive to growth, a flush of growth developed and matured in about eight weeks. Plants were then decapitated above the lowest leaf on the scion stem. The leaf was removed and growth regulator treatments were applied to the axillary bud on a daily basis until bud burst occurred. All other buds which sprouted on the stem were removed.

In the first part of the experiment the effect of gibberellin A₃, kinetin and abscisic acid on shoot flush growth, was compared with the water-treated controls. In the second part, a similar comparison was made between the effect of CCC dosage and the control. All solutions were constituted and applied as described in Experiment 1, above. The concentration of CCC was also

one milligramme per millilitre and the dosage was a daily application of a 0,02 ml droplet (= 0,02 mg active ingredient) applied to the quiescent scion bud. Treatments were applied for approximately eight days before sprouting was evident. Seven plants were used per treatment. About eight weeks later the new shoot was considered to have matured. Various parameters of leaf and stem growth were measured and, in some cases, derived statistics were calculated.

Results

Deviations from the controls in the overall leaf and stem characteristics of the new flushes on plants treated with the growth regulators are presented in Table 5.2. In the case of both the adult and juvenile flushes growing from buds treated with gibberellin A₃ an overall inhibition of leaf growth and enhancement of stem growth was evident. Measurements of leaf area and leaf mass were significantly reduced from the levels in the control plants, while stem length and mass was significantly increased. The same response was evident from gibberellin treatments in adult shoots.

Kinetin applications to the quiescent buds had no significant effect upon the leaf, stem and overall shoot characters in both adult and juvenile scion growth. Abscisic acid had the effect of producing a significantly greater number of leaves and a greater stem mass in juvenile scions, while no apparent alteration to the overall shoot mass was recorded. In adult scions, however, a greater leaf number, total leaf area and total leaf mass than in control plants was produced following abscisic acid treatments.

The individual variation in shoot growth of the plants treated with the growth regulators was relatively high. Standard errors of the mean, which were used to assess significant differences, were excessive and therefore a true reflection of meaningful treatment effects was not forthcoming.

The significant reduction in leaf area of adult shoots especially, following gibberellin application to quiescent buds is reflected clearly when the pattern

Table 5.2 Parameters of shoot growth in juvenile and adult scions of *Citrus sinensis* cv. Pickstone Valencia orange, which had been treated with gibberellin A₃ (GA₃), kinetin (KIN) and abscisic acid (ABA). The growth regulators were applied to quiescent shoot apical buds as droplets for as long as the bud remained quiescent and up to the time of sprouting. Results are presented as a percentage difference from the respective juvenile and adult control plants. The responses which were significantly different from the control treatments are indicated with an asterisk. (+ = greater than control; - = less than control)

| PARAMETER MEASURED | JUVENILE | | | ADULT | | |
|-------------------------|-----------------|-----|------|-----------------|-----|------|
| | GA ₃ | KIN | ABA | GA ₃ | KIN | ABA |
| Leaf number | -4 | -8 | +20* | -1 | -12 | +20* |
| Leaf area | | | | | | |
| Total per stem | -50* | +2 | +8 | -56* | +1 | +18* |
| Mean per leaf | -35* | -7 | -11 | -34* | 0 | +7 |
| Leaf mass | | | | | | |
| Total per plant | -34* | +7 | +2 | -50* | 0 | +19* |
| Mean per leaf | -40* | -1 | -15 | -30* | -2 | -6 |
| Leaf mass per unit area | +10 | +5 | -6 | -7 | -1 | 0 |
| Stem | | | | | | |
| Total mass | +28* | +19 | +31* | +20* | +1 | +19 |
| Total length | +29* | -12 | -7 | +28* | -3 | +1 |
| Total shoot mass | -17 | +11 | +9 | -20 | 0 | -9 |

of change in unit leaf area over the flush stem length was plotted (Figure 5.4 A). The first leaves to emerge and expand from the adult buds were less than 50 percent of the area of similar leaves from adult control plants. The disparity from the controls was reduced in leaves produced subsequently, but all were significantly smaller than the leaves of the adult control plants. Regions of depressed leaf size in juvenile gibberellin-treated plants were evident at 20 and 90 percent of the stem length with peaks in size at the basal end of the stem and at 60 to 80 percent of the stem length. At no stage did the juvenile or adult scions which were treated with gibberellin, produce leaves of equal size with the respective control scions.

Trends or significant differences in leaf area from the respective controls were not apparent in flushes of growth produced by kinetin- or abscisic acid-treated buds of either juvenile or adult scions (Figure 5.4 B; C). Leaf areas did show a slight decline towards the distal end of both juvenile and adult flushes emanating from buds treated with abscisic acid, but no significant differences from the respective controls were determined.

As might have been expected, the trends in leaf mass along the length of flush stems followed a similar pattern to those of leaf area (Figure 5.5). The leaf mass of adult scion flushes emanating from gibberellin-treated buds was significantly reduced below that of the control with the exception of a small peak at 40 to 50 percent of the stem length (Figure 5.5 A). Leaves of similarly treated juvenile scions were consistently reduced in comparison with the control treatment.

The comparatively small changes in leaf mass along the stems of kinetin-treated scions (Figure 5.5 B) were not statistically significant and probably mirrored the high variability of the experimental material. The abscisic acid treatment also did not provide any statistically significant differences, but a definite trend of declining relative leaf mass was discernible in juvenile shoots (Figure 5.5 C).

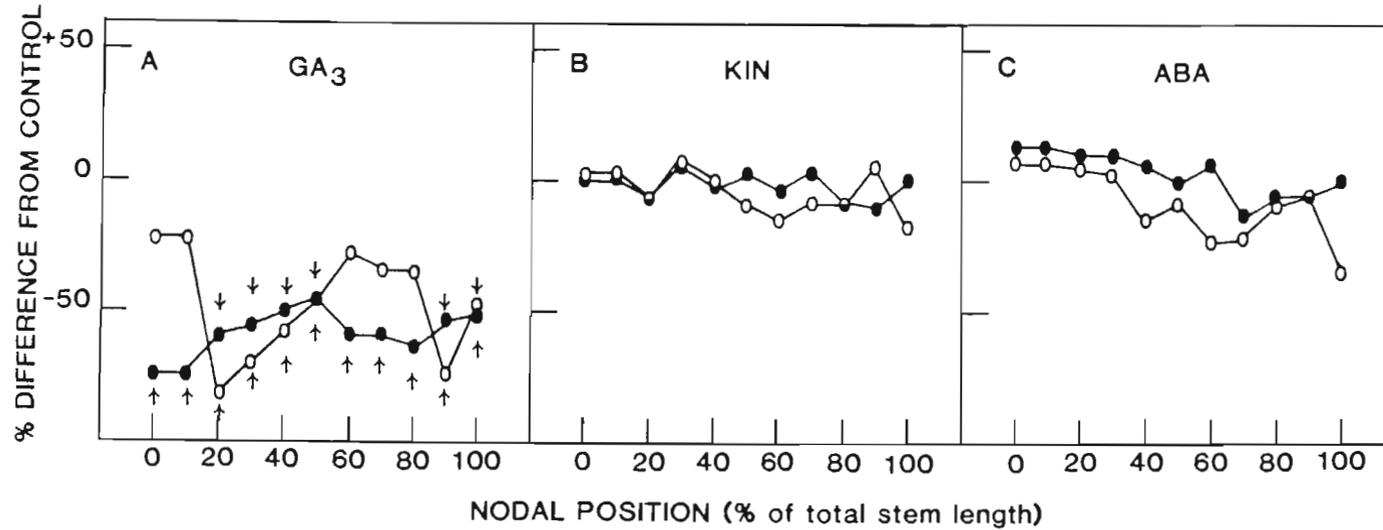


Figure 5.4 The effect of gibberellin A₃ (GA₃), kinetin (KIN) and abscisic acid (ABA) on the variation of unit leaf area along the length of a shoot flush. The compounds were applied daily to dormant apical buds of *Citrus sinensis* cv. Pickstone Valencia orange scions, up to the time of bud burst. Results are presented as a percentage difference from the respective juvenile (o) and adult (●) control treatments. Points significantly different from the respective control values are indicated by arrows.

