FLUCTUATIONS IN THE ENDOGENOUS ABSCISIC ACID CONTENT OF *ALNUS GLUTINOSA* (L.) GAERTN.

BY

SUSAN H. WATTS BSc.

1985

A dissertation presented to the University of Glasgow for the degree of Doctor of Philosophy.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

To M and D
with thanks

CONTENTS

ACKNOWLEDGEMENTS

This study was carried out in the Botany Department, University of Glasgow. I wish to thank Professor J. R. Hillman for continuous supervision of the project and Dr. C. T. Wheeler for his tremendous help and guidance throughout the course of the work. Professor M.B. Wilkins is thanked for the use of facilities in the Department. The following members of staff are warmly thanked for their generous advice which was often sought :

Dr. A.M.M. Berrie
Dr. V.B. Math
Dr. A. Crozier

James McMurray and Richard McGowan are thanked for their expert technical assistance; the gardeners, Robert Cuthbertson and Donald Hamilton are also thanked for their invaluable help with growing plant material and alder nodule and seed collecting expeditions.

This work was supported by the University of Glasgow.

Finally, I wish to thank Keith for the photographs presented - and for his patience.

Thanks are due also to my parents for their support during my university career.

The manuscript was skilfully typed by Mrs. M. Winship.

ABBREVIATIONS

The SI system of units has been used throughout this thesis unless otherwise stated. Other abbreviations are listed below :

ABA	abscisic acid
ABAGE	abscisic acid glucose-ester
ABAGS	abscisic acid glucopyranoside
ABAMe	abscisic acid methyl ester
AES	automatic external standard
CD	circular dichroism
cv.	cultivar
CV	coefficient of variation
d	days
d.wt	dry weight
DPA	dihydrophaseic acid
ECD	electron-capture-detector
FID	flame-ionisation-detector
f.wt	fresh weight
GA	gibberellic acid
GC	gas-chromatography
h	hours
HMABA	hydroxymethyl abscisic acid
HPLC	high performance liquid chromatography
IAA	indole acetic acid
IR	infra-red
λ	wavelength
MIM	multiple-ion monitoring
MS	mass-spectrometry
MVA	mevalonic acid
m/z	mass to charge ratio
n	number of observations
NAA	napthalene acetic acid
NMR	nuclear magnetic resonance
ORD	optical rotary dispersion

PA	phaseic acid
PAR	photosynthetically active radiation
PGS	plant growth substances
pH	$-\log_{10}[H^+]$
ppm	parts per million
	water potential
PVP	polyvinylpolypyrrolidone
R_f	distance compound has moved from origin / distance of solvent front from origin
SE	standard error of the mean
short-day plants	plants grown under short photoperiod
Σ	sum of
SIM	single-ion monitoring
TLC	thin-layer chromatography
UV	ultra violet
var.	variety
v/v	volume for volume

SUMMARY

This thesis concerns an investigation of the distribution of the plant growth substance abscisic acid (ABA) in the deciduous tree Alnus glutinosa (L.) Gaertn., a species which bears nitrogen-fixing root nodules. The study was divided into two areas: (i) the identification and quantification of endogenous ABA in Alnus
(ii) the transport of applied [2-^{14}C] ABA; results are presented in two sections accordingly.

In part 1.1 of the Results, the development of a schema suitable for the purification of ABA from Alnus tissues is described (Results 1.1.1). ABA was successfully identified in Alnus roots, leaves and nodules using the technique of combined gas-chromatography/mass-spectrometry (GCMS); this is the first report of the occurrence of ABA in perennial root nodules (Results 1.1.2). Routine quantification of ABA in extracts was by gas-chromatrography using an electron-capture detector (GCECD) ; all extracts incorporated an internal [2-^{14}C] ABA standard to correct for purification losses, and further checks of the methods employed were made (Results 1.1.2.3, 1.1.2.4, 1.1.3). The variation in plant root, leaf and nodule ABA content was investigated in greenhouse-grown plants, and it was demonstrated that whilst there were significant differences in the 'total' ('free' plus 'bound') ABA content of all three tissues, and between the 'free' ABA content of the leaves and the roots/nodules (but not between the roots and the nodules) there were no significant differences in the 'free', 'total' or 'bound' ABA content of replicate batches of 20 plants each.

In part 1.2 of the Results, experiments involving the quantification of endogenous ABA in Alnus are described. An investigation of seasonal fluctuations in nodule 'free' ABA levels in plants growing under natural photoperiod but frost-free conditions was made in 1980/1981, and correlated with seasonal changes in nodule nitrogenase activity (acetylene reduction). There was no clear relationship between nodule ABA content (which ranged from 101 - 879ng g^{-1} f.wt in this

season) and the onset of nitrogen fixation in March after winter dormancy (Results 1.2.1.2, 1.2.1.3), although there was a broad inverse correlation between rates of nodule acetylene reduction and nodule ABA levels in this season.

The possibility of diurnal fluctuations in leaf and nodule ABA content was next examined (Results 1.2.2) in greenhouse- and growth-room-grown plants. Careful analyses of 'free' and 'bound' cis and trans ABA levels failed to demonstrate any fluctuations in leaf or nodule ABA content over a 24h period.

Root-applied 0.1mol m^{-3} synthetic (±) ABA led to increased endogenous ABA levels and to the onset of bud dormancy in plants growing in water culture in long day conditions (Results 1.2.3).

In part 2 of the Results, the translocation of radioactivity and ABA following [2-^{14}C] ABA application is described. In plants grown under short photoperiods for at least 34 d, less radioactivity was translocated out of the donor leaf 24h following [2-^{14}C] ABA application than in long-day conditions and of that translocated, at least 3 times more was recovered in the tissues below the site of application than above it. In plants grown under long days, at least as much ^{14}C was transported acropetally as to tissues underlying the donor leaf. TLC of tissue extracts following petiolar [2-^{14}C] ABA application demonstrated that [2-^{14}C] ABA distribution was similar to that of ^{14}C in plants growing under both long and short photoperiods. However, [2-^{14}C] ABA levels (expressed as a percentage of the radioactivity present) in the stem and leaf tissues beneath the donor leaf were up to 12.6 and 5.9 times greater respectively in short-day treated plants than those observed in plants grown under long days, illustrating the influence of photoperiod on tissue ABA metabolism.

Experiments described in 2.1.4 indicated that ABA is phloem-translocated in Alnus shoots, although phloem : xylem exchange may occur in the root system.

Finally, in section 2.2 of the Results, the translocation of radioactivity and ethyl-acetate-soluble radioactivity following root [2-^{14}C] ABA application for 5d to young plants growing in water culture is described. In the presence of 2.52×10^{-5} mol m^{-3}

$[2\text{-}^{14}C]$ ABA,6.3 % of the total ABA available in the culture solution was taken-up by the plants (assuming that all of the radioactivity recovered in the plants entered the root system as $[2\text{-}^{14}C]$ ABA). Uptake of $[2\text{-}^{14}C]$ ABA represented only 2.3 - 2.6 % of the total synthetic ABA available to plants in root-contact with 0.1mol m^{-3} synthetic ABA 'spiked' with $[2\text{-}^{14}C]$ ABA.

The implications of the results described in this thesis are considered in the Discussion which also highlights the problems encountered, and possible directions for further research concerning plant growth substances.

INTRODUCTION

1. Plant Growth Substances

Abscisic acid (ABA) is a member of a group of plant growth substances (PGS), which have been ascribed an important role in plant development and functioning. This section reviews both the terminology which has been used in research concerning these compounds, and the significance of PGS levels in tissue extracts.

Unlike higher animals, plants show remarkable developmental plasticity, an essential adaptation to a sessile growth pattern. Without the regenerative, developmental and organizational plasticities of plant meristems, these autotrophic organisms would be incapable of both surviving adverse environmental conditions including predation by grazing animals, and responding to favourable changes (Trewavas, 1981).

Integration of plant growth occurs both at spatial and temporal levels (Wareing, 1977), although it is not always possible to distinguish fully between the two; spatial co-ordination is required to integrate events occurring in different localized regions, whilst temporal co-ordination is necessary to achieve the orderly sequence of changes that occur throughout the growth and development of the whole plant, and the organs and cells of which it is composed. An example of spatial co-ordination is the arrested development of lateral buds in many plants, in which the apical bud alone continues to grow, a phenomenon termed 'apical dominance' or more specifically 'correlative inhibition'. Information from the meristematic cells of the shoot apex is somehow conveyed to the lateral bud meristems which is interpreted according to their genome and developmental history, and leads to the inhibition of their growth (Phillips, 1975). Temporal co-ordination is required to integrate the response to perception of an important stimulus by a part of the plant (for example, the perception of short days by leaves (Wareing, 1977)).

Since the discovery that auxin was involved in phototropic and geotropic curvatures (Went, 1928), the presence of 'chemical messengers' has been deemed essential to the integrity of plant development involving both autonomic and environmental factors. These endogenous messengers have been termed 'plant growth substances' (PGS); this term has been taken to include the auxins,

gibberellins (GA's), cytokinins, ethylene and abscisic acid (ABA).

1.1 The Concept of PGS as Hormones

By analogy with animal control systems, PGS have often been termed 'plant hormones' or 'phytohormones'. This analogy is far from satisfactory, however, and has resulted in conceptual difficulties in portraying the way in which PGS may act (Trewavas, 1981, 1982). The word hormone itself is derived from the present participle of the Greek verb homao, to impel, and in broad biological terms, may be defined as a 'substance, formed by internal secretion, that passes into the blood or sap and stimulates organs to action' (Oxford English Dictionary). Clearly, whilst the term PGS includes compounds exerting inhibitory effect, the word hormone should only cover those substances known to stimulate biological processes.

Tukey et al. (1954) described phytohormones within the category of 'growth regulators', which they defined simply as 'regulators which affect growth', including compounds not found naturally within the plant. Larsen (1955) maintained that the definition of the term 'regulator' ('to adjust so as to work accurately and regularly' (Webster's Dictionary)) precludes its application to substances not produced by the organism itself, since several synthetic growth substances (e.g. 2-4 dicholorophenoxy acetic acid) do not achieve this goal.

A number of points may be described which outline the differences between hormones in the strict definition of the term and PGS. Firstly, although it is undisputed that the translocation of PGS occurs, since gibberellins, cytokinins, auxins and ABA have been detected in phloem and xylem sap (Wareing, 1977), there is little hard evidence for the localized production of these substances in specific areas, or that transport is necessary to overcome deficiencies in other parts. Even cytokinin biosynthesis, once thought to be exclusive to the root system necessitating translocation to the leaves and shoots where it is required to maintain leaf functioning, has been demonstrated to occur in excised leaves supplied with inorganic nutrients (Salama and Wareing, 1979). Localized biosynthesis is an essential feature of mammalian hormonal control systems.

Secondly, each PGS shows a wide spectrum of physiological

effects, that is, different effects may be produced by the same substance in different species, and in different parts of the same plant (Wareing, 1977). For example, auxin stimulates cell division in the elongating zone of the stem, cell division in the cambium, and even the initiation of adventitious roots on stem cuttings (Kende and Gardner, 1976). Thirdly, whilst animal hormones are often large molecules and operate over a single order of magnitude, unconjugated PGS are small molecules, (none of which are proteins), with a molecular weight of only a few hundred (Jacobs, 1959), and show dose-response curves functional over 3-6 orders of magnitude (Kende and Gardner, 1976). Response in plant systems is often related to the logarithm of the hormone concentration, which means that low doses are relatively much more effective than high ones (Kende and Gardner, 1976).

Fourthly, there have been many conflicting observations regarding the extractable levels of PGS preceding or following physiological events. Whilst no generalities between the PGS can be made, it remains likely that gibberellin levels in Pisum play a key role in the control of stem elongation (Potts and Reid, 1983); Ingram et al., 1983). Careful analysis (by MIM) of the endogenous GA_1 content of the apical regions of Pisum sativum L. shoots demonstrated that this substance was either absent or present at very low levels in dwarf plants (genotype le), whilst levels were much higher in the tall strain (genotype Le). This observation, coupled with the effect of AMO1618 (an inhibitor of gibberellin biosynthesis), which reduced the growth of both phenotypes, led these authors to suggest that in Pisum the maintenance of gibberellin levels is critical for normal growth. In many other instances, however, the importance of endogenous PGS levels in plant tissues remains an area of controversy. It must be concluded, therefore, that mechanisms responsible for co-ordinating the open-ended mode of plant growth show only few similarities with hormonal control mechanisms in higher animals.

(Trewavas (1981, 1982) feels that the failure to acknowledge the unique plasticity of plant growth, and adherence to the hormone concept, has led plant physiologists to two assumptions: 1) that 'growth substances are limiting and therefore regulatory factors in development' and 2) that plant development 'is regulated....by altering the concentration of growth substances'.

He maintains that whilst growth substances are fundamental 'integrating agents in development', they are not responsible for the ultimate control of this process, mediated by changes in their concentration.

It is possible, however, that the level at which PGS operate may have been missed by plant physiologists. The term 'control' has been described as the exercise of restraint or direction (Cram, 1976), encompassing both the physical and chemical limitations applicable to any system, in addition to the adjustment of specific processes (regulation). Whilst PGS rarely determine the way in which a tissue may respond, it has been suggested that they may indeed regulate specific processes, which occur prior to the observed physiological event. This possibility may remain unrecognized, whilst research centres on subsequent processes (Zeroni and Hall, 1980).

There remain instances that support the notion of control of a specific biological process by PGS. The control of stomatal aperture in Commelina communis by ABA, for example, (Weyers, 1978) fulfils each of the 6 rules outlined by Jacobs (1959), which were formulated to act as guidelines in answering the question 'What substance normally controls a given biological process?' The more of these that are satisfied, the greater the possibility that the hypothesis of control by a particular factor is correct. The keywords of each rule can be recalled using the mnemonic 'PESIGS', representing Parallel variation (of the amount of the structure S whose development is normally controlled by the substance under test C, with the amount of C), Excision (of the part forming C, demonstrating that S decreases), Substitution (of C or an extract containing C for the part of the organism shown to be the source), Isolation (of the reacting system and demonstration that C has the same effect as in the intact organism), Generality (of the results for other families, as well as for the development of S in different kinds of organs) and Specificity (of effect, i.e. that no naturally-occurring chemicals have a similar effect on the structure).

1.2 Why There May be no Direct Relationship Between the Levels of a PGS with a Physiological Event

Before commencing this discussion, it is necessary to explain what the terms 'level' and 'concentration' with regard to amounts of a PGS within a tissue extract or plant system imply, as these find wide usage in the literature. Concentration has been defined as 'the quantity of a substance present in a given space or defined quantity of another substance. Concentration of aqueous solutions is usually expressed in moles per cubic metre' (Uvarov and Chapman, 1971). In a system composed of cells, this definition should not be applied, strictly speaking, since the permeable nature of the plasmalemma means that water in the cells is in a continual state of flux. The static conditions required to define concentration do not thus exist in cell systems. The concept of levels within a cell system has often been adopted therefore.*

As mentioned earlier, recent years have seen the accumulation of much conflicting evidence concerning the physiological significance of differences in the amounts of PGS in plant extracts (Wareing, 1977). In this section, reasons are put forward to explain why this might be so.

1.2.1 The Concept of PGS Sensitivity

Trewavas (1981) has suggested that PGS are not designed to operate in the same way as animal hormones i.e. via an amplification process, in which, for instance, one messenger molecule may cause the synthesis of 10 product molecules. The large range of concentrations over which PGS may operate may be explained by a need to buffer tissues against unnecessary response. This author has recently developed a theory to explain the lack of correspondence between extracted PGS levels and the observed physiolological status of the tissue. This is described in several articles (Trewavas, 1981, 1982), although the idea was mentioned earlier (Wareing, 1977). Since PGS act at low concentrations, are active without metabolic interconversion, and do not seem to act as enzyme co-factors, it has been suggested that specific recognition molecules or receptor sites (R) are required as mediators between the signal and the cell in which the response is to occur (Venis, 1977, Stoddart and Venis, 1980).

* In the context of this thesis, the term 'level', has been defined as amount per unit weight (eg. ng (ABA) per g fresh weight).

This has been summarized by the following equation (Trewavas, 1982):

$$\underset{(GS)}{\text{Growth Substance}} + \underset{(R)}{\text{Receptor}} \longrightarrow (GS.R) \longrightarrow \text{Biological Response}$$

From this it may be inferred that the 'overall concentration of hormone in the cell will not necessarily be the only or the primary factor determining the physiological response' (Wareing, 1977). Thus the 'sensitivity' or availability of specific (protein?) receptor molecules, will be a determining factor in the amounts of GS.R complex formed and hence the response initiated (Trewavas, 1982). By ensuring that the amounts of PGS in a biological assay are in excess, the sensitivity of plant cells to PGS may be investigated, since this becomes effectively equivalent to biological response. This is described for all the PGS in a variety of biological systems in which variations in PGS sensitivity were correlated with changes in biological events over a period of time (Trewavas, 1982). The results of these experiments enabled this author to conclude that:-

'(a) Growth substance sensitivity by the tissue varies continuously during development.

(b) Variations in sensitivity parallel and indeed precede that of the event in the intact plants, unlike levels of growth substances'.

This theory presents a new way of looking at the influence of PGS on plant growth and development. Loss of receptor sites as cells age culminates in the requirement of larger doses of PGS to achieve similar responses. Hence the 'sensitivity' of the cell to PGS is seen as the controlling factor in determining response. The idea is both compelling and simple. However, the presence of receptor sites in plant systems remains an area of controversy, and this theory may have drawn too close a parallel with animal systems. In the following, some of the problems with the notion of PGS receptor sites are broached.

1.2.1.1 The Receptor Concept in Plant Systems

Recognition of active substances by the plant cell must occur before a physiological response can be evoked. One of the several ways in which this can be achieved in animal systems is via the presence of receptor sites, specific binding proteins associated with the cell in question. Similar sites may be present in plant systems. However, whilst a number of protein receptor molecules have been characterized in animals (e.g. for the steroid hormones), no PGS receptor has yet been isolated and characterized. Methods of assessing the interaction between a hormone and a receptor in animal systems have been developed, which under equilibrium conditions permit the evaluation of the amount of free hormone. From data obtained at a series of total PGS concentrations, the binding constant and number of binding sites can be calculated (see Stoddart and Venis, 1980). This approach has been used with PGS using membranous preparations (e.g. Hocking et al., 1978).

Kende and Gardner (1976) outline several criteria which should be satisfied in studies concerning putative PGS receptor binding. No single PGS receptor system yet described satisfies completely these points, and the authors concluded that 'the ultimate confirmation of the significance of (plant) hormone binding, will probably only be obtained by work with receptor mutants and by elucidating in vitro and in vivo the primary hormonal response'. They maintain that the isolation of a receptor in plant systems would be difficult (a) if the receptor has a low affinity to the hormone, and occurs at low concentrations in the cell (b) if large numbers of unspecific binding sites were also present (c) in the absence of a conventional interaction between a small molecule and a regulatory protein.

Pauls et al. (1982) consider the possibility that some of the physiological effects of GAs can be explained through their interaction with the lipid portion of cell membranes without the involvement of a receptor. These authors using prepared liposomes noted that a GA_4/GA_7 mixture (physiologically active), perturbed the bilayer complex with the phospholipid molecules, and have suggested that this may cause vertical displacement of functional membrane proteins, leading to changes in their exposure and orientation, and hence to an alteration in transport processes and membrane-bound enzyme systems.

Finally, the effective concentration range of plant hormones has been likened to that of olfactory stimulants in animals (Kende and Gardner, 1976), which also spans several decades. It is possible that PGS act in a similar manner. It has been suggested that olfactants interact with the sensory tissue by partitioning into epithelial membranes; stimulation occurs when an adsorbed odorant molecule desorbs from the cell leaving a hole through which ions flow to initiate nervous impulses.

1.2.2 Compartmentation and Carriers

Amounts of PGS in plant extracts may also show no obvious relationship with physiological events due to compartmentation of the active substance. Thus the level of PGS seen in extracts may represent an average of the levels present either within specific cells of a tissue, or within a subcellular compartment of some or all of the cells.

Dela Fuente and Leopold (1972) were the first to apply 'compartmentation analysis' techniques, widely used in ion-transport experiments, to the study of auxin localization in sunflower stem. They found that two transport components, a fast one and a slow one, were responsible for the efflux of IAA [1 - ^{14}C] out of sunflower stem segments. They concluded that these probably represented efflux from cytoplasmic (fast component) and vacuolar (slow component) regions, (although they could represent radioactivity eluting from two forms of the applied IAA). Active transport of IAA was proposed to occur at the plasmalemma, since both components of auxin transport were susceptible to inhibition by TIBA.

Behl *et al.* (1981) using compartmental analysis, discovered that the efflux of ABA from root cells of *Hordeum distichon* also consisted of two components. They, too, suggested that the fast-exchanging component represented the cytoplasm, and the slowly-exchanging ones, the vacuoles. Longitudinal analysis of the uptake capabilities of root segments demonstrated strong accumulation of [^{3}H] ABA in the root tip, where the cells were small, and had a large cytoplasm volume. Further away from the root tip, cells were increasingly vacuolated, and contained less ABA. This distribution pattern for ABA in *Hordeum* roots, which

had also been observed by Astle and Rubery (1980) in Phaseolus coccineus roots, led to suggestions that the cytoplasm, at a higher pH than the vacuole, was acting as an 'anion trap' for the undissociated (and membrane-permeable) form of the molecule, ABAH.

Ohlrogge et al. (1980) also concluded that the uptake and distribution of radiolabelled GA_1 within cowpea leaves was partially mediated by a carrier system. These authors determined the subcellular localization of applied [^{3}H] GA, by a sucrose-density centrifugation technique; organelles were analyzed for the presence of radioactivity. Unlike ABA, 50-100% of the [^{3}H] GA was localized in the vacuole, whilst less than 5% was associated with the chloroplasts, mitochondria, nuclei or membranes.

Immunocytochemistry has recently been used in determining the localization of a cytokinin in Zea root tips (Zavala and Branden, 1983). Antibodies or antibody fragments were directed against dihydrozeatin riboside, labelled with rhodamine or colloidal gold, and used to investigate the localization of the cytokinin. The authors found that meristematic and root-cap cells only were specifically labelled using immunofluorescence. Antibody fragments labelled with colloidal gold were located only in cytoplasmic regions and at intracellular membranes, and were absent from the nuclei and vacuoles.

The study of PGS localization remains very much in a state of infancy. The studies described here demonstrate that as cells age and their cellular components alter, so the uptake capacity of the cell to ions and PGS is likely to change in accordance with the new physical gradients exerted across membranes. Whilst we do not yet know the precise physiological effects of PGS localization, it remains important to appreciate that tissue extracts composed of a population of cells, or indeed heterogeneous cell types with different subcellular anatomy and chemical requirements are likely to contain individuals with widely differing PGS levels sequestered in different subcellular compartments.

1.2.3 Interactions Between PGS

There is a great temptation in the study of PGS physiology to neglect the possible interactions between these agents and to deal with a single substance only. The analysis of PGS requires sophisticated physical and chemical procedures, often making studies of more than one in a single plant system difficult. Moreover, accurate analytical techniques for identification and quantification have only been available latterly. The importance, or otherwise, of PGS interactions, has often thus received little attention, and those studies which have been made, have often centred around exogenous application. There can be little doubt, however, that the influence of, and balance between several PGS on a particular event may have added to our failure to observe direct correlations between individual levels of a single PGS and the physiological event in question.

At a morphological level, interactions between PGS have been described as qualitative or quantitative (Wareing, 1977). The former describes an alteration in the direction of the response, whilst the latter refers to antagonistic and synergistic effects between PGS.

There is a considerable body of literature concerning PGS interaction, and no attempt will be made here to review it. However, the types of responses that have been shown to occur in some plant systems may be summarized as given below, and examples of each point are presented in the following text.

Known Interactions Between PGS

(i) The balance between the levels of two PGS may influence the direction of a response.

(ii) Application of a combination of PGS may lead to a greater response than the application of a single PGS (but in the same direction). Applications may need to be sequential.

(iii) Application of combination of PGS may act antagonistically towards a response normally influenced by a single PGS.

(iv) Application of one PGS may stimulate the production of another.

Examples

Skoog and Miller (1957) noted that kinetin and IAA interacted to alter the differentiation of tobacco callus or stem-tissue cultures. Kinetin alone stimulated bud formation (0.2ng dm^{-3}) whilst 0.3-3.0mg dm^{-3} IAA caused root formation. Low cytokinin to auxin ratios suppressed bud growth in favour of callus growth, whilst high cytokinin to auxin ratios caused bud formation and suppressed root formation.

Catalano and Hill (1969) demonstrated synergism between GA_3 and kinetin applied to axillary buds of decapitated Lycopersicon plants which had been treated with IAA on the cut surface. Lateral bud outgrowth was 4.9 and 3.1 times greater than applications of GA_3 and kinetin respectively.

An antagonism between ABA and IAA was observed by Snaith and Mansfield (1982) in Commelina communis epidermis. In their experiments, the effectiveness of IAA in reducing stomatal response to CO_2 was antagonized by ABA. Thus a balance between ABA and IAA probably governs the behaviour of stomata in this species.

That treatment of seeds of Phaseolus vulgaris, Zea mays and Pinus sylvestris and young plants of Phaseolus with kinetin increased the levels of extractable IAA, was shown by Saleh and Hemberg (1980). However, application of the synthetic auxin NAA had no effect on the levels of endogenous cytokinins in internodal bark segments of Salix babylonica (Choveaux and van Staden, 1981).

Interactions between PGS may take several forms. To appreciate more fully the meaning of the levels of one PGS in a tissue extract, it may be necessary to construct a framework of similar information concerning other PGS.

1.2.4 Biosynthesis and Metabolism

Following the observation that in the long-day rosette plant spinach (in which stem elongation and flowering are induced by GA application in short-days, or by long-days) levels of GAs were only two-fold higher when plants were maintained under long-days than when kept in short-days, Zeevaart (1971) suggested that turnover rates of GA were important. He demonstrated that the rate of GA biosynthesis was indeed much greater in long-day plants than short-day plants, and concluded that the increased availability of GA to long-day plants, coupled with increased

sensitivity to GA was responsible for increased stem elongation. It is possible that similar mechanisms exist for other PGS and even that transient increases of a metabolite could initiate the response to a stimulus.

We assume that PGS are in the same chemical form in vivo as following extraction from the plant material, yet this may not be so. Liberation of active forms from conjugates inactive in vivo may occur when the contents of subcellular compartments hitherto distinct come into contact with each other or with the extraction medium. Similarly, inactivation of active forms may occur.

Turnover rates and the nature of active forms of PGS should also receive consideration, therefore, whilst assessing the significance of changing levels of PGS in tissue extracts; physiological events are often accompanied by changes in cellular metabolism.

1.2.5 The Importance of PGS Isolation, Identification and Quantification: Some Conclusions

In the preceding discussion, some problems involved with the interpretation of PGS levels in tissue extracts have been described. An understanding of the role of these substances requires further work in each of the areas mentioned.

The importance of developing complex technology for PGS analysis must not be underated, however, since even in the absence of simple correlations between PGS levels and the growth and development of plants, it is clear that precise methods of isolating, identifying and indeed quantifying PGS are a pre-requirement to the establishment of a foundation sound enough for future work. To 'throw the baby out with the bathwater' in regarding the analysis of PGS as a waste of time amidst the confusion concerning the importance of PGS levels, would be foolish indeed (Horgan, 1980).

2. Abscisic Acid

2.1 The Molecule

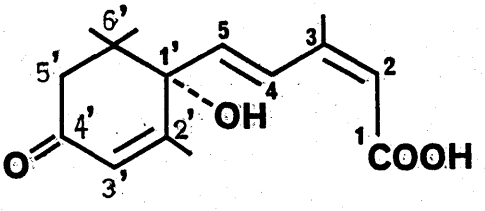
A history of the discovery of ABA may be found in a review by Addicott and Lyon (1969). The structure of the molecule was first accurately described by Ohkuma *et al.* (1965), who used nuclear magnetic resonance. They named it 'abscisin II' since a compound termed 'abscisin I', from which it was seemingly chemically distinct, had already been isolated from the mature fruit wall of cottom (Liu and Cairns, 1961). Cornforth *et al.* (1965a),however, in collaboration with Professor Wareing's group at Aberystwyth, who subsequently isolated the same compound from the leaves of *Acer pseudoplatanus,* referred to it as 'dormin'. The 'trivial' name 'abscisic acid' was chosen in 1967, to facilitate the naming of derivatives, and to reduce the confusion arising from a substantial change in the term first proposed by Ohkuma *et al.* (1963) (Addicott and Lyon, 1969).

The full chemical name for abscisic acid is:

3-methyl-5-(1'-hydroxy-4'-oxo-2',6',6'-trimethyl-2'-cyclohexen-1'-yl)-*cis*, *trans*, 2,4,-pentadienoic acid (Addicott and Lyon, 1969).

2.1.1 Physical Properties

ABA is a carboxylic acid, with the structure shown below



(+)ABA

The molecule has one asymmetric carbon atom at C-1', and can thus exist in two forms which show optical activity. The naturally-occurring enantiomer is dextrorotatory (+), (rotating plane - polarized light of wavelength 589nm in a clockwise direction) exhibiting an intense Cotton effect in acidified

ethanolic solution, with a peak at 289nm (+24,000), a trough at 246nm (-69,000) and zero rotation at 269nm and 225nm (Milborrow, 1967). Application of the 1966 version of the Cahn, Ingold and Prelog sequence rules for (+) ABA gives the configuration of the tertiary hydroxyl at C-1' as sinister (S) (Milborrow, 1974).

Synthesis of ABA was first reported in 1965 by Cornforth *et al.*[1965b]; synthetic ABA is a mixture of the (+) and (-) enantiomers (and is thus optically inactive), with a melting point of 191°C, some 30°C higher than crystalline natural (+) ABA (m.p. 160-161°C).

In solution, (+) ABA shows ultraviolet absorption, although the position and intensity of the absorption maximum are affected by pH (Cornforth *et al.*, 1966). The spectrum of the methyl ester of ABA (ABAMe) is unaffected, however, by changes in pH, with a maximum (ϵ = 20,900) at 264-265nm, (Milborrow, 1974). The side chain around C-2 can exist in a *cis* (z) or *trans* (e) configuration: 2 *cis*, 4 *trans* ABA is simply termed ABA, whilst the *trans* form (2 *trans*, 4 *trans* ABA) is referred to as 2-*trans* ABA (Milborrow, 1974). In light, solutions of ABA isomerize to a 1:1 equilibrium with the 2-*trans* isomer (Milborrow, 1974, Brabham and Biggs, 1981).

2.1.2 Biological Activity of (+) ABA and 2-*trans* ABA

The R and S enantiomers of ABA can show equal, or almost equal activity in growth assays, such as coleoptile growth in excised wheat embryos (Milborrow, 1980). However, whilst rapid stomatal response of isolated epidermal strips of *Commelina communis* and *Tropaeolus majus* to applied ABA solutions occurred with (+) or (±) ABA, the (-) enantiomer was totally ineffective (Milborrow, 1980). This author suggested that the near symmetry of the ABA molecule permitted accommodation of (-) ABA in the active site of the 'slow' growth responses, by C-2' and its methyl lying in the position normally occupied by C-6' and one of its methyl groups. Alternatively, the tertiary hydroxyl group may not interact at all with the active site, but serve to stabilize the molecule (Milborrow, 1974). A different more restricted receptor site possibly requiring an interaction between the C-2' double bond and part of the receptor was proposed to explain the inactivity of the (-) enantiomer in 'fast' responses.

Early reports concerning the biological activity of applied 2-trans ABA were contradictory; Nitsch (1967) for example, reported that both ABA and the 2-trans isomer had similar inhibitory effects on the elongation of oat mesocotyl and wheat coleoptile sections, whilst Milborrow (1966) using a similar system found that 2-trans ABA had only 1/30th of the activity of natural ABA. Today it is generally thought that 2-trans ABA has little effect in biological systems, and that its apparent activity in biological assays resulted from interconversion to the active 2 cis, 4 trans form (Milborrow, 1974). Nevertheless, 2-trans ABA is known to occur naturally in plants (see reports by Lesham et al.,1974;Jones et al.,1976) although it is unlikely that this is an intermediate in ABA biosynthesis, since the immediate precursors of the active molecule in Cercospora rosicola are already in the cis form (Neill et al., 1982; Bangerth, 1982). It has been suggested that 2-trans ABA is a product formed from the metabolism of ABA, since this isomer was isolated from apple fruits, which had been carefully handled to prevent light isomerization (Bangerth, 1982).

2.1.3 Biosynthesis

ABA is classified amongst the sesquiterpenes by virtue of the number of carbon atoms in the molecule, and the characteristic methyl branching (Sembdner et al., 1980). Two possible pathways of ABA biosynthesis are (a) direct synthesis via a C_{15}-intermediate, and (b) degradation of a C_{40} carotenoid to a C_{15} compound which can then be converted to ABA. Both involve the general pathway of terpenoid biosynthesis.

Mevalonic acid (MVA) is the parent molecule in the terpenoid pathway, giving rise to farnesyl and geranyl geranyl pyrophosphates, which are the immediate precursors of sesquiterpenoids and diterpenoids (of which the gibberellins are a member) respectively. Only the (+) form of MVA which has a 3R configuration, is biologically active, and in enzyme-catalyzed reactions, the H atoms at each of C-2, C-4 and C-5 are distinguished stereospecifically to permit the synthesis of compounds with cis and trans bonds. The C_{15} carbon-skeleton of ABA is formed by three isoprene units derived equally from MVA (Milborrow, 1974). Experiments using different stereospecifically labelled species

of MVA to ascertain the derivation of the H atoms of ABA (Milborrow, 1972) have shown that ABA arises from an intermediate with a trans Δ^2 double bond, probably farnesyl pyrophosphate, which is converted into the cis Δ^2 double bond of ABA. Additionally, it was shown (Milborrow, 1974) that three of the four methyl groups of ABA are derived from the 3' methyl groups of MVA, whilst the pro (S) and pro (R) 6' methyl groups are derived from the 3-methyl and C-2 of MVA. These experiments did not, however, permit distinction between the formation of ABA directly from farnesyl pyrophosphate, or via a carotenoid precursor (Walton, 1980). ABA is structurally similar to the end portion of carotenoids which led to suggestions that violaxanthin was a possible precursor (Taylor and Smith, 1967). However, although compounds such as the plant growth regulator xanthoxin 5-1',2'-epoxy-4'-hydroxy-2',6',6'-trimethyl-1'-cyclohexyl)-3-methyl pentadienal could be derived from violaxanthin by photooxidation or chemical reactions in vitro (Milborrow, 1974) , the operation of a similar pathway in vivo has not been demonstrated. Xanthoxin may itself be converted to ABA in shoots of tomato and dwarf bean (Taylor and Burden, 1973). However, without establishment of the links between xanthophylls and xanthoxin in vivo, operation of this pathway in higher plants remains hypothetical.

Recent work by Neill et al. (1982) on the biosynthetic pathway of ABA in cultures of the plant pathogenic fungus Cercospora rosicola, has demonstrated that a route from MVA via the immediate precursor 1'- deoxy ABA occurs in the micro-organism. These authors have suggested that ABA is directly synthesized from a farnesol or farnesyl derivative, and that a pathway involving xanthoxin or a carotenoid precursor is unlikely in C. rosicola.

2.1.4 Metabolism

Knowledge concerning the metabolism of PGS both occurring in vivo and following exogenous application, is clearly necessary to permit an assessment of the levels of active compound (Walton, 1980). Although ABA is the youngest of the PGS, much is known already about its biochemistry. Two classes of ABA metabolite may be distinguished (Loveys and Milborrow, 1981).

Firstly, conjugates of ABA may be formed when a moiety (e.g. a sugar moiety, methyl group, or even 'gluten-like' protein and carbohydrates other than glucose (Dewdney and McWha, 1978)) becomes attached to the molecule. 'Free' ABA can be released generally, by alkaline hydrolysis, from the conjugated form. Secondly, a number of compounds may be produced following oxidation of ABA, and formation of an unstable intermediate, 6' hydroxymethyl ABA, which may themselves become conjugated. The structures of the metabolites of ABA are shown in Figure 1.

2.1.4.1 Conjugates

The first ABA metabolite to be characterized was β-D-glucopyranosyl abscisate (ABAGE) (Koshimizu et al., 1968), which has been given the systematic name β-D-glucopyranosyl (1'S,2Z,4E) -5-(1'-hydroxy-2',6',6'-trimethyl-4'-oxocyclohex-2'-enyl) 3 methyl penta 2,4-dienoate (Loveys and Milborrow, 1981), and is often referred to as the glucose ester of ABA. This polar, water-soluble metabolite has been positively identified in lupin seeds (Koshimizu et al., 1968), Xanthium leaves (Zeevaart, 1980), pear-receptacles (Martin et al., 1982), and in orange and avocado fruits and leaves of Phaseolus vulgaris (Neill et al., 1983). Milborrow (1970) identified ABAGE in the pseudocarp of Rosa arvensis both as a natural metabolite and also following exogenous ABA application.

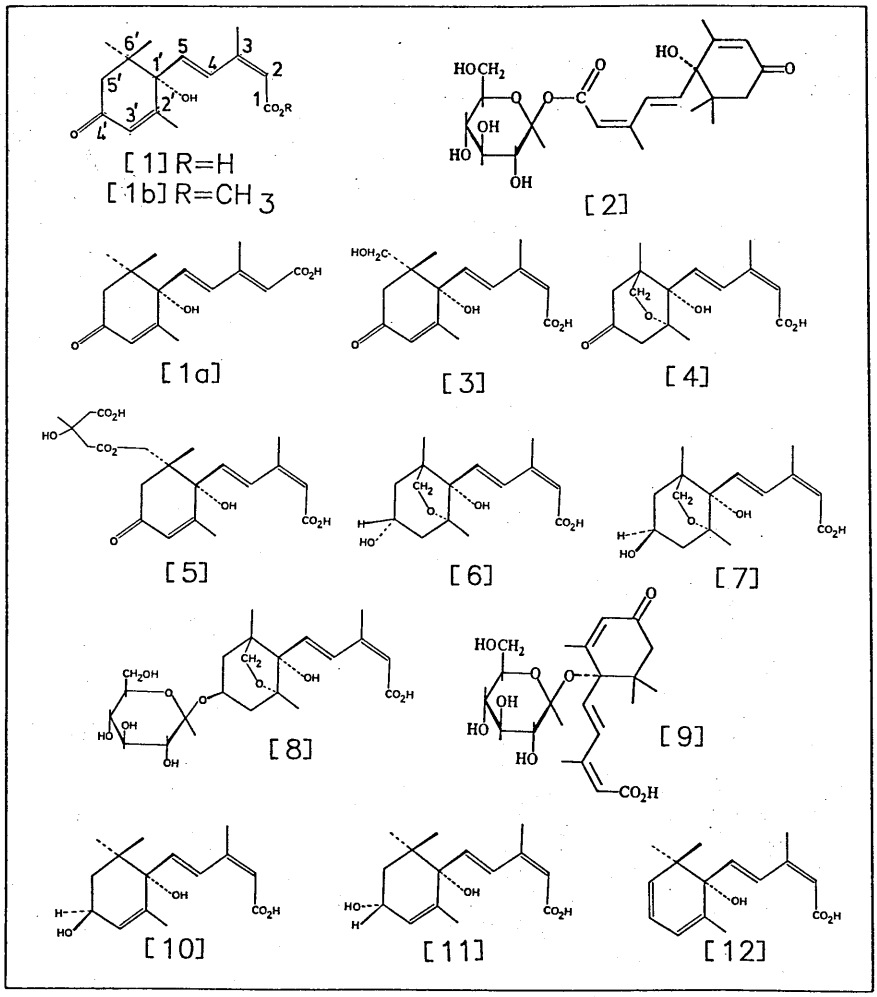
Many studies have assumed that conjugated ABA is this glucose ester, although recent research has confirmed the identity of a second glucose conjugate (Milborrow and Vaughan, 1979; Loveys and Milborrow, 1981).

ABAGE often occurs at levels 10-50% of the 'free' acid (Neill et al., 1983), although in turgid Xanthium leaves, Zeevaart (1980) noted that the content of the glucose ester was higher than that of 'free' ABA.

Formation of the glucose ester of both the (+)S and (-)R enantiomers has been shown to occur following the application of racemic ABA to Lycopersicon shoots (Milborrow, 1970). It has been demonstrated recently, however, that in this species, (R) ABAGE is 5.8 times more abundant following (RS) ABA feeding than the ester formed from natural (S) ABA (Vaughan and Milborrow, 1984). In addition, metabolism of racemic 2-trans ABA into a glucose ester by Lycopersicon shoots occurs ten times more rapidly than ABA (Milborrow, 1970).