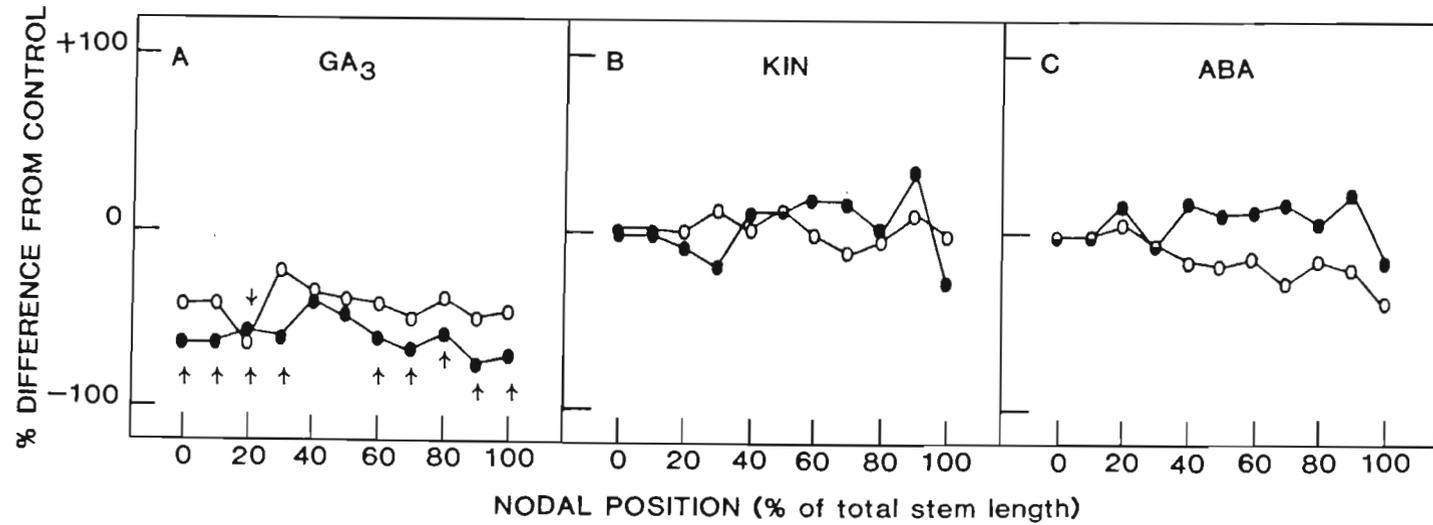


Figure 5.5 The effect of gibberellin A₃ (GA₃), kinetin (KIN) and abscisic acid (ABA) on the variation of unit leaf mass along the length of a shoot flush. The compounds were applied daily to dormant apical buds of *Citrus sinensis* cv. Pickstone Valencia orange scions, up to the time of bud burst. Results are presented as a percentage difference from the respective juvenile (o) and adult (●) control treatments. Points significantly different from the respective control values are indicated by arrows.

Since only the gibberellin treatment appeared to affect leaf shape in the previous experiment (Figure 5.3), the variation of this parameter (leaf width: length ratio) over the length of the flush stem was only determined for the gibberellin treatment in this experiment (Figure 5.6). As has been noted, a reduction in this parameter relative to the respective control, indicates that leaves are comparatively longer and narrower. Figure 5.6 indicates therefore, that the gibberellin treatment causes a significant change in leaf shape of all leaves produced from treated juvenile and adult buds. Leaves of adult and juvenile scions were narrower and/or longer at the base of the new flush than leaves from the control plants, but the effect of the gibberellin appeared to diminish towards the distal end of the flush where differences from the control were reduced. Juvenile scions did appear to regress in this respect and produce long/narrow leaves in the terminal 10 percent of the stem length. CCC applications to quiescent apices had no significant effect upon the total fresh mass accumulated by the new flushes of growth of juvenile and adult scions (Table 5.3). Important differences were however apparent in parameters of leaf and shoot growth. While the stem length was significantly reduced and the total stem mass was decreased in both juvenile and adult shoots, only the adult shoots experienced a significant increase in leaf area and leaf mass parameters above the levels found in the adult control plants. The responses to these applications of CCC are of major significance, but will be discussed later.

Experiment 3 Trunk injection of growth regulators

The possibility that the hormones in organs which are distant from the shoot apical meristem, may play a role in the differentiation mechanisms of apices is worth considering. This role may be of a primary or secondary nature, i.e. the hormones may themselves be translocated to the apices and exert their influence within the meristem, or they may act locally in the distant organs and cause a response there which will affect the growth or differentiation of the

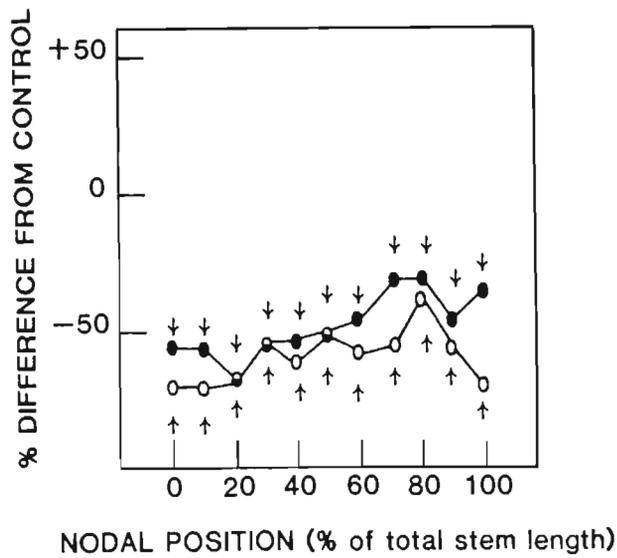


Figure 5.6 The effect of gibberellin A_3 on the variation of the leaf width:length ratio (expressed as a percentage) along the length of a shoot flush. The gibberellin was applied daily to the dormant apical buds of *Citrus sinensis* cv. Pickstone Valencia orange scions, up to the time of bud burst. Results are presented as a percentage difference from the respective juvenile (o) and adult (●) control treatments. Points significantly different from the respective control values are indicated by arrows.

Table 5.3 Parameters of shoot growth in juvenile and adult scions of *Citrus sinensis* cv. Pickstone Valencia orange, which had been treated with CCC. The growth regulator was applied to quiescent shoot apical buds in droplet form for as long as the bud remained quiescent and up to the time of sprouting. Results are presented as a percentage difference from the respective untreated juvenile and adult control scions. The responses which were significantly different from the control treatments are indicated with an asterisk. (+ = greater than control; - = less than control)

| PARAMETER MEASURED | JUVENILE | ADULT |
|----------------------------|----------|-------|
| Leaf number | 0 | +1 |
| Leaf area | | |
| Total per stem | 0 | +20* |
| Mean per leaf | +3 | +15* |
| Leaf mass | | |
| Total per stem | +5 | +17 |
| Mean per leaf | +6 | +11 |
| Leaf mass per unit area | -6 | -2 |
| Stem | | |
| Total mass | -22 | -9 |
| Total length | -31* | -25* |
| Total shoot mass per flush | -9 | +11 |

meristem. Whichever way the effect may be exerted, it seemed logical to investigate the differences found in endogenous hormone levels in the wood and bark of juvenile- and adult-budded plants (Chapter IV). The fact that both gibberellins and cytokinins may be produced in roots and translocated to aerial parts (JONES and PHILLIPS, 1967; KENDE, 1965) should be regarded as additional motivation for examining the effects of stem applications of growth regulators on new shoot growth.