Figure 1. Abscisic acid and its metabolites. From Loveys and Milborrow, 1984.

1. ABA
1. (a) 2-trans ABA
1. (b) ABAMe
2. ABAGE
3. 6'-hydroxymethyl ABA
4. PA
5. 3-hydroxy-3-methyl glutarylhydroxy ABA
6. Epi-DPA
7. 4'-dihydrophaseic acid
8. DPA-4'-O-β-D-glucopyranoside (DPAGS)
9. ABAGS
10. 1',4' trans diol ABA
11. 1',4' cis diol ABA
12. 4'-anhydro ABA


[1] R=H
[1b] R=CH3
[2]
[1a]
[3]
[4]
[5]
[6]
[7]
[8]
[9]
[10]
[11]
[12]

Neill et al., (1983) demonstrated that the increase in 'free' ABA levels in bean leaves that occurred following wilting was not accompanied by a fall in the ABAGE content of the tissue, and concluded that the glucose ester was not acting as a source of 'free' ABA. Pierce and Raschke (1981) also failed to observe any differences in the rate of conjugation in Phaseolus following the onset of water-stress, or alleviation of the stress, which suggests that ABAGE also does not act as a sink for 'free' ABA following rehydration (Neill et al., 1983). Furthermore, Milborrow (1978) has suggested that 'free' ABA cannot be released from the glucose ester in vivo i.e. that conjugation is irreversible, although it has been pointed out that it is difficult to distinguish this possibility from a situation in which an equilibrium exists between biosynthesis and degradation (Zeevaart, 1983). A glycosylating enzyme has been described in cell suspension cultures of Macleaya microcarpa (Lehman and Schutte, 1980), whilst Neill et al., (1983) demonstrated that ABAGE could be hydrolyzed enzymatically in vitro by β-glucosidase (although α-glucosidase was ineffective).

Weiler (1980) has demonstrated by radioimmunoassay, that the highest levels of conjugated ABA are found in senescent leaves, and it has been suggested that in vivo, ABAGE represents an end-product of ABA metabolism, formed from a small proportion of total ABA pools only (Neill et al., 1983). This is supported by the observation that the glucose ester acts as a cumulative index of the degree of water-stress experienced by an individual leaf (Zeevaart, 1983). This author noted that in intact plants of Xanthium, ABAGE content more than doubled following the onset of water-stress, and did not fall for over 34 d following rehydration. Levels continued to rise as plants were subjected to successive stress recovery cycles.

Another naturally-occurring ABA conjugate was isolated from extracts of tomato plants, and has been identified as the 1'-O-β-D-glucopyranoside of ABA (ABAGS) (Loveys and Milborrow, 1981). The systematic name is (1'S,2Z,4E)-5-[β-D-glucopyranosyloxy]-2',6',6'-trimethyl-4'-oxocyclo-hex-2'-enyl)-3-methyl penta-2,4-dienoic acid (Loveys and Milborrow, 1981). The compound is acidic, soluble in butan-1-ol, slightly soluble in ethyl acetate, and can be hydrolyzed by glucosidases. Some scepticism has been expressed

concerning the occurrence of a glucoside at the C-1' (Walton, 1980), since the 1'OH group of ABA is difficult to substitute. Loveys and Milborrow (1981) have demonstrated, however, using 'space-filling' models, that a glucosyl residue can be accommodated without steric interference at the 1'OH position, and have suggested that the glycosylating enzyme can negate the influences which cause the chemical unreactivity of the 1' hydroxyl. Later experiments have shown that when racemic ABA is fed to tomato plants, ABAGS formed from the unnatural (-) enantiomer is found at levels 29.4 times higher than that formed from (+) ABA (Vaughan and Milborrow, 1984). Thus it probably represents a minor metabolite of ABA in vivo.

The methyl ester of ABA (ABAMe) in which a methyl group is attached to the carboxyl, is a conjugate which does not occur in vivo, and has little activity in growth assays (Milborrow, 1974). Derivatization of 'free' ABA to ABAMe is important, however, in the analysis of ABA in extracts, since it permits separation of the molecule from impurities remaining after chromatography as a free acid.

2.1.4.2 Metabolites Formed Following Oxidation

The major pathway of ABA metabolism in green plants involves the formation of the acidic compound 6'-hydroxy methyl ABA (HMABA) by oxidation, followed by rearrangement to phaseic acid (PA) and reduction to 4'-dihydrophaseic acid (DPA) and the 4'epimer of DPA (epi-DPA) (Walton, 1980). HMABA, PA and DPA all show low or no growth regulatory activities and may themselves become conjugated (Sembdner et al., 1980). Only (+) ABA is metabolized via the HMABA route; radiolabelled (-) ABA fed to excised bean axes was not metabolized by this pathway (Sondheimer et al., 1971; Mertens et al., 1982).

HMABA was first isolated by Milborrow (1970) in crystalline form after feeding tomato plants with racemic ABA; the compound rearranged to PA upon methylation, and subsequent attempts to reisolate it were unsuccessful, demonstrating that it is extremely unstable. Whether or not it shows more biological activity than ABA itself has thus yet to be determined (Walton, 1980). A particulate enzyme catalyzing the hydroxylation of one of the geminal C-6' methyl groups of ABA, forming HMABA, has been

isolated from cell-free extracts of Echinocystis lobа liquid endosperm, which has a high substrate specificity for (+) ABA (Gillard and Walton, 1976). A stable conjugated form of HMABA has also been reported in the immature seeds of Robinia pseudoacacia and identified as (+)-3-methyl-5-[1(S), 6(R)-2, 6-dimethyl-1-hydroxy-6-(3-hydroxy-3-methyl-4-carboxybutanoyloxymethyl)-4-oxo-cyclohex-2-enyl] -2-Z-4-E-pentadienoic acid (also termed β-hydroxy-β-methyl glutaryl hydroxyabscisic acid) (Hirai et al., 1978; Hirai and Koshimizu, 1981). HMABA is cyclized by the formation of a cis oxymethylene bridge (Sembdner et al., 1980) leading to PA.

The correct structure of PA was published by Milborrow (1969). The compound may have some activity in the control of stomatal aperture in several species (Sharkey and Raschke, 1980).

DPA was identified as a major ABA metabolite by Tinelli et al. (1973), and its 4' epimer was isolated by Zeevaart and Milborrow in 1976. Both epimers are formed enzymatically from PA by reduction of the 4' keto group without loss of the 4' oxygen (Sembdner et al., 1980) although levels of epi DPA only amounted to less than 2% of those of DPA (Zeevaart and Milborrow, 1976). Characterization of a glucoside of DPA was achieved by Milborrow and Vaughan (1982) using NMR and mass-spectrometry, following partial identification of a 4'-O-aldopyranoside of DPA by Setter and Brun (1981), which they termed DPA 4'-O-β-D-glucopyranoside (DPAGS). It was confirmed that this highly polar molecule, resistant to hydrolysis, was a natural metabolite of (+) ABA (Milborrow and Vaughan, 1982). These authors reported the formation of an even more polar acidic metabolite, following feeding of tomato plants with ^{14}C-labelled samples of DPAGS.

Further ABA metabolites have been characterized such as (S)-3-methyl-5- (1'-hydroxy-2'-hydroxymethyl-6'-dimethyl-4'-oxo-cyclohex-2'-enyl) penta-2Z,4E-dienoic acid, which was identified by NMR and IRS and MS techniques, in cell suspension cultures of Lycopersicon peruvianum, Nigella damascena, Papauer alpinum and Portulaca grandiflora after the application of [2-^{14}C] ABA (Lehman et al., 1983). It was also detected as an endogenous compound in the leaves of Vicia faba.

Other uncharacterized ABA metabolites have been reported; two acidic metabolites of (-) ABA were recorded by Mertens et al. (1982) which were also produced in smaller quantities by the (+)

enantiomer, whilst di and tri-hydroxy derivatives of ABA were tentatively identified in methanolic extracts of lettuce seeds (Robertson and Berrie, 1977). Six unidentified acetone-soluble metabolites were reported in wheat plants (Dewdney and McWha, 1978), whilst in apple-seeds, decarboxylation of [1-^{14}C] ABA was observed (Rudnicki and Czapski, 1974), although this may have been as a result of the activities of micro-organisms.

The biological significance of all these compounds and their positions in the biochemical degradation of ABA (whether or not they have been fully characterized) are not yet known. Neill et al., (1983) have cautioned that many of the unknown 'polar metabolites' of ABA reported, may represent rearrangement products of ABAGE.

2.1.4.3 The Differential Metabolism of (+) and (-) ABA

Differences in the biological degradation of the 2 enantiomers of ABA may be summarized as follows:

(i) the two enantiomers are metabolized via different routes, at least, when radiolabelled ABA is fed to plants. The naturally-occurring compound, (+) ABA, is metabolized via HMABA, DA and DPA, and conjugated in small amounts to ABAGE and ABAGS. The (-) enantiomer, however, is not metabolized via the HMABA pathway, and is found as ABAGE and ABAGS at levels far greater than the same conjugates derived from (+) ABA;

(ii) the metabolism of (+) ABA occurs at a faster rate than (-) ABA (Mertens et al., 1982; Sondheimer et al., 1971). The biological half-life of (+) ABA in leaf discs of Vicia faba, for example, was only 6-8h, whilst that of (-) ABA was 30-32h (Mertens et al., 1982).

2.2 The Analysis of ABA

The analysis of plant extracts for the presence of compounds associated with certain physiological effects was the starting point in the isolation of PGS. Following the identification of auxins, cytokinins, gibberellins and ABA, experiments in which synthetic or pure compounds were applied directly to plants or plant parts were possible. However, it has always remained necessary to establish firstly the presence of a PGS in specific plant systems, and secondly their physiological levels. The

extraction and purification of crude extracts leading to the subsequent resolution and identification of their component PGS remains thus an essential area of research.

Complex chemical procedures are required to permit the analysis of PGS, since levels in a typical plant extract rarely exceed one part in 10^6 (Crozier 1980). In recent research, prior to the detection of PGS, a multistep analytical sequence is usually employed, in which chromatographic techniques offering high sample capacity are used in the early stages of purification, followed by different separatory procedures (Crozier 1980). A format of this nature excludes many interfering compounds.

2.2.1 Extraction and Purification

The extraction of ABA from plant tissues following harvest and maceration in the extraction medium, is usually carried out with aqueous acetone or methanol, as with the extraction of other PGS. The efficiency with which this removes ABA from cellular fragments remains unknown, although it has been shown that 592 times as much GA_9 can be extracted from leaf material by the non ionic detergent Triton X100 than by 80% methanol (Browning and Saunders, 1977).

Extracts are generally first filtered to remove cellular debris, and reduced *in vacuo* to an aqueous phase. This may be diluted with phosphate buffer (Crozier 1980) prior to preparative chromatography and partitioning against organic solvents. The order in which general 'clean-up' procedures are applied is variable, and often a function of the material undergoing purification. Solvent partitioning is often used in early stages to reduce the weight of the extract sufficiently for further chromatographic techniques. Partition coefficients for ABA (K_d (ABA)) which have been expressed as a-b/b where a = initial concentration of ABA in the aqueous phase, and b = concentration of ABA in the organic phase after partitioning, have been described for equal volumes of a variety of solvents and water, at different pH values (Ciha *et al.*, 1971). Ethyl acetate (K_d(ABA) = 0.100 at pH 2.5) and diethyl ether (K_d(ABA) = 0.302 at pH 2.5) have found most frequent use, although a partitioning step against methylene chloride at pH 9 (K_d(ABA) = 123), toluene at pH 8, or hexane at pH 2.5 (K_d(ABA) = 93.0), with retention of the aqueous phases has also been recommended (Ciha *et al.*, 1977; Crozier 1980). Warning

has been given, however, that the application of absolute partition coefficients should be made with care in the purification of crude plant extracts (Horgan, 1980).

Charcoal-celite (1:2) or just charcoal adsorption chromatography has often been used to reduce the dry weight of extracts (Yokata et al., 1981); contaminants bind to the column packing, whilst ABA can be removed with aqueous acetone. Recovery of ABA can be variable however (Crozier, 1980).

Column chromatography using Sephadex G25 (Sweetser and Vatvars, 1976) and non-exchange chromatography on Dowex IXI-100 or DEAE Sephadex G-25, originally devised for the separation of gibberellins, can also be used with ABA (Browning and Saunders, 1977, Crozier, 1980).

Insoluble polyvinyl pyrollidone (PVP) adsorption chromatography is widely used in the purification of PGS; the elution profiles of ABA, IAA and GAs on PVP columns has been described by Glenn et al., (1972). PVP greatly reduces the dry weight of plant extracts by forming insoluble complexes with phenols, under appropriate conditions (Yokota et al., 1980). Latterly, the use of a PVP slurry with the extract (pH 8.0), which is a much speedier technique, has been found nearly as effective as column chromatography (Crozier, 1980).

Thin-layer chromatography (TLC) may be used in the purification and isolation of ABA, although today this method is being replaced by high-performance liquid chromatography (HPLC) (Horgan, 1980). In TLC the absorbent or carrier of the stationary phase is often silica gel (although alumina, kieselguhr, and cellulose powder are also used) (Yokota et al., 1980). The method gives rapid and good separation, and permits the use of many agents for detection, although recovery may be variable (Horgan, 1980). Use of silica gel containing an unorganic fluorescent indicator (silica gel GF_{254}) is applicable to the anlysis of ABA which absorbs strongly in the ultraviolet (UV); the ABA bands on the thin-layer plate appears as a dark spot under ultra-violet light (Yokota et al., 1980).

Recently, small disposable cartridges (SEP-PAK cartridges, Waters Associates) prepacked with a silica gel support, or silica with a bonded octadecyl silane (ODS) stationary phase (permitting normal phase and reverse phase partition chromatography respectively) have been used to purify extracts for ABA (Hubick and Reid, 1980,

Crozier, 1980). These permit rapid sample throughput and may remove sufficient impurities to allow final quantification with few additional purification steps (Hubick and Reid, 1980).

Conventional liquid column chromatographic techniques are normally time-consuming and do not give good separation. High-pressure (or performance) liquid chromatography, in which pressure is applied mechanically to a finely-packed column to force through the eluting solvent, was developed to improve the technique, and has recently proved valuable in the anlysis of ABA, as well as gibberellins, cytokinins and IAA. HPLC is a particularly useful analytical tool in that there are many different column packings commercially available for adsorption, partition (including reverse phase, in which silica gel particles have bonded phase coatings consisting of hydrocarbons such as C_{18} (octadecyl silane or ODS)), ion-exchange and gel filtration, and samples can be recovered for further purification.

Use of reverse-phase HPLC is often made in the initial analysis of partially purified extracts, since bonded-phase materials have high sample capacities (Horgan, 1980, Crozier, 1980) and permit the concentration of PGS present in only trace amounts. The most serious limitations of HPLC lie with the detectors currently available for organic compounds following sample separation.

ABA lends itself to analysis by HPLC since it is readily soluble in many organic solvents and has acidic properties making it suitable for partition chromatography (Yokota et al., 1980). Additionally, the strong UV absorbance shown by ABA permits detection of<1ng with an absorbance monitor, although this detector does not approach the sensitivity or selectivity of the electron-capture detector for ABAMe (used in conjunction with gas chromatography).

The advent of capillary gas-chromatography coupled with more specific detectors available with this technique, have resulted in the use of HPLC more as a very useful preparative tool rather than in the identification or quantification of ABA.

2.2.2 Identification and Quantification

In the present context, as in most research, the identification of PGS refers to the "confirmation of the presence in a plant extract of a compound the structure of which has already been established" (Horgan, 1980). The elucidation of chemical structures hitherto unknown, of compounds associated with biological activity is a far more complex process, and occurs relatively rarely.

Whilst tentative identification by co-chromatography with authentic compounds is the only available technique for PGS that occur at very low levels within a specific tissue or localized area, spectroscopic techniques are preferred where more of the PGS is available for analysis (Horgan, 1980). Combined gas-chromatography-mass-spectrometry (GCMS) remains a particularly powerful method of identifying PGS in plant extracts even in the presence of impurities, unlike most spectroscopic methods available for PGS analysis, which require relatively clean extracts.

The UV absorption, ORD, CD, IR and NMR spectra of ABA have been published (Milborrow and references therein, 1974), but only ORD and CD find occasional use today in the identification of this compound (Yokota et al., 1980).

In GCMS , chromatographic separation of extract components is made prior to mass-spectrometric analysis. Column effluent is introduced into the ionization chamber of the mass spectrometer which is maintained at a pressure of less than 10^{-5}mm Hg, following removal of the carrier gas by a molecular separator. Ionization of the compounds present occurs by electron impact, or via the use of a reagent gas (chemical ionization). The resultant molecular and fragment ions (which have specific mass to charge ratios) interact with magnetic fields within the spectrometer to produce a mass-spectrum characteristic of the compound. The mass spectrum of each GC peak can be obtained by rapid scanning. Today, the availability of an on-line computer to store spectral data as it is produced can permit the detection of contaminants within single GC peaks, and can hence afford a measure of sample purity. Horgan (1980) has warned, however, that the use of data-handling systems may lead to the production of artifactual spectra in certain situations.

ABA is usually derivatized to the volatile methyl ester

(ABAMe) prior to GCMS analysis. The mass spectrum of ABAMe and its fragmentation pathway was published by Gray et al. (1974). Whilst in most cases comparison of the mass-spectrum of a plant extract with that of the authentic PGS will permit the identification of the PGS within the extract, occasionally further information is necessary. For example, the mass-spectrum of trans ABA is too similar to that of ABAMe to permit distinction between the two by this method alone, and hence chromatographic retention times must be used to permit definitive identification (Horgan, 1980). This author also stresses that the best way of identifying a PGS within an extract is by 'utilising wherever possible, all the data available.....in addition to the matching of mass-spectra'. Thus a series of techniques is substantially more valuable than relying on one or two, no matter how powerful they are individually.

Once the identitiy of a PGS in an extract has been established, quantification may be attempted. A measure of purification losses is clearly required, which relies on the addition of a compound at the outset of the procedure, which is expected to behave in the same manner as the endogenous PGS, yet can be easily distinguished from it. Even despite careful use of an internal standard, both the release of free ABA from conjugated ABA and the transesterification to ABA-Me and glucose of the glucose ester which may occur in methanolic solution, may still create problems in ABA quantification, particularly where small values are involved. Three types of internal standard have been used in the analysis of ABA. Milborrow (1967, 1971) used a 'racemate dilution' technique in which he added a known amount of racemic ABA (RS (±) ABA) to crude extracts. Following purification the (±) ABA content of the extracts was determined by spectropolarimetry (UV absorption in 0.005N ethanolic sulphuric acid, λ = 260nm), whilst the (+) ABA content was measured by spectropolarimetry (ORD); these measurements then permitted evaluation of the true (+) ABA extract content. Lenton et al., (1972) added known amounts of synthetic 2-trans ABA (amounts of which were re-determined by GC following purification) to methanol preparations of birch, maple and sycamore seedlings, to enable calculation of extraction losses. This method is clearly suitable only if (a) there is no naturally occuring trans ABA in tissue extracts (b) precautions are taken to prevent photointerconversion of the 2 isomers and (c) no mechanism catalyzing the inter-

conversion of the isomers occurs in the absence of light, as has been reported by Bangerth (1982).

Finally, isotopes of ABA may be used as internal standards to quantify ABA losses. Both [2-^{14}C] and [^{3}H] ABA are readily available today and find wide usage (e.g. Milborrow and Robinson, 1973). The use of radiolabelled isotopes is particularly applicable to ABA since few chemical changes have been reported to occur in this PGS during the usual extraction and purification methods (Horgan, 1980). A mass isotope of ABA, hexadeuterated ABA (ABA H_6) (which may be distinguished from ABA by mass spectrometry) has been described as a 'near perfect internal standard' (Rivier et al., 1977). The isotope can protect small levels of endogenous ABA against degradation and absorption during purification, although care must be taken to keep extracts at a pH below 8.0 as the location of the deuterated label is not stable above this (Milborrow, 1971).

HPLC and GC are the most widely used physical detection techniques today in the final analysis of extracts for ABA quantification. However, radioimmunoassay, permitting distinction between the R and S enantiomers and ABA metabolites (Mertens et al., 1982; Weiler, 1980) is becoming increasingly popular where speedy analysis of multiple extracts is required, and the information yielded by chromatographic techniques is superfluous to the study in hand.

2.2.2.1 Electron-capture-detector-gas-chtromatography

The methyl ester of ABA was shown to possess strong electron-capturing (or electron absorption) properties (Seeley and Powell, 1970) and is hence ideally suited to analysis with an electron-capture detector (ECD) following GC. The ion-chamber of the detector incorporates a radioactive source (usually ^{63}Ni) which emits ionizing radiation. The chamber itself is connected to a source of low potential which is initially adjusted to collect all the electrons liberated by ionization from the stream of an inert gas (e.g. N_2 or H_2) which enters the detector from the GC.

Electron-absorption is described by two chemical equations: $\bar{e}+AB \rightarrow \bar{AB}$ or $\bar{e}+AB \rightarrow A+\bar{B}$ (Lovelock, 1963). Following the introduction of a vapour capable of electron absorption into the chamber, the standing current falls, (a) since the negative

molecular ions formed by electron absorption more easily recombine with positive ions present in the chamber than free electrons and (b) since negative ions are much less mobile than free electrons. A record of the alterations in standing current which result during the passage of column effluent through the detector forms the basis of GCECD analysis (Lovelock, 1963). The value of the technique for the analysis of multi component extracts for ABA, lies in the capacity of the ECD to selectively respond to volatile derivatives capable of electron absorption.

2.2.2.2 Gas-chromatography with Single or Multiple-ion Monitoring (GCSIM/GCMIM)

In addition to providing full mass-spectra, the mass-spectrometer can be used as a selective and sensitive GC detector by the techniques of GCSIM or GCMIM. In SIM, a single ion is monitored by 'focussing' the mass spectrometer on that ion (i.e. by selecting the correct ion-accelerating voltage at a fixed, magnetic field), and the intensity of the ions is recorded as a function of time. To optimize the sensitivity of the technique, ions with high mass values (hence more likely to be characteristic of the compound than ions with low mass values) are chosen, which occur at high intensities (Horgan, 1980). SIM has been used successfully to quantify ABA in plant extracts (see Hillman et al., 1974 and Berrie and Robertson, 1976). MIM (which is also termed multiple-peak monitoring (MPM) or mass-fragmentography (MF)),involves rapid switching of the accelerating voltage to enable monitoring of more than one ion during any one sample run permitting discrimination between mass isotopes. The technique was used by Rivier et al. (1977) to quantify ABA in maize roots. These authors, who used hexadeuterated ABA as an internal standard, monitored m/z 190 and m/z 194 to distinguish ABA from its hexadeuterated analogue, respectively.

2.2.2.3 Immunological Methods

Immunoassay for ABA was first attempted by Fuchs and co-workers (Fuchs et al., 1972). ABA in itself is too small to be antigenic per se, and must be covalently linked as a hapten to a protein carrier, which can then be used to raise antibodies with specificity towards the hapten. Fuchs et al.(1972) raised

antibodies in rabbits (termed anti ABA antibodies) to ABA conjugated with bovine serum albumin (BSA) or hemocyanin (HC). The specific binding of free ABA antibodies (and hence the level of extract ABA) was estimated by adding ABA conjugated to a T_4 bacteriophage; this was normally inactivated by the presence of anti ABA antibodies, but released from inactivation by anti ABA antibodies bound to free ABA.

The technique has been greatly improved and developed, culminating in the use of radio immunological methods, in which antibodies are reacted with a fixed quantity of radioactively labelled PGS and extract containing an unknown amount of unlabelled PGS. Fractionation of the PGS remaining free and that bound to the antibody following the establishment of equilibrium and measurement of the radioactivity ascribed to each fraction then permits an evaluation of the PGS content of the extract by reference to a standard calibration curve (Reeve and Crozier, 1980).

Chemical groups via which a PGS is linked to the protein carrier determines the specificity of the hapten; with ABA it has thus been possible to distinguish between the free acid and conjugates such as the glucose ester, by creating haptens linked via the C_1 of the molecule (for total ABA determination) or C_4' (for free ABA determination) (Weiler, 1980). Distinction between the two ABA enantiomers is possible, and little cross-reactivity with metabolites structurally similar to ABA (such as PA and DPA) has been reported (Weiler, 1980, Mertens et al., 1982). The technique requires little equipment apart from a scintillation counter, although it does require large amounts of standards for preliminary immunization work and standards of high specific radioactivity for the assays themselves.

2.2.3 Verification of Accuracy

In the quantification of PGS by chromatographic methods, it is desirable to establish the accuracy of the analysis. Reeve and Crozier (1980) have defined accuracy as the non-random or systematic error associated with analysis. (The term precision, in contrast, refers to random errors which occur during sample analysis, and a measure of which can be relatively easily obtained).

Two methods have been detailed by these authors to verify the accuracy of chromatographic analysis. The first is termed

'successive approximation', and is concerned with the selectivity of the analysis. Extracts are analyzed, purified further and re-analyzed, until there is no difference in successive estimates of extract PGS levels. At this point, the selectivity of the analysis has been improved such that random errors are larger than non random errors, and the estimate can be considered accurate.

The second method concerns the uncertainty associated with sample purity (Crozier, 1980, 1981). The method involves making an objective quantification of the total information yielded by the chromatographic analysis of a plant extract by applying the concepts of information theory. The binary digits (I) or bits of information, which are calculated from the relationship

$$I = \frac{\log_{10} n}{0.3}$$

where n is the number of components in the system, may be compared with the amount of information required to guarantee the accuracy of the analysis at a given probability value. Thus Reeve and Crozier (1980) have calculated that in a typical plant extract, there are 10^{42} compounds potentially present at a probability level of 0.9, and hence at least 140 bits of information are required for an analysis to be termed accurate. Clearly the fewer impurities that are present in an extract, the greater the information content yielded.

Detection limits, selectivity and information content yielded by physiocochemical detectors are often related (Reeve and Crozier, 1980); high selectivity provides low detection limits but may result in low information content. Selective detectors are to be preferred, however, in the analysis of impure samples when generally more information is obtained than through the use of less specific methods (Crozier, 1980).

In the analysis of ABA, an additional test of accuracy may be obtained by re-analysis of a sample following UV irradiation (Crozier, 1980). This should result in the appearance or enhancement of a trans ABA peak, and a diminution in the cis peak (in the same proportion as a cis ABA standard similarly treated).

2.3 Physiology

2.3.1 Summary of the putative physiological roles of ABA

ABA has been ascribed a number of physiological roles, many of which are illustrated in Table 1 . The importance of ABA in bud dormancy is described in more detail however.

2.3.2 The Putative Role of ABA in Bud Dormancy

Dormancy has been defined as 'the ability to retain viability while having restricted metabolic activity, and no observable growth' in the presence of unfavourable conditions (Berrie, 1984). In temperate woody perennials, bud dormancy permits shoot apices to overwinter, and the formation of dormant flower buds in some species, is necessary to protect floral structures which are already differentiated. In a physiological sense, a dormant bud is not subject to inhibitory effects imposed by the apex (i.e. to correlative inhibition), but will not necessarily recommence growth following restoration of a favourable climate. Dormancy in such buds may be imposed (or bud-break prevented) by the transmission of a stimulus arising elsewhere in the plant; this has been termed correlative dormancy. A bud may also be innately dormant, when conditions within the bud itself prevent outgrowth; differentiation within the bud may continue following the onset of either correlative or innate dormancy however (Saunders, 1978).

Shoot growth in some species such as pines follows a pattern predetermined by primordia laid down within last season's dormant bud; terminal buds are formed once shoot growth has ceased. In species that show indeterminate shoot growth however, such as *Betula pubescens* and *Acer pseudoplatanus*, changes in photoperiod control shoot growth and hence the appearance of dormant buds (Saunders, 1978).

Plant growth substances were first implicated in the control of bud dormancy, in studies with *Acer pseudoplatanus* made by Wareing (1954). Defoliation of the plant except for two pairs of mature leaves, followed by a short-day treatment to the leaves alone, led to the appearance of a terminal bud. This did not occur if the leaves (but not the apex) were given long days, or the plant was completely defoliated. Furthermore, the correlative stimulus from the leaves also resulted in a period of innate bud dormancy, since shoot extension did not recommence if long days were restored (Wareing, 1954). Whilst there was (and still is) no *a priori* reason to suggest that PGS are involved directly in the

Table 1. (a) Some putative physiological roles for ABA inferred from work with exogenous applications. (b) Some putative physiological roles for ABA inferred from endogenous measurements. B indicates measurement of ABA levels by bioassay.

PUTATIVE PHYSIOLOGICAL ROLE	LITERATURE THAT SUPPORTS A ROLE FOR ABA	LITERATURE THAT DOES NOT SUPPORT A ROLE FOR ABA
Correlative inhibition	Van Onckelen et al., 1981 Phaseolus vulgaris (L.) 100ng ABA injected into the cotyledons caused 75% inhibition of axillary shoots.	
Geotropism	Wilkins and Wain, 1975 Zea Mays (L.) 0.1mol m^{-3} ABA applied to intact roots caused downwards curvature when roots were held horizontally in darkness. (The root was not normally geotropic in darkness).	Hartung, 1976 Phaseolus coccineus (L.) There was no asymmetric distribution of ^{14}C ABA applied to geotropically-stimulated roots held horizontally, regardless of the site of application.
	Pilet, 1978 Zea mays (L.) cv. Anjou 210 (Geotropic in light and dark) 0.1mol m^{-3} ABA applied to the lower side of the apical end of a decapitated root held horizontally in the dark, caused downwards curvature.	Mertens et al., 1983 Zea mays (L.) cv.Anjou (Geotropic in light and dark) No induction of curvature 4h after application of 0.001 - 1.0mol m^{-3} ABA to roots held vertically in the light, despite asymmetric distribution in favour of the side of application.
Stomatal regulation	Jewer et al., 1981 Kalanchoe daigremontiana (Hamet et Perr) Stomatal aperture declined linearly in isolated epidermal strips incubated on buffer solutions containing 10^{-8} - 10^{-2} mol m^{-3} ABA	Kubik and Antoszewski, 1983 Fragaria grandiflora (Duch.) cv. Red Gauntlet Stomatal sensitivity to foliar-applied 7.5×10^{-6} mol m^{-3} ± ABA (the level to which the transpiration rate fell) was reduced by repeated applications.

Table 1a

Table 1a

PUTATIVE PHYSIOLIGICAL ROLE	LITERATURE THAT SUPPORTS A ROLE FOR ABA	LITERATURE THAT DOES NOT SUPPORT A ROLE FOR ABA
Stomatal regulation	Jones and Mansfield, 1970 Xanthium pennsylvanicum (Wall.) 0.1mol m^{-3} ABA applied to the intact leaf surface caused stomatal closure.	
Acceleration of leaf senescence	Back and Richmond, 1971 Rumex pulcher (L.), Taraxacum megallorhizon (Forsk.) The presence of 0.019mol m^{-3} ABA (Rumex) or 0.038mol m^{-3} ABA (Taraxacum) increased the senescence of leaf discs.	Colquhoun and Hillman, 1972 Raphanus sativus (L.) var. Cherry Belle 0.1mol m^{-3} ABA RS ABA retarded the senescence of leaf discs.
	Lindoo and Nooden, 1978 Glycine max (L.) (Merrill) var. Anoka Foliar sprays of 0.1mol m^{-3} ABA accelerated the leaf senescence of podded plants (monocarpic senescence).	
Fruit abscission	Weaver and Pool, 1969 Vitis vinifera vars. Carignane, Muscat of Alexandria Dipping clusters of flowers and berries at the fruit-set stage into 4mol m^{-3} ABA or 4×10^{-3} mol m^{-3} ABA respectively resulted in abscission.	

PUTATIVE PHYSIOLOGICAL ROLE	LITERATURE THAT SUPPORTS A ROLE FOR ABA	LITERATURE THAT DOES NOT SUPPORT A ROLE FOR ABA
Leaf abscission	Dörffling *et al.*, 1978 *Coleus rehneltianus* Berger Applications of 10^{-4} to 10^{-5} μg petiole $^{-1}$ accelerated abscission of explants directly without influencing ethylene production.	Jackson and Osborne, 1972 *Phaseolus vulgaris* (L.) cv. Canadian Wonder No increase in petiolar abscission in explants (1cm long abscission-zone segments) by 2 mol m^{-3} ABA and 2nl m^{-3} C_2H_4 applied together over that caused by C_2H_4 alone.
		Sagee *et al.*, 1980 *Citrus sinensis* (L.) Osbeck var. Shamouti 0.5 mol m^{-3} ABA applied to explants with 0.12 mol m^{-3} aminoethoxyvinyl glycine (an inhibitor of C_2H_4 biosynthesis) did not accelerate petiole abscission.
Flower induction	Fujioka *et al.*, 1983 *Lemna pauciostata* Flowering induced by either 0.5 ppm benzoic acid or short days was inhibited by 4×10^{-4} mol m^{-3} ABA.	Krekule and Kohli, 1981 *Chenopodium rubrum* (L.) 0.3 mol m^{-3} ABA applied to the plumule of intact plants could not substitute for the effect of photoperiod in inducing changes in the growth and differentiation of the apical meristem, necessary prior to flower induction.

Table 1a

Table 1b

PUTATIVE PHYSIOLOGICAL ROLE	LITERATURE THAT SUPPORTS A ROLE FOR ABA	LITERATURE THAT DOES NOT SUPPORT A ROLE FOR ABA
Correlative inhibition	Tucker and Mansfield, 1973 B Xanthium strumarium High levels of ABA were present in inhibited axillary buds (12,200 μg g^{-1} f.wt.). Low levels (855 μg g^{-1} f.wt.) were found following removal of the youngest 3 leaves and perceptible bud outgrowth.	Taylor and Rossall, 1982 Lycopersicon esculentum No significant difference between the stem and leaf ABA content of the lateral suppressor mutant (lacking axillary buds) and the wild type.
	Van Onckelen et al., 1981. Phaseolus vulgaris (L.) Lateral bud outgrowth following decapitation and increased cotyledonary metabolism of ABA.	Dörffling, 1976 Acer pseudoplatanus, Syringa vulgaris No absolute decrease in axillary bud ABA content following defoliation and decapitation and bud outgrowth.
	Everat-Bourbouloux and Charnay, 1982 Vicia faba (L.) Decrease in ABA content of inhibited axillary buds following decapitation, from 200ng g^{-1} f.wt. to 30ng g^{-1} f.wt.	
Root geotropic curvature	Wilkins and Wain, 1974 B Zea mays (L.) ABA is released and translocated from the root cap prior to the inhibition of root elongation by white light.	Feldman, 1981a, 1981b Zea mays (L.) var. Merit (Geotropic after light stimulation) Absence of ABA in caps from roots grown and detached in darkness following illumination, despite their ability to induce a geotropic response in roots kept in darkness.

PUTATIVE PHYSIOLOGICAL ROLE	LITERATURE THAT SUPPORTS A ROLE FOR ABA	LITERATURE THAT DOES NOT SUPPORT A ROLE FOR ABA
Root geotropic curvature	El-Antably and Larsen, 1974 Vicia faba (L.) ABA was present in the lower side of roots held horizontally in the dark for 30 minutes in amounts 3.08 times those present in the upper half of the root.	Suzuki et al., 1979. Zea Mays (L.) cv . Golden Cross Bantam (geotropic only after light-stimulation) There was 1.6 times as much ABA present in the upper halves of roots held horizontally for 1h following red-light illumination as in the lower halves (bending had just commenced).
		Mertens et al., 1983 Zea Mays(L.) cv. Anjou, Vicia faba (L.) cv. Hangdown grünkernig (whole seedlings). No asymmetry in ABA distribution in roots held horizontally in the light or when it would be required to induce the graviresponse (25-30 minutes after horizontal orientation).
Regulation of stomatal aperture	Boussiba and Richmond, 1976 Nicotiana rustica (L.) Stomatal aperture and leaf ABA content were inversely related in the recovery of plants subjected to mineral deprivation or salination of the root medium.	Bengston, et al., 1979 Triticum aestivum (L.) cv . Weibulls Starke II Despite an increase in leaf ABA following root-cooling and decrease in leaf transpiration, ABA levels were reduced to pre-stress values prior to the restoration of normal transpiration

Table 1b

PUTATIVE PHYSIOLOGICAL ROLE	LITERATURE THAT SUPPORTS A ROLE FOR ABA	LITERATURE THAT DOES NOT SUPPORT A ROLE FOR ABA
	Loveys et al., 1974 Vitis vinifera (L.) cv. Cabernet sauvignon An increase in stomatal resistance was positively correlated with a rise in leaf ABA content following a change in photoperiod length from short to long days.	Ackerson, 1980 Gossypium hirsutum (L.) cv. Stoneville 2B Although young leaves had higher levels of ABA than older ones, they did not show a greater degree of stomatal closure.
Leaf senescence	Even-chen and Itai, 1975 Nicotiana rustica (L.) There was a sharp increase in leaf free ABA content prior to significant chlorophyll loss.	Samet and Sinclair, 1980 Glycine max (L.) (Merrill) cvs. Corsoy and Chippewa Leaf chlorophyll and protein levels had declined by over one half prior to increases in 'free' ABA levels.
		Colquhoun and Hillman, 1975 Phaseolus vulgaris (L.) var. Canadian Wonder There was no consistent change in leaf ABA content with progressive senescence
Fruit abscission	Davis and Addicott, 1972 Gossypium hirsutum (L.) cvs.Acala 4-42 and Acacia SJ-1 High levels of ABA correlated positively with the abscission of young leaves and the senescence and dehiscence of mature fruit.	

Table 1b

PUTATIVE PHYSIOLOGICAL ROLE	LITERATURE THAT SUPPORTS A ROLE FOR ABA	LITERATURE THAT DOES NOT SUPPORT A ROLE FOR ABA
	Guinn, 1982 B Gossypium hirsutum (L.) cv. Deltapine 16 Changes in both ethylene and ABA correlated positively with % abscission of fruit. Dim light increased fruit ABA content only after the abscission of young fruits.	
Leaf abscission	Bottger, 1970 Coleus rehneltianus Petiolar diffusates from adult leaves contained less ABA than senescent leaves.	Dorffling et al., 1978 Coleus rehneltianus (Berger) ABA levels per petiole (and on a f.wt. basis) declined with progressive senescence despite the ability of diffusates from senescent petioles to accelerate explant abscission.
Flowering	Dunlap and Morgan, 1981 Sorghum bicolor (L.) (Moench.) cvs. 60M and 90M At the commencement of flower induction in cv. 60M, shoot 'free' ABA levels were lower than in 90M in which flowering was delayed.	

Table 1b

imposition of innate bud dormancy, these experiments led to the hypothesis that the translocation of an inhibitor arising in short-day leaves to the apex was responsible for correlative dormancy, which could then lead to the development of innate dormancy (Saunders, 1978).

Eagles and Wareing (1964) demonstrated that an extract from the leaves of Betula pubescens grown in short-days could induce the formation of resting buds in actively growing plants maintained in a 14.5 hour photo_period, when applied to young expanding leaves. One of the compounds active in the extracts was ultimately isolated and identified as abscisic acid (Cornforth et al.,1965a). Much subsequent research concerning the role of PGS in bud dormancy has centred on the quantification of endogenous ABA levels in buds and leaves, and the effects of exogenous applications. In this resumé, examples of the work aimed at elucidating the role played by ABA during the onset, maintenance and breaking of bud dormancy are presented.

2.3.2.1 Conclusions from Changes in Endogenous ABA

Several studies have reported a close correlation between bud dormancy and endogenous bud ABA levels, although no causal relationship between the two has ever been established (Laver, 1978). Dumbroff et al. (1979) reported that the high levels of ABA in dormant Acer saccharum buds in October to January, which declined prior to bud-burst, implicated a role of ABA as part of the mechanism responsible for maintaining dormancy. However, the highest levels of all were recorded in November and December, and deemed more likely to be associated with leaf senescence than responsible for the-induction of bud dormancy. Wright (1975) demonstrated that at bud-burst in Ribes nigrum and Fagus sylvatica, low free ABA values in the buds were associated with high levels of the glucose ester, and suggested that the interconversion of the free to the conjugated form was of importance in the release of these buds from dormancy. Similarly, Harrison and Saunders (1975) concluded that an increasing ability of birch buds to conjugate ABA was associated with their emergence from winter dormancy, although this capacity did not lead to a reduction in absolute free ABA levels in this species. However, Taylor and Dumbroff (1975) reported that there was a relatively constant level of β-inhibitor activity in buds of Acer saccharum during the winter period, and the suggestion was made that an increase in endogenous cytokinin

levels was responsible for the release of the buds from dormancy. Cytokinins and IAA were implicated in a study made of bud-break in pecan (Wood, 1983); ABA was ascribed a secondary role in the inhibition of post bud-break growth.