Accordingly, the effect of gibberellin and cytokinin pressure injection into stems, on scion shoot growth was assessed in juvenile- and adult-budded plants.

Experimental procedure

Plants which were produced and cultured as described in Chapter II, were used in this experiment. Thirty each of the juvenile- and adult-budded Pickstone Valencia orange plants were induced to the dormant state in a growth chamber as described in Experiment 1, above. After four weeks of dormancy induction in the growth chamber, plants were transferred to a glasshouse where conditions were conducive to active shoot growth (30°/20°C alternating diurnal temperature regime; 16 h daylength with supplemental lighting from five 300W Hg-vapour lamps). Eight weeks after placement in the glasshouse, growth flush had emerged and matured on all plants.

In addition to the 60 plants to be used for measuring shoot growth responses to trunk-injected hormones, six plants (three adult- and three juvenile-budded) were incubated as were the above plants. The latter plants were to be used for a brief examination of the translocation of injected growth regulators.

The methods of DE LANGE (1974) and AKAO and TSUKAHARA (1981) for pressure injecting chemicals into the trunks of *Citrus* plants were adapted for introducing the growth regulators into the rootstock stem of the experimental plants. A hole (2,5 mm diameter; ca. 2,5 mm depth) was drilled midway up the rootstock stems (ca. 100 mm from ground level) and the nozzle of a

one millilitre plastic disposable syringe was forced into the cavity. Solutions to be injected were dissolved in one millilitre of distilled water and this was injected under pressure into the wood of the rootstock. Pressure was applied by connecting tensioned elastic bands between the plunger and the syringe base. The time required for the injection of one millilitre of solution was approximately one hour.

In the preliminary study of growth regulator translocation, the movement of [^{14}C]-labelled gibberellin A_3 was monitored. A six millilitre aliquot of [1, 7, 12, 18- ^{14}C] gibberellin A_3 (radioactive concentration 50 $\mu\text{Ci/ml}$ or 1,85 MBq/ml) (Radiochemical Centre Ltd., Amersham, England) in ethyl acetate was mixed with six millilitres of distilled water. The ethyl acetate was then evaporated off under a stream of air, with continuous stirring. Each of the six plants was then injected with one millilitre of the aqueous radioactive gibberellin solution. Twenty-four hours later the scion stem was defoliated and cut into 100 mm sections; the rootstock stem and the tap root were also sectioned into 100 mm segments. The centrifugation method of GOLDSCHMIDT and MONSELISE (1968) for the extraction of hormones from *Citrus* stems was adapted for use in this study. Segments were placed in centrifuge tubes so that they dipped into the one millilitre aliquots of absolute methanol placed in the tubes. After centrifugation at 3000 g for 30 minutes, the segments were discarded and the methanolic centrifugates were decanted into glass scintillation counting vials. The vials were placed under a stream of air at room temperature so as to evaporate the extracts to dryness. After this was achieved, one millilitre of absolute methanol was added and 10 ml of scintillation cocktail (4,0 g l^{-1} of PP (2,5-diphenyloxazol) and 0,2 g l^{-1} of dimethyl-POPOP (2,2'-phenylbis 4-methylphenyloxazol)) (HENSON and WHEELER, 1977). Radioactivity was then determined using a Tri-carb liquid scintillation spectrometer (Packard Instruments (Pty.) Ltd., Johannesburg). Correction for quenching was achieved by the channels ratio method.

Injection of the labelled gibberellin in the preliminary study, described above

and of the growth regulators used in the growth study, described below, was carried out as soon as bud sprouting was apparent in the apical buds of scion shoots. Aqueous solutions of gibberellin A₃ (one milligramme per millilitre) and benzyladenine (one milligramme per millilitre) were injected and a control treatment of distilled water was injected into the ten plants allocated to each treatment of the juvenile- and adult-budded plants, respectively. One millilitre aliquots were injected into each plant.

The effect of these treatments on the emerging scion flush growth was evaluated after the new flushes of growth had matured. Parameters of leaf and stem growth on the new flushes only, were recorded and certain derived statistics were calculated.

Results

One millilitre aliquots of the [¹⁴C]-labelled gibberellin A₃ were used as standards and the recovery of radioactivity from the centrifugates was compared with the standards. About one percent of the injected [¹⁴C]-labelled gibberellin A₃ was recovered in the centrifugates. The proportions of the total amount of radioactivity obtained from the three components of the plant's central axis, is presented in Table 5.4. There appeared to be a gradient of recoverable radioactivity from the scion stem down to the tap root. No major differences were apparent in recovery percentages of components from either juvenile- or adult-budded plants.

The comparison of scion flush growth of gibberellin- and benzyladenine-injected plants with that of water-injected plants is shown in Table 5.5, where measured parameters are expressed as a percentage of the same parameters in control plants. From the results it seems that the amount of gibberellin introduced into the plant was excessive as all aspects of shoot growth were reduced and the overall mass gained by the new shoot of both juvenile and adult scions was significantly lower than that of the respective controls. The variability of

Table 5.4 Recovery of radioactivity from the tap roots, rootstock stems and scion stems by centrifugation after injecting [^{14}C]-gibberellin A₃ into the rootstock stems of juvenile- and adult-budded *Citrus sinensis* cv. Pickstone Valencia orange plants. Results are presented as percentages of the total recovered from the above mentioned plant parts.

| Tissue Analyzed | Percentage of Total Radioactivity Recovered | |
|-----------------|---|---------------------|
| | Juvenile-budded plants | Adult-budded plants |
| Scion stem | 3,7 | 1,3 |
| Rootstock stem | 44,5 | 40,1 |
| Tap root | 51,9 | 58,6 |

Table 5.5 Parameters of shoot growth in juvenile- and adult-budded plants of *Citrus sinensis* cv. Pickstone Valencia orange which had been injected with water (control), benzyl-adenine (BA) or with gibberellin A₃ (GA₃). Results are presented as a percentage difference from the respective juvenile and adult control plants. The responses which were significantly different from those in the control treatments are indicated with an asterisk. (+ = greater than control; - = less than control)

| PARAMETER MEASURED | JUVENILE | | ADULT | |
|----------------------------|-----------------|------|-----------------|------|
| | GA ₃ | BA | GA ₃ | BA |
| Leaf number | -30 | +12 | -27 | +6 |
| Leaf area | | | | |
| Total per stem | -58* | -7 | -7 | -9 |
| Mean per leaf | -46* | -16 | -28* | -4 |
| Leaf mass | | | | |
| Total per stem | -63* | +16 | -10 | +14 |
| Mean per leaf | -40* | -10 | -31* | -9 |
| Leaf mass per unit area | -14 | +25* | -17 | +26* |
| Stem | | | | |
| Total mass | -16 | +4 | -20 | +25 |
| Total length | -40* | -3 | -29* | -2 |
| Total shoot mass per flush | -54* | +14 | -35* | +16 |

the response was very high but significant differences in leaf number, unit area and unit mass and in the total flush stem lengths were detectable in adult and juvenile scion flushes. Total leaf area and mass of the juvenile gibberellin treatments were also significantly depressed below the levels in the control treatments.

The injection of benzyladenine, on the other hand, produced a more uniform response in the ensuing scion flush (Table 5.5). Although it appeared from the measurement of the total shoot flush mass that the benzyladenine may cause enhanced shoot growth relative to the control, only the parameter of leaf mass per unit area was significantly greater than the control value for both adult and juvenile flushes. It seems that the benzyladenine may have caused an increase in the number of leaves and a concomitant reduction in unit leaf mass. Statistical differences did not confirm such an hypothesis, probably because of the high inherent variability in such plants. The 25 percent increase in total stem mass of adult plants, as compared to a four percent increase for juvenile plants, in response to the benzyladenine injection was the only major observable difference between juvenile and adult scion growth.

The extreme variability in plants treated with gibberellin was partly due to the necrosis of two of the experimental plants, twig die-back in others and partial defoliation on new flush growth of some plants. It was not possible to determine the changes in shoot conformation for this treatment as had been done for treatments in other experiments of this chapter. The benzyladenine treatment did, however, induce consistent effects in the ten replicates of juvenile- and adult-budded plants, respectively, and the analyses of patterns of change in shoot conformation are presented in Figure 5.7.

Leaf area and leaf mass were caused to change significantly in both juvenile and adult scion flush growth (Figure 5.7 A; B) but the leaf width:length ratio appeared to be unaffected by the benzyladenine treatment (Figure 5.7 C). Basal leaves of new adult flush growth were larger, in terms of area and mass, as a

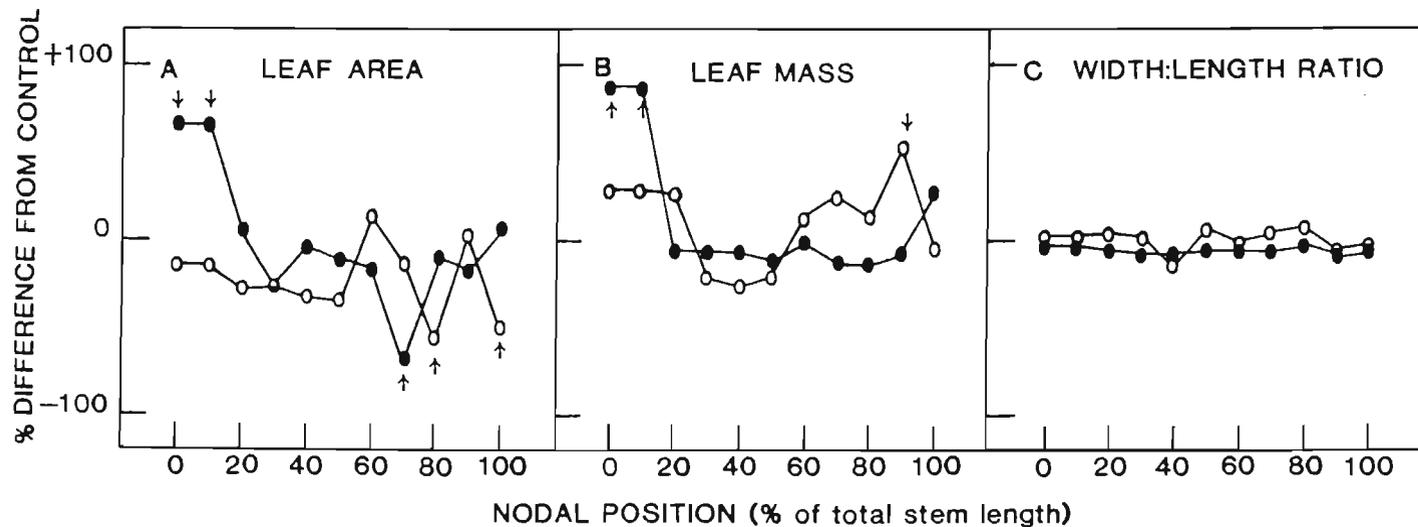


Figure 5.7 The effect of benzyladenine on the variation of unit leaf area (A), unit leaf mass (B) and the width:length ratio (C) along the length of a shoot flush. The benzyladenine was injected into the rootstock stem of juvenile- and adult-budded *Citrus sinensis* cv. Pickstone Valencia orange plants. Results are presented as a percentage difference from the respective juvenile (o) and adult (●) control treatments. Points significantly different from the respective control values are indicated by arrows.

result of the benzyladenine treatment. Towards the terminal end of the shoot flush, leaf area was depressed by the application of benzyladenine to juvenile plants while a point of depression of leaf area was evident only at 70 percent of the flush length in adult scion flush growth (Figure 5.7 A).

Apart from the initial difference in leaf mass caused by the benzyladenine application, no significant response was evident in the leaves of the adult scion flushes (Figure 5.7 B). The situation was somewhat different in juvenile scion growth. Leaf mass was not significantly different from the control although it was reduced in leaves between 30 and 50 percent of the stem length. Despite the indication in Table 5.5 that total leaf mass per stem was slightly higher in benzyladenine-treated adult and juvenile plants, the only point of significant difference was at 90 percent of the shoot length, where leaves of the juvenile scion flush were significantly greater than those of the control treatment (Figure 5.7 B).

Discussion

There are two important concepts underlying the experiments conducted with synthetic growth regulators, as described above. In the first place, it is clear that shoot development is controlled largely by the stem apex. Secondly, although it may not be as clear, shoot meristems are known to have developmental, organizational and regenerative plasticity (TREWAVAS, 1981). This means that it is through the ability of the plant itself to manipulate shoot differentiation from an apical meristem, that differences such as are evident in juvenile and adult shoot growth, become possible. In order to understand the role played by plant growth regulators in these manipulations, the differences in endogenous hormones should be established first and then the effect of synthetic changes to specific classes of hormones should be observed. The purpose of the current series of experiments was to execute the latter of these requisites.

In the first two experiments described above, growth responses affected by growth regulator applications to apical buds, before the commencement of and during rapid growth and differentiation, were measured. When the supply of gibberellin A₃ to the apex was kept at a high level artificially throughout the period of active shoot growth, there appeared to be a reduction in the restriction on the number of leaves differentiated by the apices. This effect was absent when gibberellin levels were only augmented at an early stage in shoot differentiation. Individual leaves were invariably reduced in area and mass by gibberellin applications to apical buds, though, while the converse was true for dimensions of stem growth. It seemed that the balance between leaf and stem growth was mediated by the gibberellin levels. In all three of the above experiments gibberellin applications resulted in a reduction of leaf area and mass. The exact nature of gibberellin mediation in this balance will be discussed below. No consistent discrepancies in leaf, shoot and overall stem growth responses resulting from gibberellin applications were evident between juvenile and adult scion growth. Yet it seems, from the results of Chapter IV, that gibberellin-like hormones do play a role in the differences of shoot conformation and growth between juvenile and adult scions. The dilemma may be resolved, either partly or wholly, by repeating the experiments with a range of gibberellin concentrations.

The type of reciprocal allometric growth relation suggested above, between stems and leaves, may also be found between other parts or organs of plants and there is some evidence that hormones play a regulatory role in the growth of such plant components. RICHARDS (1980) found that cytokinin applications to the leaves of apple seedlings encouraged shoot growth at the expense of root growth, for example. He found that cytokinin applications to the roots reversed the situation and decreased the shoot:root ratio.