Some research has implicated a role for ABA in the correlative control of bud dormancy imposed by other parts of the plant. Davison and Young, (1974) suggested that the fall in xylem ABA levels observed in peach, at bud swell, might be responsible for the release of bud dormancy. A similar relationship was observed in willow (Alvim et al., 1976). However, an elegant study with two races of maples, which both showed an increase in leaf ABA content following removal to short days, although only the northern race showed signs of bud dormancy, led Perry and Hellmers (1973) to conclude that ABA was not the causal agent in the development of winter rest. Additionally, these authors demonstrated that the stock and scion on interracial grafts behaved quite independently, and in accordance with whole plants of their race when transferred to conditions that induced dormancy in the northern race. They concluded that the development of internal rest and cold resistance in this species occurred as a result of local changes within a bud, and that the transmission of a stimulus was not involved. In birch, endogenous leaf ABA levels were lowered following the onset of short days (Loveys et al., 1974 although no alteration in the metabolism of shoot [2 - ^{14}C] ABA was observed. These authors suggested that ABA was unlikely to play a role in the photoperiodic control of extension growth.

2.3.2.2 Conclusions from Exogenous ABA Applications

Despite early claims that synthetic ABA applied to the leaves and apices of Betula pubescens and Acer pseudoplatanus, and to the leaves alone of Ribes nigrum caused the formation of terminal buds in an 18h photoperiod (El-Antably et al., 1967), subsequent work has not endorsed the view that changes in ABA levels alone are responsible for initiating bud dormancy. Hocking and Hillman (1975) reported that as much as 2 x 10^{-4}M (+) ABA applied to upper expanded leaves of Alnus glutinosa or Betula pubescens failed to induce bud dormancy in long days. Additionally, very little radiolabelled ABA applied in a similar manner, remained unmetabolized in the apical tissues. Juntilla (1977) and Proebsting (1983) working with Salix pentandro and Cornus respectively, also reported that exogenous ABA applications did not have any effect on the onset of

dormancy. Hocking and Hillman (1975) concluded from their studies that subtle interrelationships between endogenous growth regulators including ABA may be important *in vivo*, but may not be reflected in changes in whole tissue ABA levels. Moreover, a mechanism of this nature would make it unlikely that exogenous PGS applications could in any sense mimic the natural pattern of events leading to dormancy.

2.3.2.3 General Conclusions

Besides the problems involved with exogenous applications of PGS (including penetration, compartmentation, distribution and metabolism (Hocking and Hillman, 1975)) and meaningful interpretation of endogenous PGS levels, Saunders (1978) has pointed out that studies often fail to describe adequately the development of buds and shoots, in metabolic and cytological terms. This author has suggested that it is unlikely that correlative control (when it occurs) of apical growth during the onset of dormancy, is mediated by a single PGS, or even a balance between growth promoters and inhibitors; instead it may be influenced by a conglomeration of raw materials which arise from the leaves, and even physical stimuli. Bud-burst may be under innate control of the buds themselves; the mechanisms underlying this event remain largely unclear.

2.4 Transport

Studies of the transport of ABA are important to complement investigations concerning endogenous tissue ABA levels. ABA transport has been studied (a) through the application of radiolabelled ABA to leaves, roots and tissue segments, and (b) through the examination of the endogenous ABA content of phloem and xylem exudates.

In a study with *Helianthus annuus* seedlings, Hoad (1975) suggested that the ABA present in xylem sap collected from the roots following a water stress treatment, had originated in the leaves. However, other reports have demonstrated that roots can synthesize ABA (Walton *et al.*, 1976; Hartung and Abou-Mandour, 1980) and hence may represent a source of this PGS for other parts of the plant. Additionally, the translocation of ABA within roots may participate in the geotropic response. This account covers therefore, studies involving the ABA translocation mechanisms of both roots and shoots.

2.4.1 The Translocation of Radiolabelled ABA

The translocation of [2 - ^{14}C] ABA applied to a mature foliage leaf of Phaseolus vulgaris to the remainder of the plant was shown to occur via the phloem at speeds of some 20 cm h^{-1} (Everat-Bourbouloux, 1982). In this study, 90 minutes after the application of 7.8×10^{-6}g of [2 - ^{14}C] ABA to the fourth leaf, 1% of the radioactivity had been transported out of the donor leaf, reaching all parts of the plant, both above and below ground. In wheat, rapid transport of [1-^{14}C] ABA applied to the flag-leaf also took place, although this was accompanied by a substantial metabolism of the label in the developing grains (Goldbach and Goldbach, 1977) which has also been observed in other actively growing parts (such as plant apices).

The profile of ABA translocated throughout the plant following foliar application may alter with time, showing that redistribution may occur (Everat-Bourbouloux, 1982; Hocking et al., 1972). Thus it has been demonstrated that although movement of [2 - ^{14}C] ABA applied to the fourth leaf of Phaseolus vulgaris plants was mainly basipetal after 90 minutes, a profile was obtained 7½ hours after treatment in which the radioactivity associated with ABA accumulated above the donor leaf (Everat-Bourbouloux, 1982). Some 60% of the translocated radioactivity remained as ABA at this time.

In Phaseolus coccineus, a water-stress treatment sufficient to stop growth has been shown to reduce (by 40 times) the amount of ABA exported over a 24h period from the leaf of application; no alteration in ABA metabolism was recorded however (Hartung , 1976).

In addition to phloem-translocation, ABA may be removed from the site of application or synthesis by diffusion. The apices of pea and bean plants transport applied [2 -^{14}C] ABA poorly and in a manner characteristic of diffusion transport (Bellandi and Dorffling, 1974; Everat-Bourbouloux, 1982). In a study with 20mm sections of cotyledonary petioles, Davenport et al. (1977) recorded non-polar transport of [1 -^{14}C] ABA at rates of 0 - 2.0 mm h^{-1} and concluded that this too was facilitated by passive diffusion. These authors ascribed the rapid movements of ABA applied to cotton petioles recorded by Ingersoll and Smith (1971) to the use of short (3 or 6mm) tissue segments, rather than to a mechanism proposed by these authors involving cytoplasmic streaming.

In intact plants, basipetal transport of [2 -^{14}C] ABA applied to the roots has been demonstrated (Hartung, 1977 (Phaseolus coccineus); Chanson and Pilet (Zea mays), 1982), although a substantial amount of the radiolabel may be retained within the root segments of Phaseolus coccineus, acropetal movement of ABA has also been observed (Hartung and Behl, 1974,1975; Astle and Rubery, 1980). In Phaseolus coccineus, a zone of elongation (in which the cells show increasing vacuolation with age) extends from 2 to 10mm behind the root apex. Radioactivity applied to root segments 40mm long that included the zone of elongation, but lacked the apical 2mm, accumulated at the apical end from a basal donor block, or was retained at the apex when applied at this end (Astle and Rubery, 1980). Similar results were obtained by Hartung and Behl (1974) and attributed to the transport mechanisms of stelar tissue. Astle and Rubery (1980) suggested however, that the accumulation of ABA by the cells of the apical end of the segments was due to their large cytoplasm (pH 7-8) to vacuole (pH 4-6) ratio, which favoured the uptake of the undissociated species of ABA (ABAH).

Apparently contradictory results concerning the movement of ABA in P. coccineus root segments lacking the zone of elongation have been observed; whilst Astle and Rubery (1980) failed to demonstrate any polarity of transport in this tissue, Hartung and Behl (1975) recorded acropetal ABA movement which was located in the stele.

The uptake of ABA from an external solution over a range from 0-160μmol dm^{-3} by short tissue segments from all root regions of P. coccineus is mediated by diffusion (Astle and Rubery, 1980). A saturable uptake component functional over an external ABA range from 0-0.5μmol dm^{-3} was also observed in the apical 4-6mm, and may represent an ABA carrier.

In a very different experimental system with soyabean grown in water-culture, Markhart (1982) concluded that penetration of the roots (which were exposed to a 3 bar hydrostatic pressure difference by racemic ABA, was not controlled to any degree by a carrier mechanism. This author suggested that the transpirational water column was responsible for conveying ABA to the shoot. Differential transport of the two isomers of ABA was aseribed to their relative hydrophobicity; cis ABA may have penetrated the

roots more readily than the trans isomer since it is less hydrophilic and may easily traverse lipid cell membranes.

Fiscus et al., (1982) have determined ABA transport coefficients for Phaseolus root systems; the equations that they describe enable the amount of ABA translocated to the shoot for a given root dosage to be calculated.

2.4.2 The ABA Content of Phloem and Xylem Exudates

ABA was first detected in xylem sap by Lenton et al., (1968) and in phloem sap by Hoad (1967). Some values of the levels of ABA which have been recorded in phloem and xylem exudates are presented in Table 2 . Metabolites of ABA have been detected in the phloem (dihydrophaseic acid, hydroxy or oxo-dihydrophaseic acid (Hoad and Gaskin, 1981, Cocos nucifera);phaseic acid, dihydrophaseic acid (Zeevaart, 1977, Ricinus communis); phaseic acid (Belke et al., 1980, Lycopericon esculentum)). Since it is unlikely that the sap itself contains the enzymes necessary for ABA degradation, it is probable that the metabolites are loaded directly into the sieve tubes from their sites of synthesis (Hoad and Gaskin, 1981).

2.5 The Occurrence of Diurnal Changes in Tissue ABA Levels

Before the importance of long-term changes in PGS levels can be fully appreciated, it is essential to have some knowledge of the cyclical changes in endogenous levels which may occur on a daily basis. The possibility that endogenous PGS levels undergo diurnal fluctuations has received attention only relatively recently, however.

In view of the influence of plant water status on endogenous ABA, much of the work concerning daily fluctuations in ABA levels in leaf tissue, has been linked with observations of changes in leaf water potential, and stomatal conductance. Changes in leaf ABA content on a diurnal basis, have been examined in Prunus (Xiloyannis et al., 1980), Gossypium (McMichael and Hanny, 1977), Sorghum (Kannangara et al., 1982), Pisum (Kobriger et al., 1982), Pennisetum (Henson et al., 1982) and Arbutus unedo (Burschka et al., 1983). In all cases, tissue ABA content fluctuated daily, although the magnitude of the peak ABA levels recorded (over the minimum observed), and the time(s) at which they occurred, differed between experiments. Henson et al., (1982) recorded a 2-3 fold

Table 2. Some examples of ABA levels which have been recorded in phloem and xylem exudates.

SPECIES	NOTES ON EXPERIMENT	PHLOEM OR XYLEM EXUDATE	ABA LEVELS ($\mu g\ dm^{-3}$)	REFERENCE
Acer saccharum (Marsh.)	Commercial syrup	Xylem	0.63 *cis*	Davidson and Young 1973
Actinidia chinensis (Planch.)	Collected as bleeding sap in spring	Xylem	0.09 *cis*	
Malus sylvestris (L.) Mill.	) Collected by	)	0.25 *cis*	
Prunus persica (L.) Batsch.	) applying vacuum to	) Xylem	2.17 *cis*	
Salix fragilis (L.)	) the base of cut	)	2.19 *cis*	
Tecomaria capensis (Spach.)	) shoots in winter	)	11.04 *cis*	
Salix	Sap collected at 2 week intervals from field-grown plants	Xylem	20 - 100	Alvim *et al.*, 1976
Ricinus communis (L.)	Plants subjected to water stress. Representative data from one specimen	Phloem	Pre-drought 125 Drought 1420 Post-drought 290	Hoad, 1973
Ricinus communis (L.)	Plants subjected to water stress	Phloem	Stressed 1700 Non-stressed 1000	Zeevaart, 1977
Yucca flaccida (Haw.)		Phloem	195 (*cis* + *trans*)	Hoad and Gaskin, 1981.
Cocos nucifera (L)		Phloem	90 (*cis* + *trans*)	

change in field-grown, severely water-stressed plants during the photoperiod; maximum ABA occurred at 1030h and declined to a minimum at 1500h. Similar, but small fluctuations occurred in irrigated plants.

The significance of the observed changes in leaf ABA remains unknown; only one publication mentions a correlation between ABA levels and plant xylem potential (Kannangara et al., 1982). Circadian or other endogenous rhythms may influence ABA biosynthesis, metabolism or sequestration. These observations all illustrate, however, that any studies involving long term changes in PGS levels, their biosynthesis or degradation, should include both investigation and consideration of diurnal patterns.

2.6 Occurrence and Role of ABA in Roots and Root-nodules

Phloem-ringing experiments by Walton et al. (1976) demonstrated that it was likely that stress-induced synthesis of ABA could occur in the roots of Phaseolus vulgaris seedlings. More conclusive proof that roots can synthesize ABA independently of a shoot system came from a study by Hartung and Abou-Mandour (1980) with Phaseolus coccineus roots grown in culture. These authors also consolidate the literature concerning relative root ABA levels in other species.

A role for ABA has been implicated in several aspects of root physiology; it may control water and ion uptake (Behl and Jeschke, 1979) and play a part in root geotropism (Table 1). In the context of this thesis however, more important is the involvement of ABA on aspects of general root growth. Exogenous ABA has been shown to both suppress lateral root formation in decapitated roots of Pisum sativum (Wightman et al., 1980) and stimulate this process in root cultures of soyabean (Yamaguchi and Street, 1977). Mulkey et al., (1983) demonstrated that 0.1mM ABA will successively stimulate and then inhibit main axis root growth in Zea mays, whilst in intact seedlings of Phaseolus coccineous 10^{-7} - 5 x 10^{-6}M ABA stimulated adventitious root production by 169.5% (Abou-Mandour and Hartung, 1980).

Seasonal patterns of endogenous root free ABA levels correlated well with periods of root growth in Acer saccharum (Cohen et al., 1978) and Philipson and Coutts (1979) have shown that the application of 10^{-4} M ABA to roots of Sitka spruce cuttings from 12-year old plants, grown in culture solution, can both inhibit root elongation

and induce dormancy. These studies implicate a role for ABA in the winter dormancy of roots of mature temperate tree species, which occurs in response to an unfavourable environment, rather than an innate mechanism. In Sitka spruce, applications of exogenous ABA may have a direct effect on root growth since Philipson and Coutts (1979) demonstrated that the photoperiod to which the shoot was exposed did not influence the response of the roots to ABA. However, shoot-applied ABA can lead to an increase in the ratio of roots to shoots, and this may be attributed in part to an increase in root growth (Watts et al., 1981).

Exogenous ABA was found to inhibit root nodulation in Pisum sativum (Phillips, 1971), possibly by inhibiting cortical cell division required for nodule formation. Endogenous ABA has been identified in nodules of Glycine max (Williams and Sicardi de Mallorca, 1982) and probably arises in most plant tissues. Osmotically-induced leaf water-stress (Ψ_{leaf} = -5.5 bars) which resulted in a 4-fold increase in root ABA levels in this species, was shown to both delay nodule initiation and subsequent nodule development (Williams and Sicardi de Mallorca, 1984).

3.0 Alnus Glutinosa (L.) Gaertn.

Young saplings of Alnus glutinosa were used in all of the experiments described in this thesis. The species was chosen (a) in view of the relative paucity of knowledge concerning ABA physiology in tree species and (b) to permit the examination of endogenous ABA levels in perennial root nodules formed in association with an actinomycete.

3.1 Morphology, Habitat and Economic Importance

Alnus glutinosa (L.) Gaertn. (the black alder), a member of the Betulaceae, is a deciduous tree normally attaining heights of 3 - 12m. It is found in most of Europe except the Arctic, Mediterranean and the Russian Steppes, and throughout the British Isles. Pure alderwood or carr is found in the Norfolk Broads, and mixed alder-birch and alder-ash woods are common in the Great Glen area of Scotland. Other habitats include stream and lakesides, and soils with impeded drainage (McVean, 1953).

The leaves are glutinous when young, orbicular in shape, and have tufts of hairs in the angles of the principle veins beneath. Leaf buds are purple with two stipular scales. Male and female catkins are produced in early July; anthesis and stigma extrusion occur in the following year before the leaves appear. By autumn, the female catkins have become hard and woody, and shed some 60 one-seeded fruits, leaving the empty cones to blacken on the tree. There is considerable variation in leaf and catkin size in Britain; generally both diminish from south to north and from east to west (McVean, 1953).

The black alder is one of about 200 species classified in 22 genera and 8 families (Bond, 1983) that form root nodules capable of fixing atmospheric dinitrogen, N_2, in an association with actinomycetes of the genus Frankia. Actinorhizal nodules are perennial structures and have been described as adventitious lateral roots, induced by an appropriate Frankia strain between the normal points of lateral root production (Angulo Carmona, 1974). They can fix nitrogen at nearly the same rate as legume nodules, although as they age, nitrogenase activity declines (Akkermans and Roelofsen, 1980). Nodule biomass in a variety of alder species grown in the field varies from 5.6 to 9.8% of the leaf biomass

(Binkley, 1982). Alder leaf litter contains 2-3% nitrogen which may be easily mineralized (Mikola et al., (1983)). The ability of alder to improve soil fertility was noted as long ago as 1630 (Tarrant and Trappe, 1971), and today, the species finds use in silviculture and land reclaimation and soil amelioration programmes (Tarrant, 1983).

In forestry, the use of plants capable of symbiotic nitrogen fixation has potential advantages over the application of fertilizers, at sites where total nitrogen supplies and soil organic matter content are low, and early in the development of a plant stand. In contrast, the potential for fertilizer-application is greatest when the rate of mineralization is slow, leading to low inorganic nitrogen levels. Maximum benefits are obtained with application to an established stand, with a rapidly-growing canopy, and extensive root development. Various nitrogen-fixing systems which offer potential in forestry are described by De Bell (1979). One such system is the inclusion of nitrogen-fixing plants with the commercial crop. Hansen and Dawson (1982 (cited by Tarrant, 1983)) recorded that the height attained by 3y old Populus saplings was increased by the inclusion of Alnus glutinosa in the stand to produce mixtures which comprised 66% of the nodulated plant. The nitrogen accretion of the plantation was estimated to be 40kg $ha^{-1}y^{-1}$.

Alnus glutinosa has also been used successfully throughout its range to reclaim or improve land in the following situations in which both soil humus and nitrogen content are low:

> Mining spoils, exhausted farmland, degraded forest land, peatland, sandy soil, high water-table (Tarrant and Trappe, 1971).