In the current experiments cytokinin applications to the shoots were also found to increase shoot growth above that of the controls, but the effect on

root growth was not observed. In most instances leaf mass per unit area and total stem mass per flush were increased by the cytokinin treatments but the effects were not always statistically significant. There did not appear to be any related changes in the growth of shoot components as a result of the cytokinin applications, as there were with gibberellin applications.

Although abscisic acid is generally regarded as a stress hormone, some evidence has been presented for its role as a growth stimulator (TIETZ, 1973; WESTON, 1976; ABOU-MANDOUR and HARTUNG, 1980). No significant change in the total shoot mass of scions was achieved by applications of abscisic acid before or during the active extension growth of shoots. In those cases where leaf growth was stimulated by abscisic acid applications, there was generally a concomitant increase in stem mass. Where parameters of leaf growth were not stimulated, stem growth appeared to be depressed. These effects were, however, inconsistent and of dubious significance. They did provide a rare example of a clear difference in response of the juvenile and adult scions, when the regulator was applied repeatedly during flush growth. In this case leaf growth, generally, was stimulated in the juvenile scions, while it was not in adult scions. The adult scions did respond by developing lighter and shorter stems.

By availing of the current knowledge on the subject, it can only be assumed that the majority of the effects of the growth regulators in the above experiments were mediated through differential effects on assimilate partitioning. No major change seemed to be effected in the behaviour of shoot apices by the applications of growth regulators, as was evinced by the stable phyllo-taxis of all treated plants in the first experiment.

The concept of differential assimilate partitioning will only be considered in its entirety, however, when the effects of the growth regulators on aspects of shoot conformation have been deliberated upon. In doing so it is essential to relate changes in structure (presented in this study as deviations of

parameters from those of control treatments) to the differences in aggregate leaf and stem production of the different treatments. For example, when gibberellin A₃ was applied continuously to the apices of developing flushes a gradual reduction in leaf area, mass and width:length ratios relative to the controls was observed. Yet the mean response from the whole shoot for these parameters was only significantly affected in the case of the adult scions. Nevertheless, a trend was apparent in which basal leaves were larger and heavier than those of the control treatments and terminal leaves were significantly smaller and lighter. This trend may have indicated that, in common with the findings of MULLIGAN and PATRICK (1979), gibberellin A₃ was able to attract assimilates to the site of growth regulator application, while at the same time the augmented gibberellin levels in the apex caused a continuous bud sprouting and differentiation of new shoot tissues (COOPER, YOUNG and HENRY, 1969). Inevitably the supply of assimilates became limiting and the end product was an elongated flush of growth with small narrow leaves near the terminal end of the flush.

The cytokinin treatments were not found to reduce aggregate leaf growth under any of the experimental circumstances in juvenile or adult scions. In many cases the treatments had the effect of promoting aggregate parameters of leaf growth and of promoting leaf growth in certain regions of the new stem. The continuous application of abscisic acid to buds during shoot growth had the effect of stimulating growth of the more distal leaves, but in other respects and in the limited early application of abscisic acid, no major response was noted.

The understanding of laws governing assimilate partitioning is beset by problems of numerous interacting factors. In none of the experiments conducted here, was it possible to isolate a system of absolute response which was unaffected by other features of shoot regeneration. In these studies, the effects of gibberellin treatments in the postulated role of enhancing sink strength at

the site of application (MULLIGAN and PATRICK, 1979), were irregular and may have been greatly affected by the time and concentration of applications. The interaction of these applications with the natural variation in components of shoot conformation (e.g. leaf size or mass) and the natural control mechanism of these properties, needs to be given due consideration. The trends induced by growth regulator applications were of greater significance than the aggregate or localized significant differences, for two reasons: 1) the trends provided some indication of the influence of a factor limiting growth or varying in its limitation of growth and 2) because the interaction with natural trends of regulation can be shown to be synergistic or antagonistic. For example, by continually augmenting gibberellin levels in the apex during shoot growth a synergistic response, in terms of gradually decreasing unit leaf area in the distal direction, was found. A similar treatment with abscisic acid acted contrariwise by increasing the unit leaf area closer to the distal end of juvenile shoot flushes. The possibility of interaction can be of use in identifying the regulating effects of specific growth parameters.

Whether or not photosynthetic reserves are limiting factors in the above considerations, they do undoubtedly influence the growth of new shoots and especially so when leaves reach maturity and change from being sinks to sources of photosynthetic reserves, at different times on the same flush, as in *Citrus*. The results of the first two experiments differ somewhat from the natural situation in that reserves normally available from or supplied by leaves of the shoot, were not available due to decapitation of plants. Other effects of decapitation may also have confounded results. As stated above, gibberellins and abscisic acid are able to direct differential growth probably by acting through preferential partitioning of assimilates. MOR, SPIEGELSTEIN and HALEVY (1981) have provided evidence of a similar type of regulation by cytokinins in rose shoot growth. QUINLAN and WEAVER (1969) and MULLIGAN and PATRICK (1979) showed that cytokinins and gibberellins, respectively, are able

to promote metabolic transport toward the treated organ independently of any other effects they may exert on sink or source tissues. In the case of the above experiments, two kinds of response to the specific types of regulators appear to be operative. The degree of usurpation of reserves, as expressed by the extent of reduction or enhancement of aggregate shoot growth relative to the control treatments, represents the reserve mobilizing ability of regulators. The differential promotion of growth in one organ type (e.g. leaves) at the expense of growth in another (e.g. stem) by specific growth regulators, represents the second type of regulatory mechanism which appears to be operative. Evidence that gibberellins may act to promote greater stem growth comes from the experiment where CCC was applied to apices. Enhanced leaf growth in adult flushes and reduced stem growth in both adult and juvenile scions probably resulted from the inhibition of gibberellin synthesis in apical leaflets (JONES and PHILLIPS, 1967). Note, however, that this treatment did not affect aggregate shoot growth and, therefore, possibly did not affect gibberellin mediated reserve mobilization.

Although the growth responses of juvenile and adult growth flushes were in some cases simultaneously enhanced or depressed by the growth regulator treatments, the small discrepancies in magnitude of response could indicate a difference in capacity for storage of reserves. The differences may reflect a discrepancy in the ability to cope with modifications of assimilate partitioning, expressed for example by a resistance to the changes effected by growth regulators. Adult plants may, for example, have a greater capacity to inactivate growth regulators through glucosylation or esterification.

The growth responses of juvenile and adult scions, after gibberellin and cytokinin trunk injection were confusing and inconsistent with those of the apical bud treatments. The incongruous results may have been caused by the high dosage. The method of application also resulted in unexpected movement of the gibberellin, at least, into the root system. The recovery of radioactive

tracers, as detected in the above experiment, provides no information with regard to relative apportioning of the growth regulator to shoots or roots, but it does show that gibberellins injected into the trunk move into shoots as well as into the roots. The growth responses were, therefore, affected by augmented gibberellin levels in shoot and root tissues.

Two processes appeared to be operative in the chemical growth regulation of plants as investigated here. Both the mobilization of reserves and the assimilate partitioning abilities of growth regulators seemed to affect growth. Further investigation is necessary to determine the extent of possible discrepancies in reserves, as reported in Chapter III, and the effect of such discrepancies on aggregate shoot growth in juvenile and adult scions. In all plants, however, resources are limited and it is the manipulation or redirection of growth which can, with further understanding of the processes, be put to best use in improving stem conformation for maximum efficiency of growth. This may be achieved by enhancing specific features of shoot growth through manipulating growth regulator levels.