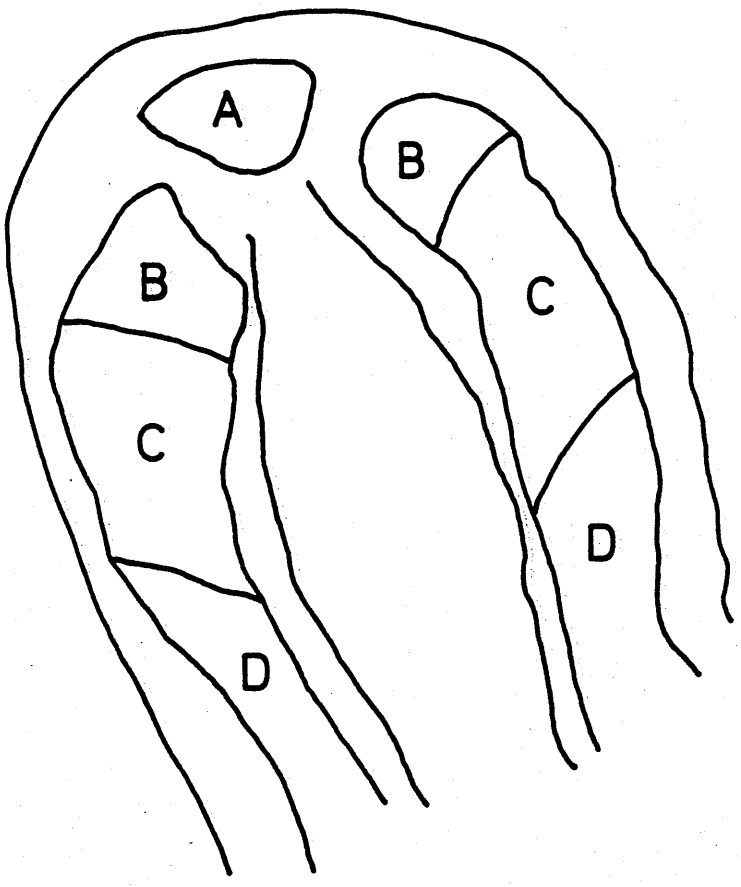
3.2 The Endophyte of Alnus glutinosa Nodules

The initiation of Alnus glutinosa root nodules, which is explained in detail by Lalonde (1977), is similar to that which occurs in other actinorhizal plants such as Comptonia peregrina (L.) (Callaham and Torrey, 1977). Penetration of the endophyte into the root system occurs via the root hairs of the tap-root and laterals, which become deformed. The endophyte at this stage resembles bacterial cells. The root hair is stimulated to produce 'blebs' of polysaccharide material which encapsulate the endophyte and form the exocapsulation thread. As the endophyte

traverses the root hair cell wall it changes to become a free hypha, which is quickly encapsulated by further polysaccharide material originating from the host-plant cell. Electron microscopy has shown that this is enclosed by a membrane which is formed from an invagination of the host-cell plasmalemma. During active growth of the endophyte, the cytoplasm of the living cell is always separated from the actinomycete by a membrane envelope. Multiple hyphal threads are formed by the endophyte, which then spread from the root-hair base into cortical cells, dissolving radial and tangential cell walls. The cortical cells in close proximity to the invading hypae proliferate, forming a structure which has been termed the 'prenodule', the cells of which house the endophyte (Callaham and Torrey, 1977) and which is visible externally as a slight swelling. Primary nodule primordia arise at the same time as the prenodule, initially by pericyclic and endodermal proliferation, and later involving cortical cell division. These structures arise in a manner similar to that of lateral roots in that they are initiated opposite the protoxylem poles, and that development involves pericyclic and endodermal cell proliferation. However, the production of lateral roots does not involve cortical cell divisions, as in the initiation of nodule primordia. The endophyte enters the cortical tissues of each of the developing nodule primordia, and vesicles, which may be 30-50 μm in diameter (Akkermans and Roelofsen, 1980) are formed at the tips of the hyphae. These are the site of nitrogen fixation (Van Staden et al., 1977).

The fully-developed nodule is composed of distinct regions (Figure 2 (from Van Dijk and Merkus, 1976)), associated with different stages of the endophyte life-cycle. Hyphae and vesicles are always present in actively-fixing Alnus glutinosa root nodules (Van Dijk, 1978), but not all Frankia strains are capable of producing spores, a resting stage of the endophyte in vivo (Van Dijk and Merkus, 1976) although all Frankia strains are able to produce sporangia in pure culture. In spore (+) nodules, areas C and D (Figure 2) contain sporangia which develop intracellularly via local transverse growth of thick endophyte hyphae. Nodule homogenates of spore (+) Frankia strains from Alnus glutinosa may be 100-1000 times more infective than spore (-) strains (e.g. Houwers and Akkermans, 1981). It has been demonstrated, however, that the biomass of Alnus seedlings successfully inoculated with

Figure 2. Distribution of distinct endophyte regions in a longitudinal sectioned nodule lobe of Alnus glutinosa (L.) Gaertn. Drawing after a light micrograph. From Van Dijk and Merkus, 1976.



A : Apical meristematic cells without signs of infection
B : Area of young growing cortex cells invaded by thin, curved hyphae. In this region thick hyphae often straight and running from cell to cell are sometimes visible. These thick hyphae show internal division.
C : Area with an abundance of mature vesicle clusters in living host cells.
D : Remnants of vesicle clusters and hyphae abundant.

spore (-) strains was greater after a 3 month period, than those inoculated with the spore (+) type, i.e. that the spore (-) inoculum was more 'effective' in nitrogen fixation (Normand and Lalonde, 1982).

Frankia was first isolated from Comptonia in 1978 (Callaham et al.) and strains from Elaeagnus umbellata, Alnus viridis (Baker et al., 1979) and Alnus rubra (Berry and Torrey, 1979) soon followed. Frankia isolates grown in appropriate (nitrogen-free) culture media demonstrate nitrogenase activity associated with the formation of spherical vesicles (Tjepkema et al., 1980). It has been shown that in vivo, the Frankia strains AVC I1 (isolated from Alnus viridis spp. crispa) and CpI1 (isolated from Comptonia peregrina) are unable to grow on media containing glucose as the sole carbon source (Blom and Harkink, 1981) and cannot complete glycolysis (Huss-Danell et al., 1982). This contrasts with an Alnus glutinosa isolate (LDAgp1) which is able to utilize mono and disaccharides, and shows some growth on arabinose and starch (Shipton and Burggraaf, 1982). Additionally, this Frankia strain did not grow on tricarboxylic acids, although the others, AVC I1 and CpI1 were able to use these carbon sources. This was attributed to the absence of a suitable uptake mechanism in AgI1 rather than the absence of tricarboxylic acid enzymes. All the isolates, however, were shown by these authors to utilize volatile fatty acids or their salts, such as propionic acid and acetate. It has been suggested that the sucrose delivered to the nodule via the phloem is partially metabolized to fatty acids by the host plant, permitting the synthesis of acetyl CoA and hence the generation of energy in the citric acid cycle by the endophyte (Blom and Harkink, 1981).

3.3 Nitrogen Fixation in Actinorhizal Nodules

The known properties of nitrogenase, the enzyme responsible for nitrogen-fixation, isolated from a diverse range of organisms (free-living anaerobic bacteria, the legume symbionts, micro-aerophiles, facultative and strict aerobes, photosynthetic bacteria, blue-green algae and non-legume symbionts) show many similarities (e.g. Lowe et al., 1980). Of importance to work on nitrogenase, was the discovery that the two protein subunits of which it is composed are oxygen-labile, one more so than the other (Kelly, 1969).

One of the proteins is a non-haem iron protein with iron-sulphur clusters (the Fe protein) whilst the other contains iron-sulphur clusters and an iron-molybdenum co-factor (the MoFe protein) (Dixon and Wheeler, 1983). The Fe protein is reduced by an electron donor, and then binds ATP and Mg. The MoFe protein combines with the substrate, and is then reduced by the MgATPFe protein complex, with the hydrolysis of ATP. The substrate is reduced in turn by the transfer of electrons from the MoFe protein. Generally, one electron is transferred per 2 ATP hydrolyzed.

Nitrogenase will reduce a number of substrates (Sprent, 1979) besides the two natural ones, protons and nitrogen, including acetylene, C_2H_2. Carbon monoxide inhibits nitrogen reduction, but again, proton reduction remains unaffected (Dixon and Wheeler, 1983). Both the reduction of protons (to H_2) and nitrogen (to NH_3) occurs by nitrogenase in the absence of inhibitors. Under atmospheric nitrogen, the reaction can be summarized thus:-

$$N_2 + 8e + 8H^+ \longrightarrow 2NH_3 + H_2$$

The ammonium ion, NH_4^+ occurs in reactions at physiological pH values (Sprent, 1979).

The reduction of acetylene to ethylene by nitrogenase has provided a cheap, simple method for assaying the enzyme activity in intact and excised nodules (Hardy et al., 1968), although the technique is not without problems when absolute measurements of nitrogen-fixation are required (Silvester, 1983). A concentration of 10% acetylene inhibits both nitrogen reduction and proton reduction, and thus ethylene produced represents the total electron flux through nitrogenase (Dixon and Wheeler, 1983). However, the theoretical 3:1 ratio of acetylene to nitrogen reduced, which can be deduced from the relationships:-

$$3C_2H_2 + 6H^+ + 6e \longrightarrow 3C_2H_4$$

$$N_2 + 6H^+ + 6e \longrightarrow 2NH_3$$

is seldom realized. Firstly, proton reduction normally occurs alongside nitrogen reduction, reducing the electrons available for nitrogen reduction. (In the presence of C_2H_2, all electron

flow is used in C_2H_2 - reduction). Secondly, hydrogen-uptake may occur in some symbionts, and may vary seasonally (Silvester , 1983). Additionally, in many legume nodules, nitrogenase activity may decline after a few minutes of exposure to acetylene, with a concurrent decrease in respiration rate (Minchin et al., 1983). The effect was attributed to the cessation of ammonia production. These authors (who used a continuous flow system to make a kinetic study of nitrogenase activity) determined that the maximum rate of acetylene reduction, which was observed for only 2 minutes in white clover roots, most nearly represented pre-assay rates of nitrogenase activity as determined by $^{15}N_2$-uptake. It is likely therefore, that measurements of nitrogenase rates based on cumulative ethylene production may underestimate actual rates of nitrogen fixation. Even assays made for comparative purposes using closed vessels, may give misleading results if the rate of decline of ethylene production varies between assay measurements. There remain, unfortunately, other situations which can lead to errors in the estimation of nitrogenase activity, some of which are specific to the C_2H_2-reduction assay, others to any assay made in an enclosed space; an account of these may be found in Silvester, 1983.

Ammonium formed by nitrogen-reduction is both toxic and will inhibit further nitrogen fixation, and must be quickly metabolized. Blom et al. (1981) have shown that vesicle cluster suspensions from root nodules of Alnus glutinosa show no ammonium-assimilating enzymes, and the process is thought to occur in the cytoplasm of the host cell. Three enzymes are generally associated with ammonium-assimilation in nitrogen-fixing symbioses; glutamate dehydrogenase (GDH), glutamine synthetase (GS) and glutamine-2-oxoglutarate-amino-transferase (GOGAT). The reactions catalyzed by these enzymes may be summarized as follows (Dixon and Wheeler, 1983):

$$\text{Glutamate} + NH_4^+ + \text{ATP} \xrightarrow[\text{GS}]{} \text{Glutamine} + \text{ADP} + \text{Pi} + H^+$$

$$\text{2-oxo-glutarate} + \text{glutamine} + \text{NAD(P)H} + H^+ \xrightarrow[\text{GOGAT}]{} 2\ \text{glutamate} + \text{NAD(P)} + H_2O$$

$$\text{2-oxoglutarate} + NH_4^+ + \text{NAD(P)H} \xrightarrow[\text{GDH}]{} \text{glutamate} + \text{NAD(P)} + H_2O$$

GS and GOGAT act sequentially to produce glutamate; the advantage of the two-step process is that GS has a much greater affinity for NH_4^+ than GDH, permitting rapid assimilation. GDH is possibly used in ammonium assimilation when carbon is limited (Dixon and Wheeler, 1983). In Alnus glutinosa nodule-homogenates, the cytoplasm of the host cells was shown to possess GS activity, whilst GDH activity was associated with cytoplasmic organelles (Blom et al., 1981). These authors were unable to detect any GOGAT activity at all, which may have been due to the presence of inhibitory compounds. The precise mechanism of ammonium assimilation in alder nodules requires further work (Akkermans et al., 1983).

To reduce the amount of carbon lost to the nodule per unit nitrogen transported, compounds with lower C:N ratios than glutamine (C:N ratio of 5:2) may be synthesised for translocation. In alder nodule tissue, the principle amino acid is citrulline (1.17μmol g f.wt^{-1}) whilst citrulline (C:N ratio of 2:1) and glutamine are prominent in the bleeding sap (4.04μmol g f.wt^{-1} and 1.5μmol g f.wt^{-1} respectively) (Dixon and Wheeler, 1983). The pathway of citrulline synthesis is described by Dixon and Wheeler, 1983; the last step is catalyzed by the enzyme ornithine carbamoyl transferase (EC 2.1.3.3). This enzyme has been located cytochemically in the mitochondria of the host cell cytoplasm (Scott et al., 1981).

Any environmental factors that influence the growth of the host plant will affect nitrogen fixation (such as moisture, temperature, light, root-pH, and nutrient supplies) (Wheeler and McLaughlin, 1979). Many plants bearing perennial actinorhizal nodules are deciduous; in these species, nodules exhibit seasonal patterns of nitrogen-fixation corresponding to growth periods of the host plant, which have been ascribed to death of the vesicle-containing cells and endophyte in autumn, and the formation of new tissues in spring (Schwintzer et al., 1982). In field-grown Alnus glutinosa in Scotland, nitrogenase activity declines in September when internode extension ceases, is not detected over the winter dormant period following leaf fall, and recommences in spring (Wheeler and McLaughlin, 1979). The initiation of nitrogenase activity occurs prior to bud-burst, leading to suggestions that it may be triggered by the removal of an inhibitor from the nodules

to the stem. Nitrogen-fixation recommenced in 2y old plants that were decapitated or bark-ringed when close to breaking dormancy in February, but when plants were similarly treated in December, and then transferred to long days (dormancy-breaking), no nitrogenase actvity was detected (Wheeler and McLaughlin, 1979). Length of photoperiod is a major factor influencing seasonal variations in nitrogenase activity in this species, although the partitioning of photosynthetic assimilates throughout the period in which the plant is foliated may also influence nitrogen-fixation. Pizelle (1984), demonstrated that in mature trees of Alnus glutinosa, seasonal changes in nodule-homogenate nitrogenase activity (in vitro activity) fluctuated less than the in vivo nitrogen-fixation measured on nodular lobes. This, and a number of other observations, led the author to suggest that growth of the female reproductive structures in early summer was responsible for reducing the photosynthate supply to the nodules, and hence for the observed decline in nitrogenase activity at this period.

Diurnal fluctuations in nitrogen-fixation in Alnus glutinosa have also been observed both in the field (McNiel and Carpenter, 1979) and in the greenhouse (Wheeler, 1969, 1971). Maximal nitrogen-fixation in one year old plants maintained at near constant temperature but natural illumination, occurred at midday (Wheeler, 1969). The peak in nitrogenase activity corresponded with a maximum influx of photoassimilates into the nodules, although this was not reflected by a change in total carbohydrate levels (Wheeler, 1969, 1971). However, older (2 - 3y) plants did not show similar daily fluctuations when grown under similar conditions (Wheeler and Lawrie, 1976). It has been suggested that soil moisture content may be an important factor in influencing diurnal changes in acetylene reduction: changes in temperature and solar radiation may have the most significant effects under conditions of ample soil moisture (Wheeler, 1969).

4.0 Aims and Rationale

There are few studies concerning endogenous ABA levels in whole plants of tree species. Additionally, little is known about any PGS in perennial actinorhizal nodules. The work described in this thesis, therefore, has aimed to produce a careful study of fluctuations in the ABA levels of *Alnus glutinosa* with special attention to the root nodules.

In the light of the current debate concerning the mode of action of PGS, it was deemed essential to develop a suitable technique for the identification and accurate quantification of ABA in woody root nodules and leaves. From this foundation, fluctuations in the endogenous ABA content of root nodules were examined at intervals throughout the year, and correlated with nitrogen-fixing activity. The possibility of a diurnal pattern in nodule and leaf endogenous ABA levels was also investigated, in controlled conditions and in the greenhouse. Additionally, the effect of root-applied ABA on the morphology, growth and endogenous ABA levels of plants grown in water-culture under long days was determined.

The movement and metabolism of leaf-applied [2-^{14}C] ABA was followed in plants grown under long and short-day conditions, to elucidate the capacity of the leaves to transport ABA, and hence their possible contribution to the endogenous ABA pools of other plant parts. Finally, the sink areas for root-applied ABA were determined by immersing the roots of young plants in a culture solution containing [2-^{14}C] ABA.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. Plant Material

All experiments were carried out with young saplings of *Alnus glutinosa* (L.) Gaertn., the black alder.

1.1 Seed Sources

Woody cones bearing the one-seeded fruits were collected annually in mid-October from Milngavie (Ordnance Survey reference NS565746) and Balmaha (Ordnance Survey reference NS416911) in south-west Scotland, and Horsham (at Ordnance Survey reference TQ175305) in south England. The cones were spread out to dry on trays in the laboratory, and the fruits liberated following this treatment were collected and carefully sorted from any debris (mostly cone bract-scales and twigs) by sieving and by hand. Fruits greater than 2mm in diameter were retained and stored in small plastic bags in the dark at 4°C until required.

1.2 Plant Cultivation

Fruits were germinated, and all plants initially grown in a greenhouse bay that was heated in winter and well-ventilated and shaded in summer, leading to temperatures within the range 10-25°C. Supplementary lighting between September and April was provided for 16h daily by Atlas Kolorlux 400W MBFR/U high-pressure mercury vapour lamps, 0.8m apart, 1m above bench height. The irradiance from these lamps in the wave-band 400-1000nm varied from 5-10Wm^{-2} at pot height (Weyers, 1978). Plants were usually later transferred to various growing conditions described in 1.3.

Fruits were sown in 21.5 x 35.5cm plastic seed trays containing horticultural perlite (Tilcon, Tilling Construction Limited) that was moistened with half-strength Crone's nutrient solution (pH 5 , nitrogen-free formula, Wheeler, 1969). Three-week old seedlings were gently uprooted and inoculated with the endophyte by dipping the root-stock into an aqueous suspension of ground nodules (1g f.wt. of nodules in 100cm^3 distilled water) removed from stock plants 3-4y in age. Seedlings were then usually transplanted into plastic pots 7.5cm or 10cm in diameter containing either perlite (for material used in seasonal analyses of ABA and nitrogenase activity) or a 3:1 (by volume) perlite-sand mix (for

material used in diurnal analysis of ABA and [2-14C] ABA transport experiments), and occasionally irrigated with half-strength Crone's solution. All plants were watered with tap water when required. When large plants (3-6 months old) were required in water culture, seedlings were transplanted initially into perlite following inoculation, and later transferred to 2dm^3 porcelain culture vessels containing full strength Crone's solution (pH 5.0), once the nodules were established (2-3 weeks). Plants (3 per pot) were secured with cotton-wool and a rubber circlet, in holes cut through a wooden support that rested across the culture jar. Smaller plants required in water culture were germinated and inoculated as before, and then transferred to 25cm^3 capacity McCartney bottles (1 per bottle) covered with black polythene to exclude the light. In this case, plants were placed in a hole cut in a polystyrene disc which rested on the top of the bottle.

1.3 Growing Conditions

In addition to the heated greenhouse bay described in 1.2, two walk-in growth rooms were available for use. Growth room A contained two benches 0.68 or 1.00m above which was a bank of 5 fluorescent strip lights (Omega 'Warmlite' 65-80W).

Relative humidity was between 70-100% during the light period, and was approaching 100% in the dark period, whilst the temperature ranged between 19-21°C. Growth room B contained two benches 1.2m above each of which were 3 Thorn 'Kolorarc' 400W lamps. PAR at plant height was 0.925 mol $m^{-2}h^{-1}$. Relative humidity was between 55-70% in the light, and was nearly 100% in the dark, whilst the temperature ranged between 15-20°C.

Seasonal analyses of root and nodule ABA levels were made in 1980/1981 with plants 3-4y old that had been grown in an unheated greenhouse under natural photoperiods since transfer from the heated greenhouse at an age of 2-3 months. Nitrogenase assays were carried out with plants 2-3y old during the same season.

In an assessment of population variability in leaf and nodule ABA levels, plants 6 months old and grown as described in 1.2 in the heated greenhouse, were harvested.

Plants 5-7 months old were used in diurnal analyses of leaf and nodule endogenous ABA levels. The experiment was conducted initially in May 1982 with plants grown in the heated greenhouse (and hence under natural illumination at this time). It was later repeated with plants transferred to growth room B from the greenhouse when 5 months old (one week prior to the experiment) and then with plants transferred to growth room A when 2 months old (3-4 months prior to the experiment). Both growth rooms were maintained under 16h photoperiods (from 0700-2300 daily) prior to and during the experiment.

[2-^{14}C] ABA transport experiments (leaf application) were conducted with plants 3-4 months old and maintained in growth room A since transfer from the heated greenhouse at an age of 2 months. Initial experiments required a 16h light period; this was later reduced to 8h in experiments which aimed to examine changing [2-^{14}C] ABA translocation patterns with photoperiod length. [2-^{14}C] ABA transport experiments (root application) were conducted in growth room A under a 16h photoperiod, with plants two months old and growing in water culture.

Plants 5 months old and grown in water culture were kept in growth room A under long days (16h photoperiod) prior to and throughout the course of an experiment designed to determine the effects of exogenous ABA (applied via the culture solution) on plant growth, morphology and tissue ABA levels.

2. Chemicals

2.1 Gases

Gases were supplied by B.O.C., Glasgow, U.K. (acetylene, air, helium, hydrogen, oxygen, oxygen-free nitrogen) and B.O.C. Special Gases, London, U.K. (52 vpm ethylene standard).