GENERAL DISCUSSION AND CONCLUSIONS

There may be many cause and effect relationships allied to the disparity found between plants of different ontogenetical age. It was an interest in the non-uniformity of shoot growth and morphology and the relation of plant growth regulators to these disparities which motivated the current research. During the growth of a shoot flush in *Citrus* plants, the following processes are activated: a) the mobilization of storage or reserve materials from tissues, organs and organelles to provide the basic structural components of the new growth; b) the activation of the shoot apex to differentiate new tissues and organs and c) the development of the new organs and tissues which will be limited, to some degree, in the extent of their growth by the amount of stored and current assimilates channelled to the particular organs. Some basic observations of these three processes have been undertaken in the present study and they will be discussed below, in relation to their supposed regulation by plant growth regulators.

The aggregate mass of the new shoot flushes reflects the relative amount of reserves and current assimilates made available for the production of new shoot tissues. The direct comparison of shoot flush growth in similar juvenile- and adult-budded plants revealed that although components of shoot flush growth differed in respect of the final fresh mass values, the aggregate fresh mass of the whole shoots did not differ significantly in this respect. It is perhaps understandable that similar plants will dispense with similar proportions of stored reserves for the production of new vegetative shoot growth. Yet there are a number of indications that growth is unequal in juvenile and adult scions. Juvenile scions were found to produce a larger collective leaf area and longer stems per flush of growth than adult scions. JONES and STEINACKER (1951) reported that starch levels increased in leaves prior to the growth of a shoot flush. They implied that the starch was utilized in the production of new shoot tissues. The higher levels of starch

found in the leaves of juvenile scions in the present study would, therefore be expected to contribute to disproportionate shoot growth. This anomalous finding needs to be investigated further in order to establish whether leaf-stored starch is the major source of structural material for shoot growth, or whether stem- or root-stored starch contributes the major proportion. SINCLAIR and BARTHOLOMEW (1937) showed that the chloroplasts of mature *Citrus* leaves bind the natural starch of the leaf and that digestion by leaf enzymes is slower than in many other plants. The mobilization of reserves from different storage sites needs to be examined in more detail, but it does seem possible that plants have the capability of using only a portion of stored reserve material for new growth. In addition the amount of leaf-stored starch may affect the type of regenerative growth after a period of quiescence or dormancy (LEWIS, COGGINS and HIELD, 1963). These authors showed that the amount of leaf-stored starch was variable and was closely linked with alternate bearing in *Citrus reticulata*.

The present investigations of growth differences in juvenile- and adult-budded plants are by no means conclusive and further research is necessary to clarify important aspects. Simple tests with labelled tracers could elucidate the question of current photosynthetic contributions to growth. Extended analysis of growth would be required, however, to determine the long term effects of the longer stems and the greater leaf area in juvenile plants, for example. Although many parameters were not significantly different, there appeared to be a capacity for small excesses in growth of many shoot components of juvenile plants. These differences may increase in an exponential fashion in accordance with the compound law of plant growth (BLACKMAN, 1919) and may show highly significant differences in larger juvenile- and adult-budded plants.

It is probable that the change in patterns of growth accompanying ontogenetic ageing in certain tree species would only have persisted if the phenomenon did not confer a competitive disadvantage on species displaying juvenile/adult

differences. It must be concluded, therefore, that the differences have no effect on the survival of such plants or that the inherent growth characteristics of juvenile seedlings and adult, bearing trees actually favour the survival. HEYBROEK and VISSER (1976) and SCHWABE (1976) class juvenility in the group of characteristics with survival value. This means that a definite programme is followed in regulating the differences between juvenile and adult shoot growth. Between the genetic incitement and the production of a final growth form, is to be found the mediation of plant growth regulators. The major part of the present study was concerned, not so much with the cause of juvenility, but with a potential regulatory mechanism involved in the expression of juvenility. Inevitably, this meant analysing the endogenous hormone levels and attempting to modify manifestations of either juvenile or adult growth habits in order to relate particular types of growth regulators to specific features of ontogenetically-related growth. Although a comprehensive study of all plant parts would have been desirable, limitations of time and materials restricted the study to the differentiation of tissues from shoot apical meristems. Since it is meristems only and shoot meristems in particular, which are capable of producing adaptive developmental changes in plants, it is justifiable to consider these organs as being of primary interest in studies concerning plant development. The plasticity of meristems (TREWAVAS, 1981) is an important factor not only in permitting plants to survive in a range of environments, but also in allowing plants to cope with the increasing complexity of growth co-ordination which accompanies ageing. Most of the interest in growth regulators stems from the fact of their recognition as "agents of developmental modification" (TREWAVAS, 1981).

Although not quite appropriate to the current experimental objectives, the rules formulated by JACOBS (1959) for the determination of substances controlling specific biological processes, were used, to some extent, in tackling the problem. In this case the substances were gibberellins, cytokinins and inhibitors and the biological process was one of shoot growth which

followed different patterns in adult and juvenile scions. Endogenous growth regulators were examined to test for "parallel variation" in order to fulfill Jacobs' first rule. Growth promoters and inhibitors were applied to plants to test for their ability to "substitute" for properties inherent in one or other phase of growth, to test for fulfillment of other criteria.

In most of the cases examined gibberellins appeared either to be present in juvenile and adult apices at different levels of activity or to invoke a drastic response in growth form from juvenile or adult apices. Studies on juvenility in *Humulus* (WILLIAMS, 1961; THOMAS and SCHWABE, 1969), *Ribes* (SCHWABE and AL-DOORI, 1973) and *Hedera* (FRYDMAN and WAREING, 1973 a, b) have provided evidence of a correlation between high gibberellin levels and the juvenile phase of growth. In addition, many examples of a reinduction of the juvenile phase have been reported following gibberellin applications to plants (ROBBINS, 1957 b; GOODIN and STOUTEMEYER, 1961; BORCHERT, 1965; DE LANGE, 1974; ROGLER and HACKETT, 1975 a). Great care should be exercised in distinguishing between the "pseudo-juvenility" (SCHWABE, 1976) found in *Ribes* the unique type of juvenility in *Hedera* and juvenility of woody species, however. There also appears to be a different regulatory mechanism as far as juvenility is concerned, in gymnosperms and angiosperms (LONGMAN, 1976). Instead of inhibiting flowering generally as for example, in *Citrus* (GOLDSCHMIDT and MONSELISE, 1972), gibberellins have the effect of promoting early flowering in gymnosperms (WHEELER, WAMPLE and PHARIS, 1980).

It does not seem likely that one central control mechanism, regulated largely by gibberellins or gibberellin levels, affects all features of juvenility, even though the majority of available evidence indicates that gibberellins appear to be implicated in many expressions of juvenility. Of the effects generally ascribed to gibberellins, the following were observed to varying degrees in juvenile- and adult-budded plants: 1. changes in reserve mobilization (MULLIGAN and PATRICK, 1979; PATRICK, JOHNSTONE and WAREING, 1979);

2. changes in assimilate partitioning (BEN-GAD, ALTMAN and MONSELISE, 1979) and 3. increases in stem length (SHININGER, 1975). The results of the current experiments indicate that gibberellins may be responsible for the differences in assimilate partitioning, as reflected in the discrepancies of some components of an entire shoot flush and for the differences in length of shoots. As might be expected, though, many unanswered questions were posed as a result of the experimental findings.

The results of the CCC-application experiment were extremely useful in indicating that gibberellins seem to control some aspects of growth which were different in juvenile and adult scions. This regulation appeared to act mainly through the process of assimilate partitioning. Thus, parameters of stem growth were reduced and, in adult plants, parameters of leaf growth were simultaneously increased, presumably as a result of the inhibition of gibberellin synthesis after CCC applications. The treatments with this synthetic inhibitor did reduce symptoms of juvenility, but there was no sign that maturation or growth typical of adult scion shoots was induced. It also answers the question of whether gibberellins are a result or cause of juvenility. Gibberellins appeared to mediate the genetic expression of a shoot meristem and to determine only the extent of specific aspects of shoot growth. As a corollary, it might be questioned whether low gibberellin levels indicate the presence of the adult phase in, for example, an apical meristem, or whether artificial augmentation of gibberellin levels in apices will always cause a reversion to the juvenile status and, if so, whether the reverted plant will be subject to abrupt or normal ontogenetical ageing in new growth cycles. In all cases the answer appears to be that the hormone levels determine transient growth responses only. It is the hypothetical time measuring device (ROBINSON and WAREING, 1969), acting within its self-imposed restrictions and in conjunction with the plants' physical status and the environment, which determines the limitations of shoot growth. These theoretical "self-imposed restrictions" are the basis of the juvenility phenomenon.

and must surely be related to the selective activation and repression of genes, as was suggested by SUSSEX (1976). Nevertheless, such gene expression seems to be mediated through plant hormone activity.

Gibberellin activity was found to affect shoot growth in many ways. High gibberellin levels are known to reduce flowering in *Citrus* (GOLDSCHMIDT and MONSELISE, 1972) and in the present study supplemented gibberellin levels appeared to alter the balance between stem and leaf growth and to alter leaf shape. Whether or not such differences in behaviour of juvenile and adult scions were caused or even affected by differences in endogenous gibberellins was not always absolutely clear. When considering the apex in isolation, it is also not clear whether the apparent inherent differences of endogenous gibberellin levels might be acting simultaneously to regulate the flowering ability, the mobilization of reserves, the partition of assimilates in the balance of stem and leaf growth and also to affect leaf shape. It is not inconceivable that it might participate in all processes at the same time. WAREING (1982) proposed that it is not the absolute level of gibberellin which is the significant parameter, but the rate of turnover. That is, hormone activity is linked to a degradative pathway. He suggested that a measure of the regulation of biosynthesis and of the sensitivity of plants to growth regulators would provide better indices for correlations with developmental processes.

The effects of gibberellins can be cancelled (GRAEBE and ROPERS, 1978) or antagonised (HILL, 1973) in certain cases, by abscisic acid. Very little can be said concerning the ratio of abscisic acid to gibberellin, on the basis of the findings from Chapter IV for endogenous hormone levels. However, the growth responses resulting from abscisic acid applications to shoot apices does suggest that some of the promotive effects of gibberellins are reduced by this inhibitor.

Although there is some evidence in the literature that cytokinin treatments to plants can enhance leaf growth (SCOTT and LIVERMAN, 1956; LEOPOLD and

KAWASE, 1964; NAITO, TSUJI and HATAKEYAMA, 1978), the current experiments showed little evidence for a consistent or concerted effect on leaf and total shoot growth. Levels of endogenous cytokinins in shoot apical buds and in other tissues differ substantially at certain times. Although it may be fruitless to measure endogenous cytokinin levels in the attempt to establish correlations with developmental processes (WAREING, 1982) as stated for gibberellin determination above, the results of these determinations do raise questions concerning differences. With regard to dormant apices, where major cytokinin differences were found, the situation is somewhat different. There is no known reason to believe that a high turnover of cytokinins is taking place in dormant apices and the detected levels of activity may well reflect the sum total of the active cytokinin component. It should be noted that a vague and possibly inaccurate indication of synthesis and oxidative metabolism of cytokinins in tissues may be obtained from the detection of *isopentenyl* adenine-like and purinylglycine-like components in extracts, respectively. These two compounds are thought to be components of the cytokinin synthetic (LETHAM, 1978) and oxidative (VAN STADEN, DREWES and HUTTON, 1982) metabolic pathways, respectively. Thus it appears from Figure 4.7, that little metabolism was taking place in dormant buds, but further tests, possibly with enzyme levels, are necessary for support of such a proposal. There is extensive evidence for the involvement of cytokinins in the regulation of assimilate partitioning (SHINDY and WEAVER, 1967; VARGA and BRUINSMA, 1974; HOAD, LOVEYS and SKENE, 1977; RICHARDS, 1980; GOUSSARD, 1981). The inherent differences in endogenous cytokinins found in many of the tissues of juvenile- and adult-budded plants may have actively contributed to regulating the differences in shoot growth of the two scion types by directing assimilates to specific developing tissues. Such a mechanism has been shown to operate in *Citrus*, where kinetin applications to the fruit resulted in a greater incorporation of assimilates by the fruit (KRIEDEMANN, 1967).

Very little information was gained concerning the effect of growth regulators, in plant parts other than the shoot apex, on shoot growth. Major differences were, nevertheless, detected in cytokinin-like and gibberellin-like components of the wood and bark of juvenile- and adult-budded plants. Although it is tempting to attach significance to the large discrepancies between juvenile- and adult-budded plants, no indication of the rate of turnover of hormones was available. Attempts have been made to relate influences of plant parts other than the shoot apex, on juvenility in *Hedera helix* (DOORENBOS, 1954; FRYDMAN and WAREING, 1973 b); in *Ribes nigrum* (SCHWABI and AL-DOORI, 1973) and in *Eucalyptus grandis* (PATON, WILLING and PRYOR, 1981). With the exception of DOORENBOS, the above authors imply that translocatable factors or alternatively a root-shoot gradient of specific compounds are actively involved in expression of juvenility. Root synthesized gibberellins are the supposed juvenility inducers in *Hedera* and *Ribes* and the compound G (DHAWAN, PATON and WILLING, 1979) is purported to decrease features of juvenility. None of the above effects appear to operate in *Citrus* as the topophysis effect predominates, no matter whether adult scions are grafted onto juvenile rootstocks or whether adult shoots are rooted directly. The role of the root supplied gibberellins, if any, in shoot growth of *Citrus* is unknown and cannot be related to systems of regulation in plants exhibiting "pseudo-juvenility" or a vine-like growth as in *Ribes* and *Hedera*, respectively. In fact, any extrapolation of the knowledge on juvenility in *Hedera* might lead to erroneous conclusions with respect to tree growth, since *Hedera* appears to be unique in many respects of the juvenility phenomenon.

A number of factors may well be related to a root-shoot gradient in trees aside from hormones. Whether the differences found in growth and in hormones (specifically in detectable gibberellin levels) from the upper and lower reaches of plants examined in the current experiments relate to the different ontogenetical age of the tissues remains an unanswered, but interesting problem. Growth arising from lateral meristems will inevitably be affected by

contrasts in light, water relations, hormone and nutrient gradients and other factors. Acting within these constraints, lateral meristems might still be expected to behave and produce new growth which is characteristic of the inherent ontogenetical age of that meristem.

In processes of dedifferentiation and the establishment of new meristems, as in callus production or rooting of leaf cuttings, the specific ontogenetical age of the meristem which produced the dedifferentiating organ is instituted. In fact, it appears from the findings of DOORENBOS (1954) that juvenile and adult somatic tissues may behave differently in certain respects. He found that rejuvenation of adult shoots, when grafted to juvenile ones, was promoted by the presence of juvenile leaves and inhibited by adult ones. In a similar vein, the differential rooting ability of budless (meristemless) leaf cuttings of the present study, illustrated this point. The difference in rooting ability was not altogether changed by the presence of an attached bud either, indicating that there is some substance to the effects of the somatic tissues. The possibility of auxin involvement in these behaviour patterns and in many other features of ontogenetical age-related phenomena is interesting and is deserving of attention.