2.2 Organic Solvents

Methanol, methylene chloride, ethyl acetate and diethyl ether which were supplied in drums were redistilled as needed. Sulphur-free toluene which was required for scintillation cocktails was used directly from the drum. n-Hexane, propan-1-ol and butan-1-ol ('analar' grade) which were purchased from B.D.H. Chemicals Limited, Poole, England, U.K., were used as supplied.

2.3 General Chemicals and ABA

Water was glass-redistilled once, and stored in polyethylene containers. General reagents were purchased from BDH Chemicals Co. Limited, London, U.K. Silica and C18 SEP-PAK cartridges (containing silica and an octadecyl silane bonded phase packing respectively) which were used in the purification of plant extracts for ABA, were supplied by Waters Associates, Chester Road, Hartford, Northwich, Cheshire.

2.4 Radiochemicals

Synthetic (±) [2-^{14}C] ABA (99% radiochemical purity) and (±) [G-^{3}H] ABA (97% radiochemical purity) were supplied in methanol or ethanol by Amersham International, Amersham, Buckinghamshire, England and stored at 4°C. Several batches were obtained over the period of this study, with the specific activities given below:

[2-^{14}C] ABA	Batch 1) Batch 2)	3.57 MBq mg^{-1}
	Batch 3	3.59 MBq mg^{-1}
[G-^{3}H] ABA	Batch 1	4.63 GBq mg^{-1}

The purity of the ABA was tested on several occasions by TLC; over 90% of the radioactivity always ran in a single band which was assumed to represent labelled ABA.

^{14}C hexadecane (specific activity 14.4 kBq cm^{-3} and ^{3}H hexadecane (specific activity 39.6 kBq cm^{-3}) which also came from the Radiochemical Centre, Amersham, were used in the construction of calibration quench equations for the liquid scintillation counters.

3. Endogenous ABA

3.1 Harvest and Extraction of Plant Material

Material was harvested for experiments as outlined in Table 3. Plants were shaken free of the rooting medium and the root-system was carefully washed under running water and then blotted dry on tissue paper. (Throughout this project, the tissues below which the first lateral roots emerged from the stem have been termed the 'root system'.) Nodules were excised with forceps, and leaves removed with a razor blade with the petiole attached. When material was not homogenized directly after harvest, it was stored in small aliquots at -20°C for not longer than 48h prior to maceration.

One of three different homogenization procedures (Table 4) was selected based on sample size to thoroughly macerate the samples. A volume of methanol sufficient to cover the material in the homogenization vessel and in any case not more than $10cm^3$ per gf. wt. of material was used. Samples were then labelled with [2-^{14}C] ABA. The amount of radiolabel added was never less than 58.3 Bq, although absolute values were dependent on the endogenous ABA expected in the extract; it was always intended that amounts of the tracer should represent less than 40% of the endogenous ABA content. This was realised in all extracts except one (Table 5); generally levels of [2-^{14}C] ABA added were much less than 40%.

Material was extracted in $10cm^3$ methanol per gf. wt. at 40°C, with two solvent replacements (same volume) at 24h intervals or longer. The extract was bulked, filtered and usually split into two equal volumes which were separately treated for the quantification of 'free' and 'total' extract ABA levels.

3.2 The Purification of Extracts for 'Free' ABA

Extracts were reduced in vacuo at 35°C and redissolved in 100 mol m^{-3} pH8.0 phosphate buffer prepared with 15.6 g of sodium dihydrogen phosphate dihydrate dissolved in $1000cm^3$ distilled water, and brought to the required pH value with NaOH.
A volume of $2cm^3$ g^{-1}f.wt. material was used for nodule and root tissues, and $10cm^3$ g^{-1} f.wt. material for leaf tissue, although the following were minimum volumes required to remove the extract

Table 3. Details of plant material harvested for given ABA analyses.

Experiment	Time/Date of Sampling	Age of Plants	Growth Conditions	Number of Plants Harvested	State of Plants	Number of Replicate Batches Harvested	f.wt. Material per Extract (g)			
							Leaves	Roots	Nodules	Stem Apices
Seasonal analyses	19/01/80	3-4y	Unheated greenhouse	10-50	Dormant	1	-	-	46.05	-
	2/03/81	3-4y	Unheated greenhouse	10-50	Dormant	1	-	11.13	39.27	
	23/03/81	3-4y	Unheated greenhouse	10-50	Budburst	1	-	-	33.52	
	21/05/81	3-4y	Unheated greenhouse	10-50	Active growth	1	-	35.83	24.62	
	18/12/81	3-4y	Unheated greenhouse	10-50	Dormant	1	-	14.16	9.15	
	6/09/82	3-4y	Unheated greenhouse	10-50	Entering dormancy	1	-	20.95	5.64	
	8/01/83	3-4y	Unheated greenhouse	16	Dormant	2(nodules) 4(roots)	-	15.00	3.02-3.92	

Seasonal analyses	17/03/83	3-4y	Unheated greenhouse	16	Prior to budburst	2(nodules) 4(roots)	-	15.00	1.98-2.82	-
Comparison of techniques for the quantification of 'bound' ABA	28/10/83 4pm	5 months	Heated greenhouse	10	Active growth	1 per experiment	-	14.00-16.00	1.00-1.30	-
Assessment of population variation in ABA content	10/12/82 4pm	7 months	Heated greenhouse	20	Active growth	5	3.00	10.00	1.33-2.32	-
Diurnal analyses of ABA	Experiment 1*	5 months	Heated greenhouse	16	Active growth	1	6.69-11.04	-	1.13-1.87	-
	Experiment 2**	7 months	Growth Room B	16	Active growth	1	10.00	-	1.57-2.11	-
	Experiment 3***	4 months	Growth Room A	13	Active growth	1	5.46-10.74	-	0.58-1.38	-

* Experiment 1 : 9/5/82-10/5/82 (Nodules harvested 12am, 4pm, 8pm, 4am, 8am; leaves harvested 11am,3pm,7pm,11pm,3am,11am.)

** Experiment 2 : 7/4/84-8/4/84 (Nodules and leaves harvested 7am, 11am, 2pm, 5.30pm,10pm, 2am.)

*** Experiment 3 : 4/3/84-5/3/84 (Nodules and leaves harvested 12am, 6pm, 12pm, 6am, 12am.)

Effect of $0.1 mol\ m^{-3}$ ABA (root application) on endogenous ABA	20/03/83 2pm	5 months	Growth Room B	10	Dormancy buds present	1 per treatment	30.00	27.33	4.40-7.90	3.47-3.09
	18/03/83 2pm	9 months	Growth Room A	6	Dormancy buds present	1 per treatment	14.96-37.53	7.55-22.15	2.42-5.33	1.25-1.27

Table 4. Homogenisation techniques used for given tissue types.

MATERIAL	SAMPLE SIZE	HOMOGENIZATION PROCEDURE
Nodules	$\geqslant$ 20g	'Virtis' 2 x 30s top speed
	< 20g	Pestle and mortar
Apices	< 5g	Pestle and mortar
Roots	$\geqslant$ 10g	Wareing blender 2 x 30s top speed
	< 10g	'Virtis' 2 x 30s top speed
Leaves	$\geqslant$ 10g	Wareing blender 2 x 30s top speed
	< 10g	'Virtis' 2 x 30s top speed

Table 5. Range of ratios of endogenous ABA per extract (ng) to [2-^{14}C] ABA internal standard added for given experiments.

$$\frac{\text{Endogenous } {}^{12}C \text{ ABA in extract (ng)}}{[2\text{-}{}^{14}C] \text{ internal standard added prior-to purification (ng)}}$$

EXPERIMENT	LEAVES		APICES		ROOTS			NODULES		
	'Free'	'Total'	'Free'	'Total'	'Free'	'Total'	'Bound'	'Free'	'Total'	'Bound'
Seasonal analyses of ABA 1980 - 1983	-	-	-	-	5.9-43.1	54.4-227.5	-	3.4-287.2	91.7-138.8	-
Comparison of techniques for 'bound' ABA analysis	-	-	-	-	16.6-21.8	32.3	2.5	3.1-8.4	17.9	12.8
Assessment of population variation in ABA content	18.1-68.1	20.7-31.7	-	-	7.4-11.0	27.5-28.5	-	0.8-4.7	12.8-17.6	-
Diurnal analyses of ABA content	39.5-528.4	103.9-1,212.6	-	-	-	-	-	2.6-25.1	23.1-156.3	-
Effect of 0.1 mol m^{-3} ABA (root application on endogenous ABA)	81.0-7,340.8	117.8-46,150.1	181.3-1,775.8	369.5-23,923.8	82.2-17,541.0	181.4-212,528.1	-	38.5-2,121.7	97.9-19,100.6	-

adequately from the rotary evaporator vessel: 30cm^3 (nodules), 60cm^3 (roots and leaves). Each extract was then slurried with non-water-soluble polyvinylpolypyrrolidone (PVP: Sigma Chemicals, Poole, Dorset, England), which had been pre-washed sequentially in distilled water and twice in 100 mol m^{-3} pH 8.0 phosphate buffer, and then filtered through a Buchner filter. Amounts of PVP used were generally 1g g^{-1} f.wt. material for leaf and nodule extracts, and 0.5g g^{-1} f.wt. material for root extracts, although 5g was the minimum used. After 20 minutes, the slurry was filtered and the PVP was washed with a further volume of phosphate buffer for ten minutes. This was then filtered, and the combined filtrates were adjusted to pH 3.0 with 3 mmol cm^{-3} HCl. The aqueous extract was then passed through a 5mm thick filter pad (prepared by filtering a slurry of paper floc and distilled water) and then applied to one or more C18 SEP-PAK cartidges (Waters Associates, Northwich, Cheshire, England). Cartridges were pre-washed with 2cm^3 methanol followed by 5cm^3 distilled water. Extracts were applied with a Luer-lock glass syringe in 10cm^3 aliquots at a rate of not more than 0.167cm^3 s^{-1}. Between 50-70cm^3 of extracts was applied to each cartridge, which was then eluted with three successive 10cm^3 aliquots of 15% methanol in 100 mol m^{-3} pH8 phosphate buffer (i.e. 15 parts of methanol to 85 parts of buffer). Methanol was removed *in vacuo* at 35°C and the aqueous phase was then adjusted to pH 3.0 and partitioned three times against equal volumes of diethyl ether. The ether fraction was retained and the solvent removed *in vacuo* at 35°C. The flask was then fully dried in a desiccator over silica gel.

Small root and nodule samples (<10g f.wt.) and all leaf samples were transferred with methanol to 2cm^3- capacity capped glass vials, dried under nitrogen, and stored at -20°C. Large root and nodule samples (>10g) were redissolved in 1cm^3 methylene chloride, further purified using a single silica SEP-PAK cartridge as described by Hubick and Reid (1980), transferred with methanol to 2cm^3- capacity vials, and dried and stored as above.

All samples were methylated not more than 2 weeks prior to GC analysis with 1-2cm^3 of diazomethane in ether, prepared according to the method of Schlenk and Gellerman (1960). After the addition of the diazomethane, the vials were loosely capped and left for 1h at room temperature in a fume cupboard. If at

the end of this period they retained a yellow colour, they were dried under nitrogen and stored at -20°C. Extracts which had become colourless were dried and remethylated.

Leaf samples were further purified by TLC after methylation, using 20 x 20cm glass plates pre-coated with 250 μm silica gel GF 254 (Camlab) and pre-run in methanol. Chromatograms were developed over 15cm in ethyl acetate and hexane (1:1 v|v). The Rf band corresponding to a methylated ABA standard (which was identified under UV light) was scraped off and eluted with 10cm^3 methanol. The eluate was reduced in vacuo at 35°C, transferred to a small vial, and dried under nitrogen.

Prior to analysis by gas-chromatography, extracts were redissolved in 100-200mm^3 methanol (the absolute volume was dependent on tissue and sample size), and aliquots were removed for scintillation counting to assess the percentage recovery of [2-^{14}C] ABA.

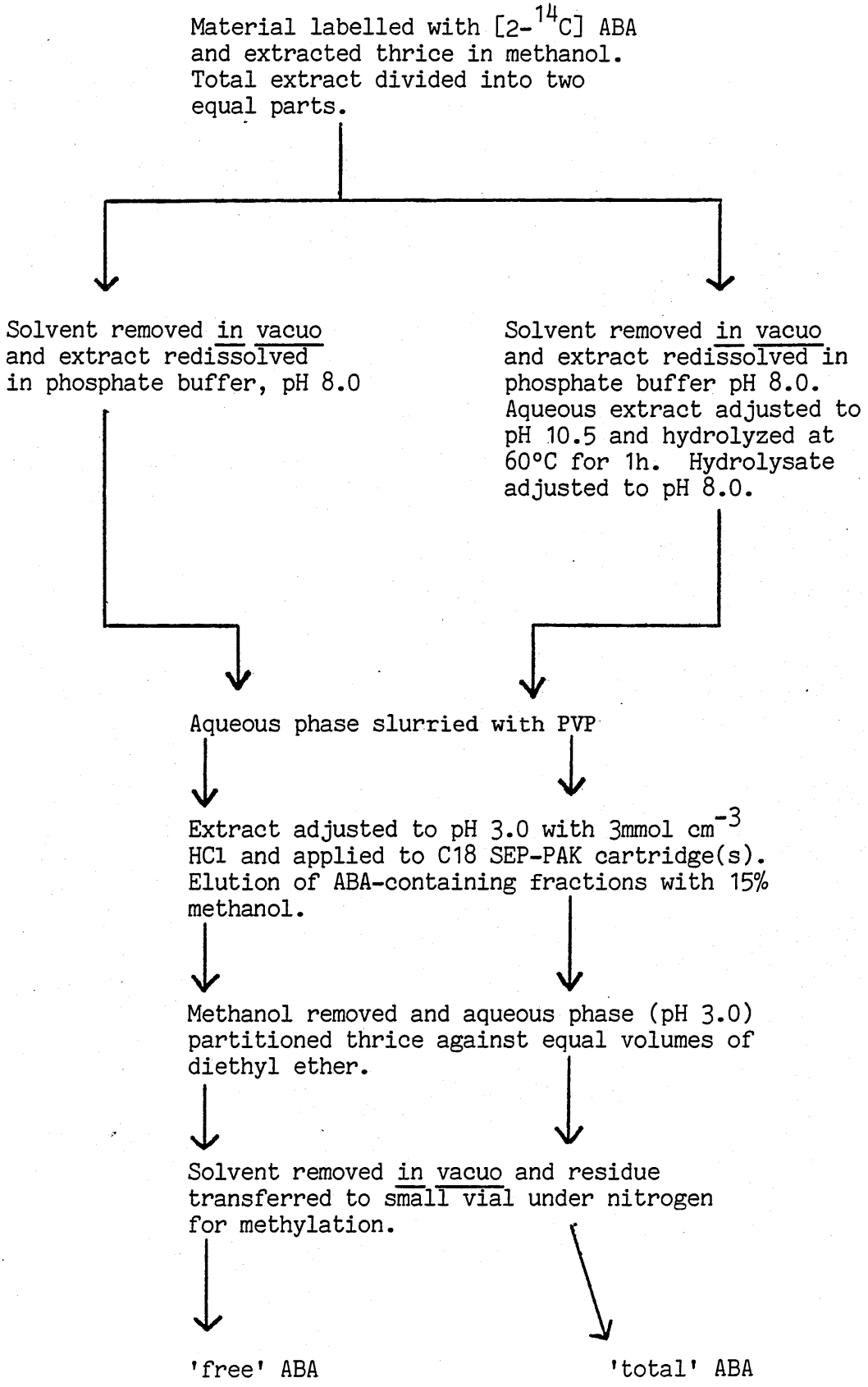
3.3 The Purification of Extracts for 'Total' ABA

Methanol was removed in vacuo at 35°C and the residue was redissolved in 100mol m^{-3} pH 8.0 phosphate buffer as before. Extracts were then adjusted to pH 10.5 with dilute NaOH and placed for 1h in a water bath maintained at 60°C. After this hydrolysis treatment, extracts were cooled rapidly to room temperature, adjusted to pH 8.0 with 3mmol cm^{-3} HCl and then slurried with PVP. The remainder of the purification procedure was identical with the analysis of extracts for 'free' ABA. All extracts hydrolyzed in this manner, however, were purified by TLC. The purification procedure normally used to quantify 'free' and 'total' ABA levels is shown in Figure 3 ..

3.4 The Purification of 'Free' and 'Bound' ABA Directly From A Single Extract

The 'bound' ABA content of extracts was usually determined indirectly by subtracting 'free' ABA levels from 'total' ABA values which were separately determined from two halves of one extract. Nevertheless, it is possible to process a single extract for 'free' and 'bound' ABA levels directly, and a comparison was made between the two techniques.

Figure 3. The purification procedure normally used to quantify 'free' and 'total' ABA levels.


Material labelled with [2-14C] ABA and extracted thrice in methanol. Total extract divided into two equal parts.
Solvent removed in vacuo and extract redissolved in phosphate buffer, pH 8.0
Solvent removed in vacuo and extract redissolved in phosphate buffer pH 8.0. Aqueous extract adjusted to pH 10.5 and hydrolyzed at 60°C for 1h. Hydrolysate adjusted to pH 8.0.
Aqueous phase slurried with PVP
Extract adjusted to pH 3.0 with 3mmol cm-3 HCl and applied to C18 SEP-PAK cartridge(s). Elution of ABA-containing fractions with 15% methanol.
Methanol removed and aqueous phase (pH 3.0) partitioned thrice against equal volumes of diethyl ether.
Solvent removed in vacuo and residue transferred to small vial under nitrogen for methylation.
'free' ABA
'total' ABA

To purify 'free' and 'bound' ABA levels directly, material was first labelled with [2-^{14}C] ABA, extracted in methanol, dried *in vacuo* at 35°C and redissolved in pH 8.0 phosphate buffer exactly as before. It was next adjusted to pH 3.0 and shaken three times against twice the volume of methylene chloride. This partitioning step theoretically separated the 'free' and 'bound' ABA pools; 'free' ABA was retained in the organic phase, whilst the aqueous phase contained 'bound' ABA. The aqueous fraction was labelled with [2-^{14}C] ABA, adjusted to pH 10.5 with dilute NaOH solution, and hydrolyzed for 1h at 60°C. It was then rapidly cooled to room temperature, adjusted to pH 8.0 with 3mmol cm^{-3} HCl and slurried with PVP.

The methylene chloride fraction was dried, redissolved in 100mol m^{-3} pH 8.0 phosphate buffer and slurried with PVP. The two extracts were then processed (separately) exactly as before (i.e. filtration; C18 SEP-PAK cartridge; ether partitioning; methylation; TLC).

3.5 The Analysis of Extracts by Electron-capture Detector Gas Chromatography (GCECD)

Methylated samples were analyzed using a Perkin Elmer Sigma 3 gas chromatograph (GC) fitted with a ^{63}Ni (367-500 MBq) electron-capture detector (ECD) and flame-ionization detector (FID). Extracts were dissolved in methanol to a volume which gave satisfactory GCECD analysis. Samples (which were usually 0.5mm^3 in size) (0.1mm^3 methanol plus 0.4mm^3 sample) were injected into the machine with an SGE 1mm^3 capacity microsyringe (needle length 7cm) and separated on either a glass open-tubular capillary column, support-coated with SP 2250 (SCOT capillary) or a flexible-fused silica (FFS) column wall - coated with methyl silicone fluid (WCOT capillary). Samples were carried directly into the SCOT capillary, but the WCOT FFS columns required a split injection to prevent column overloading. Both the split ratio and the carrier flow (which were also interrelated) had an impact on the resolution and retention of ABAMe by the column. During the period in which the column was re-conditioned prior to sample analyses, these parameters and the oven temperature programme were altered to give a satisfactory chromatogram for ABAMe standards.

The column carrier gas was hydrogen, helium or nitrogen, whilst nitrogen was used throughout all analyses to 'make-up' or supplement the gas flow to the ECD (to 1cm^3 s^{-1}). On several occasions, simultaneous analysis of samples by both ECD and FID detectors was possible, when two columns of identical length were connected in parallel to the injector. The FID was always operated at gas pressures of 124kPa (hydrogen) and 221 kPa (air) and at a detector flow-rate of 1cm^3 s^{-1}. The oven temperature was usually held constant at between 175°C and 220°C to permit isothermal elution of ABAMe , and then rapidly increased to the temperature limit of the column to remove any remaining sample. Occasionally, it was more satisfactory to programme a slow temperature increase from the start of the run. The detector and injector blocks were maintained at a constant temperature ('zone' temperature) which was between 275°C and 350°C. The Servoscribe chart-recorder was always operated at 1mV. The ECD output was maintained at amplification range 4 (least sensitive) and attenuated as required (settings from X1 to X1024 were available). More specific details of operating conditions can be found in Table 6 .