There does seem to be good reason to believe that a whole-plant contribution to growth patterns and plant behaviour is operative in juvenile and adult plants. The integrated control of these processes in plants generally, appear to be mediated through growth regulators (TREWAVAS, 1981, 1982; WAREING, 1982). However, it is argued that growth substances do not act specifically in development, but only as accelerators of an already predetermined path (SINNOT, 1960). The mediation of growth regulators in the integration of growth processes may operate via plant sensitivity to manipulation, that is, through the number of receptor sites for particular hormones (TREWAVAS, 1982). This means that there are some tissues which are more receptive to hormone mediated activation and that within a single plant receptivity to hormones in particular

organs varies with time. Notwithstanding the possibility that such an hypothesis is probable, it is important to know the aspects of growth which are modifiable by any particular growth regulator. Given optimum growing conditions, it is only through the application of growth regulating compounds that plants can be further manipulated to improve growth characteristics. Even when marginal growing conditions have to be contended with, growth regulators may be of assistance in plant culture.

Though many examples could be listed where growth regulators could be used to improve growth, the prime example in the current context is that of shoot growth in *Citrus* orchard trees. MONSELISE (1978, 1979) pronounced that it would be very desirable to be able to reduce vegetative growth, possibly without reducing leaf area, so that the same yield would be borne on a much smaller tree. He stated that size control should be attempted by reducing the length of shoots, without reducing foliage. This requires the shortening of internodes or obliteration of apical control, with the production of a larger number of short laterals. In many instances the experiments described above revealed that gibberellins may be responsible for promoting undesirable vegetative growth, while increased levels of abscisic acid and synthetic cytokinins, to a lesser degree, appeared to alter growth in the desired direction.

There are many examples of growth regulator treatments having been introduced into practical use with an insufficient understanding of their way of action or of the physiological processes involved. Although the present study has improved, to a lesser or greater extent, on the current knowledge pertaining to growth regulation in tree crops, there is undoubtedly scope for extensive further investigation.

SUMMARY

In these experiments various physiological aspects of shoot growth were examined in juvenile and adult *Citrus sinensis* (L.) Osbeck. Three sets of investigations were undertaken. In the first place, basic differences in the morphology and extent of shoot flush growth; in the anatomy and ultra-structure of shoot apical buds and differences in other features of shoot growth of juvenile and adult scions were resolved. In the second place, endogenous hormones of buds, shoot growth flushes, stems and roots of juvenile- and adult-budded plants were extracted, purified and analysed using bioassay techniques. Lastly, the response, in terms of shoot flush growth, to the application of synthetic hormones was assessed in the juvenile- and adult-budded plants.

The stability of the intrinsic differences between juvenile and adult shoot apical meristems during somatic regeneration was demonstrated after budding the respective scion material onto uniform seedling rootstocks. The plants developed distinctive scions and these plants were used in all of the experiments. Although the inherent variability of the plants was great, discrepancies in specific features of shoot growth were apparent. New scion flush growth which emerged after a period of induced dormancy revealed that in none of the features analysed, was growth of adult flushes significantly in excess of that from juvenile flushes. The reverse was true for total leaf area per plant, stem length and stem diameter. Stem mass was greater in juvenile scions, but not significantly so. Although other factors may have confounded the issue, a similar discrepancy was found between upper (ontogenetically adult) and lower (ontogenetically juvenile) shoots on single, tall plants.

Major differences in endogenous gibberellins and cytokinins were found in juvenile and adult apical buds. Whenever buds were undergoing active growth, gibberellin levels were found to be higher in juvenile or juvenile-like buds (e.g. on the lower reaches of stems of tall trees). Gibberellins were virtually undetectable in dormant and quiescent apical buds. Cytokinin levels

were higher in juvenile dormant buds but very much lower levels were detected in juvenile than adult developing buds. In expanding whole shoots the situation was very similar to the above, as far as gibberellin levels were concerned. Juvenile tissues housed very much higher levels than adult tissues.

The effect of the scions, whether adult or juvenile, appeared to extend to the uniform rootstocks, for higher levels of gibberellins were found in fibrous roots of juvenile, as opposed to adult plants which were in the process of producing a flush of growth. Gibberellins were extracted from stem tissues of dormant and flushing plants by centrifugation. In both adult- and juvenile-budded plants extremely low gibberellin levels were detected. However, in quiescent plants relatively high levels were detected in juvenile scion stems, rootstock stems and tap roots, while much lower levels were found in similar adult tissues. Cytokinin levels were higher in fibrous roots of juvenile plants, but very little difference was detected in the cytokinins extracted by centrifugation from the wood of juvenile and adult rootstock stems. The bark of adult plants yielded extremely high levels of a substance tentatively suggested to be a glucosylcytokinin-like compound. The analysis of inhibitory substances did not yield very useful information and it was concluded that more specific and sensitive techniques were necessary.

The application of gibberellins, cytokinins and abscisic acid to juvenile- and adult-budded plants did not appear to disrupt any of the basic morphogenetic processes. It was only the apparently predetermined aspects of growth which were either enhanced or retarded. Thus, it was found that by increasing gibberellin levels in shoot apices either before or during the emergence of a growth flush, stem growth was extended, but to some extent at the expense of proportional leaf growth. Synthetic cytokinins seemed to have little effect on the growth of shoot flushes and only in the case of continuous applications to growing apices of juvenile plants was unit leaf mass significantly increased.

Absciscic acid applications generally resulted in a shortening of stems, but did also result in increased total leaf and stem mass in some cases. When absciscic acid was applied continuously to apices the leaves near to the terminal end of flush stems were larger in juvenile scions. Of the growth regulators applied, gibberellin A_3 seemed to be the only compound which affected leaf shape. Longer and/or narrower leaves resulted from applications of this hormone to differentiating apices.

Total leaf number, mass per unit leaf area, and stem mass were increased by the injection of benzyladenine into the trunks of the plants, but little difference between the response of juvenile and adult plants was observed. Insufficient information was recovered from the experiments where gibberellin A_3 was injected into plants, probably as a result of the high dosage. The radioactive tracer study revealed that gibberellin injected into the trunk of plant does move in a distal direction to roots and shoots.

In the experiment where CCC (2-chloroethyltrimethyl-ammonium chloride) was applied to apical buds before bud sprouting had occurred, it was shown that gibberellins seem to control the balance between stem and leaf growth. As a result of the antigibberellin treatment, leaf growth was promoted in adult flushes and stem growth was reduced in both adult and juvenile flushes.

It was concluded that three basic processes were functional during the growth of a shoot flush: 1. the shoot apex was activated to differentiate new tissue and organs; 2. storage and reserve materials were mobilized from tissues, organs and organelles to provide structural components for the new growth and 3. new organs and tissues developed to an extent determined by the amount of stored and current assimilates channelled to these particular stem components. Plant growth regulators appear to mediate in the control of all of the above processes.

The aggregate mass of new shoot flush growth was only significantly increased by the continuous application of gibberellin A_3 . In most other cases there

appeared only to be a reapportioning of assimilates (reflected in the relative increase or decrease of growth in particular organs) as a result of growth regulator applications. The differences in endogenous hormones were, therefore, considered to be part of the growth regulatory mechanism operating to produce growth characteristic of juvenile or adult plants, respectively. In the phenomenon of juvenility, hormones appear to mediate between the genetic incitement and the production of a final growth form. The genetic incitement appears to be subject to a theoretical time or cell-division measuring device which produces shoot growth adapted either for juvenile, seedling or adult, tree canopy environments.

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