3.6 Quantification of Extract ABA Levels

Calibration curves relating the response of the ECD (peak height) to ABAMe standards analyzed using the same temperature programme, gas flows, injection volume (and syringe) and amplification range as sample analyses, were constructed on a daily basis. Methylated standards in methanol were prepared from synthetic (±) ABA (containing both *cis* and *trans* isomers) or (±) ABA (*cis* isomer only) purchased from Sigma Chemicals. The detector response was linear over the range used, and a line was fitted by eye to the calibration plot, to ease subsequent calculations. An example is shown in Figure 4. Plant samples were run at least twice; the standard error of the mean peak height recorded was never greater than ± 10%.

3.7 Combined Gas-chromatography/Mass Spectrometry (GC-MS) of Extracts

Single-ion monitoring (SIM : m/z 190) was carried out for leaf, root and nodule samples, and multiple-ion monitoring

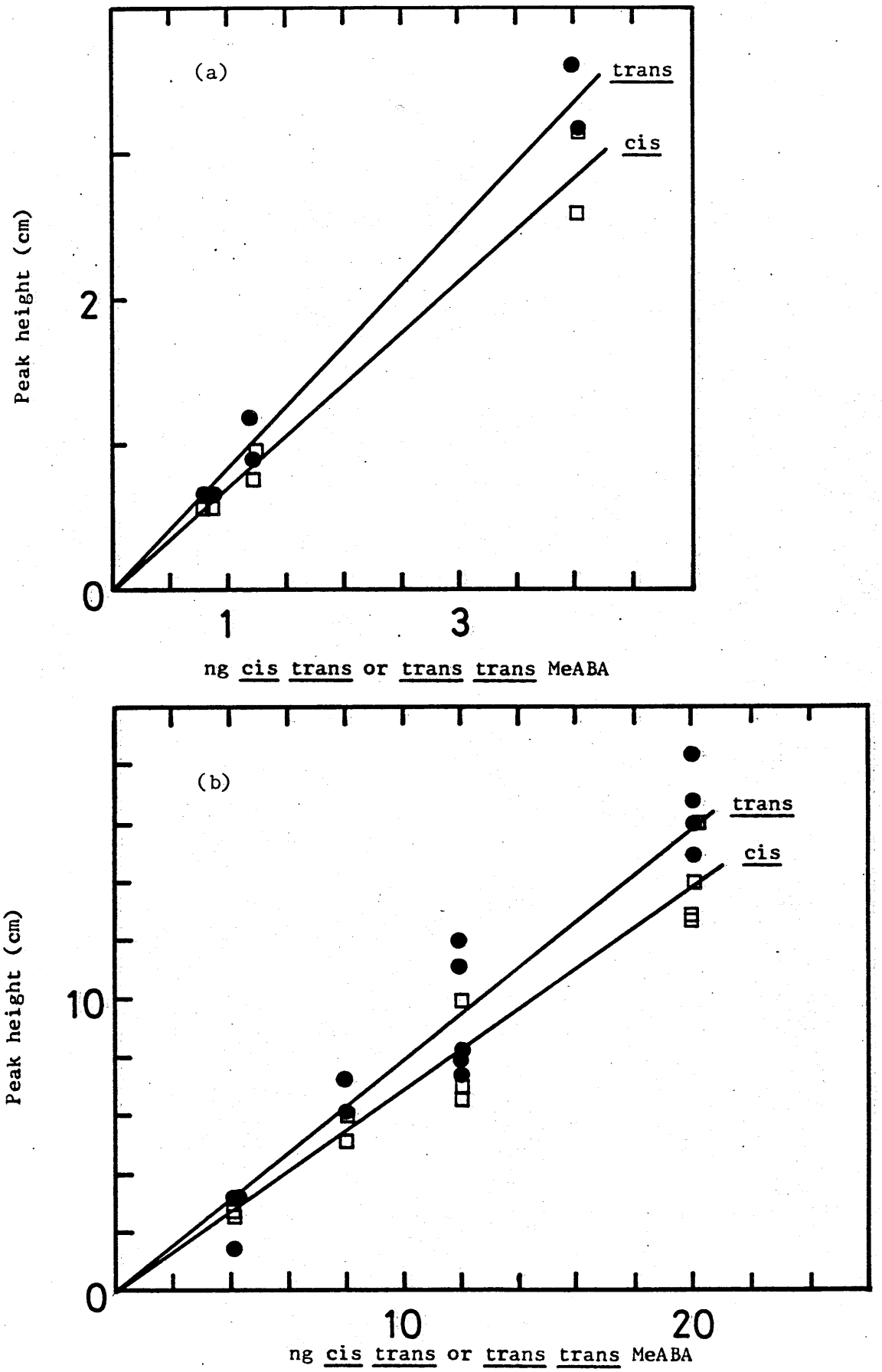
Table 6. Operating conditions for the quantification of ABA by GCECD .

EXPERIMENTS	COLUMN	OVEN TEMPERATURE	ZONE TEMPERATURE (°C)	CARRIER GAS AND FLOW RATE*	SPLITTER	RETENTION TIME OF ABAMe (s)	THEORETICAL PLATES (cis ABA)
Seasonal ABA analysis 19/1/80, 2/3/81, 21/5/81, 23/3/81.	20m glass support-coated with SP 2250	220°C for 840s then 240°C for 120s. Temperature increase 0.33°C s^{-1}	300	H_2 68.02cm s^{-1}	Splitless	cis 564 trans 684	8930
Diurnal analysis of nodule ABA content 9/5/82	25m FFS columns wall-coated with methyl silicone fluid	190°C for 120s then 280°C for 60s. Temperature increase 0.125°C s^{-1}	350	N_2 17.48cm s^{-1}	Split	cis 438	8079
Diurnal analysis of leaf ABA content 9/5/82; seasonal ABA analyses 6/9/82, 18/12/81	2 x 12.5m FFS columns wall-coated with methyl silicone fluid	190°C for 600s then 280°C for 180s. Temperature increase 0.33°C s^{-1}	350	H_2 26.59cm s^{-1}	Split	cis 320	2556
Comparison of techniques for 'bound' ABA analysis	2 x 12.5m FFS columns wall-coated with methyl silicone fluid	190°C for 600s then 280°C for 180s. Temperature increase 0.33°C s^{-1}	275	H_2 34.72cm s^{-1}	Split	cis 282 trans 378	3306
Assessment of population variation in ABA content; seasonal ABA analysis 8/1/83	10m FFS column wall-coated with methyl silicone fluid	190°C for 420s then 260°C for 120s. Temperature increase 0.65°C s^{-1}	325	H_2 33.33cm s^{-1}	Split	cis 288 trans 384	

* linear velocity

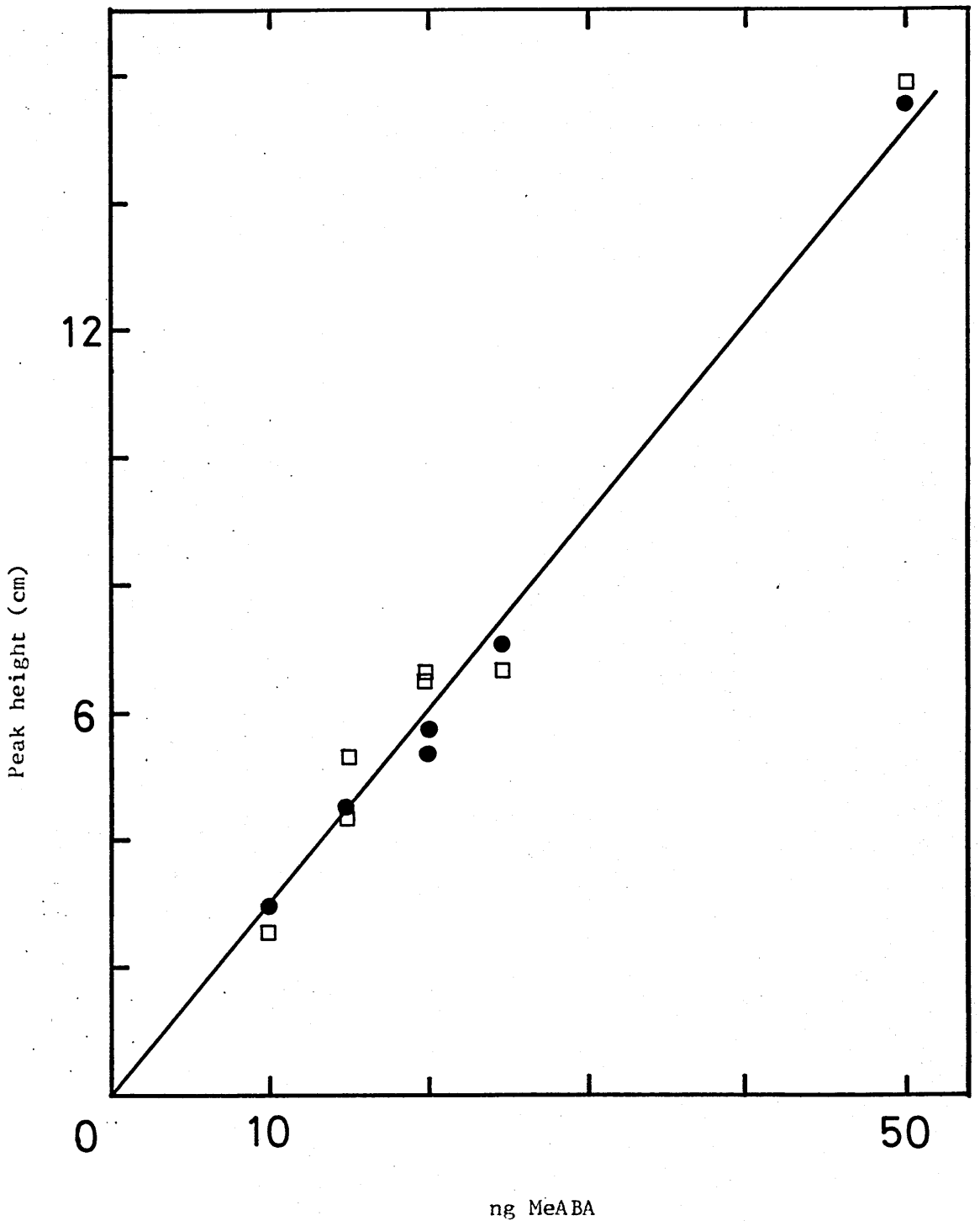
Diurnal analyses of leaf and nodule ABA content 7/4/83; effect of 0.1mol m^{-3} ABA on endogenous ABA levels (20/3/83); seasonal ABA analysis 17/3/83	10m FFS column wall-coated with methyl silicone fluid	175°C for 300s then 260°C for 300s. Temperature increase 0.65°C s^{-1}	350	H_2 58.82cm s^{-1}	Split	cis 166 trans 219	
Diurnal analysis of leaf ABA content 4/3/84	2 x 20m FFS columns wall-coated with methyl silicone fluid	220°C for 480s then 270°C for 300s. Temperature increase 0.33°C s^{-1}	300	He 83.3cm s^{-1}	Split	cis 228 trans 288	
Diurnal analysis of nodule ABA content 4/3/84; effect of 0.1mol m^{-3} ABA on endogenous ABA levels (18/3/84)	10m FFS column wall-coated with methyl silicone fluid	160°C for 0s then 240°C for 120s. Temperature increase 0.133°C s^{-1}	325	He 24.39cm s^{-1}	Split	cis 337 trans 382	

Figure 4. Typical standard curves showing the response of the ECD (peak height) to methylated synthetic cis trans and trans trans ABA standards, (a) from 0-4ng (b) from 0-20ng, using a capillary column.


(a)
trans
cis
Peak height (cm)
0
2
1
3
ng cis trans or trans trans MeABA
(b)
trans
cis
Peak height (cm)
0
10
10
20
ng cis trans or trans trans MeABA

(m/z 190, 162, 134, 125) for a nodule sample, using an AE1 MS-30 mass-spectrometer operated at 240V and connected to a Pye104 gas-chromatograph via a single-stage glass jet separator (Kratos, Manchester, U.K.). Aliquots of extracts (0.5 - 2.0mm^3 in size) were introduced on to a 3m x 4mm glass column packed with 1% OV-1 Gas Chrom Q (100-120 μm mesh) maintained at 220°C. Source and separator temperatures were 280°C and 250°C respectively. The carrier gas was helium at a flow rate of 0.4cm^3 s^{-1}. During SIM, standards were run between samples and under the same conditions to permit the quantification of extract ABAMe levels. A calibration curve was usually constructed relating levels of ABA injected with detector response to facilitate the quantification of ABA levels in samples. An example calibration plot is shown in Figure 5 . A full mass spectral scan at 70eV was taken of a nodule extract at the retention times corresponding to cis and trans ABA at a scanning speed of 10s $decade^{-1}$.

Figure 5. Typical standard curve showing the response of the mass spectrometer (tuned to m/z =190) to methylated synthetic cis trans and trans trans ABA standards.


12
6
0
10
50
Peak height (cm)
ng MeABA

4. Exogenous ABA Applications

4.1 The Application of 0.1mol m^{-3} (±) ABA to the Roots

The morphological effect of (±) ABA applied to plants growing in water culture, was examined alongside changes in endogenous ABA levels. The experiment was conducted twice; differences in any of the parameters in the second experiment (from the first) are shown in brackets.

Plants were grown for 3 (6) months as described earlier (see Materials and Methods 1.3). Half of the 2 dm^3 culture vessels then received 1cm^3 of a stock ABA solution prepared by dissolving 0.264g synthetic (±) ABA in 5cm^3 ethanol to produce a final (±) ABA concentration of 0.1mol m^{-3}. The remaining control vessels each received 1cm^3 ethanol alone.

Height increments (measured from the wooden support to the apex of the plant) were recorded every 5 - 13d on the second run of the experiment, following ABA treatment. On both occasiosn, the experiment was terminated once internode elongation had ceased and dormancy buds were developing (30 (60)d after the commencement of the ABA treatment). One plant per vessel was then harvested for leaf, stem, apex (top 5cm of the stem including the terminal bud), root and nodule fresh and dry weight measurements. Ten plants were harvested for ABA analysis.

4.2 The Application, Transport and Metabolism of [2-^{14}C] ABA in Alnus

4.2.1 Preparation of an Aqueous [2-^{14}C] ABA Stock for Application to Roots and Leaves

[2-^{14}C] ABA solutions were prepared by removing 41.2 - 66.5 kBq from stock ABA in methanol to a 2cm^3 capacity glass vial. The methanol was removed under nitrogen and the residue was redissolved in a minimum volume of redistilled methanol before the addition of 500mm^3 of distilled water. This produced an aqueous solution containing 82.5 - 132.9 Bq mm^{-3} (19.5 - 37.2 ng mm^{-3}) with a pH of 4.0.

4.2.2 Application of [2-^{14}C] ABA to the Roots

The uptake of [2-^{14}C] ABA from a 0.1mol m^{-3} (±) ABA

solution was examined using small plants (2 months old) growing in water culture. 7.2kBq of aqueous ABA stock, prepared as described above, was added to 300cm^3 of full-strength Crone's solution, (pH 7.0). Sufficient unlabelled synthetic (±) ABA was added to 180cm^3 of the labelled stock to make a 0.1mol m^{-3} (±) ABA solution. Six plants were transferred to McCartney bottles containing labelled plus unlabelled ABA, whilst four were placed in the labelled ABA solution only. Plants were maintained in growth room A for 5d in a photoperiod of 16h daily, until harvested for ^{14}C translocation profiles.

The experiment was later repeated, and the metabolism of [2-^{14}C] ABA in leaf, stem, root and nodule tissues after 5d in the presence of 0.1mol m^{-3} (±) ABA was examined.

4.2.3 Application of [2-^{14}C] ABA to Leaves

In all cases, 1.4 - 1.9 kBq (15-20mm^3) of the aqueous ABA stock was applied with a Terumo 20mm^3 capacity micro-syringe, to a scalpel-incision (0.5 - 1cm long), made in the proximal end of the petiole of the third expanded leaf from the apex. Plants were maintained in growth room A for the duration of the translocation period (24h).

4.2.4 Harvest of Material

At the end of the translocation period, plants were decapitated and the shoot portion was divided into the following parts:

donor leaf petiole
donor leaf lamina
stem above donor leaf (including apex)
stem below donor leaf
leaves below donor leaf

The root system was washed and divided into root and nodule material. When it was necessary to examine root xylem-exudate, plants were decapitated under water, 3 - 5cm above the rooting medium, the phloem (bark) was stripped back from the stump, and a piece of rubber or polythene tubing was filled over the end. Exudate was collected over a 24 - 27h period, and analyzed for radioactivity by liquid scintillation counting.

Plants fed via the roots were completely harvested at the end of the translocation period into leaves, stem, roots and nodules.

The relative merits of oxidation and methanol extraction of samples to assess levels of radioactivity were investigated in the following way. Six replicate samples of leaf, stem, root and nodule material were removed from each of two plants. Three samples from each plant were then analyzed for radioactivity by fresh methanol extraction, and the three remaining samples by dry sample oxidation. Where appropriate, material was oven-dried at 60-80°C for 24h or longer or extracted twice in $2cm^3$ methanol, which was then dried using a vacuum oven, into a $6cm^3$ capacity plastic scintillation vial and redissolved in $5cm^3$ xylene-based scintillant (Koch-Light Limited, Haverhill, Suffolk, U.K.) for liquid scintillation counting.

Dry sample oxidation yielded values 1.17 times larger than (stem and leaf material), or 1.07 times smaller than (root material) or equivalent to (nodule material) those obtained by fresh methanol extraction. For convenience, dry oxidation was the method chosen for experimental analyses. An account of the technique is given in Materials and Methods.

4.2.5 The Metabolism of [2-^{14}C] ABA

Experiments were conducted to investigate the radioactive metabolites formed after foliar and root [2-^{14}C] ABA feeding. In all cases, leaf, root, nodule and stem material from four plants was harvested after the translocation period, bulked and homogenized using a pestle and mortar, and extracted in $100cm^3\ g^{-1}$ f.wt. methanol. Extracts were left in the refridgerator at 4°C for 72h, decanted, and the remaining residue washed with $10cm^3$ more methanol. The entire solvent volume was then filtered and taken to dryness *in vacuo* at 35°C. The residue was redissolved in $30cm^3$ $100mol\ m^{-3}$ phosphate buffer, pH 8.0, adjusted to pH 2.5 with $3mmol\ cm^{-3}$ HCl and partitioned four times against $15cm^3$ ethyl acetate. The ethyl acetate phase which contained 'free' ABA was dried *in vacuo* at 35°C, taken up in $1cm^3$ methylene chloride and

applied to a silica SEP-PAK cartridge in the manner of Hubick and Reid, 1980. The radioactive fractions (methanol-containing fractions) were retained and dried in vacuo at 35°C. The extract was removed to a 2cm³ capacity glass vial, dried under nitrogen and stored at -20°C before TLC. The aqueous phase (from the ethyl acetate partitioning step) was adjusted to pH 8.0 and dried in vacuo at 35°C. The residue was then taken up in 2cm³ methylene chloride, transferred to a 2cm³ capacity glass vial, dried and stored as above.

TLC of extracts was on 5 x 20cm plastic plates (Merck, Darmstadt, Germany; one extract per plate) precoated with 200μm silica gel G254, or 20 x 20cm glass TLC plates (Merck, Darmstadt, Germany; four extracts per plate) precoated with 250μm silica gel 60 G254; both types were pre-run in methanol. Extracts were run over 15cm in two solvent system; butan-1-ol: propan-1-ol: ammonia: water (2:6:1:2 v|v) and chloroform: methanol: water (75:22:3 v|v). Authentic [2-^{14}C] ABA was run on identical TLC plates in the same TLC tank as the sample. Once the chromatograms had developed, they were dried horizontally, and either cut across into 1cm x 5cm strips (plastic plates) or scaped-off in 1 x 5cm bands (glass plates) for liquid scintillation counting (see Materials and Methods 5.1). On several occasions, [^{3}H] ABA was added to extracts before the chromatogram was run; dual-label liquid scintillation counting (^{3}H and ^{14}C) was then used to check the position of [2-^{14}C] ABA in the extract.

5. General Methods

5.1 Liquid Scintillation Counting

All samples were counted utilizing the present ^{3}H, ^{14}C or dual-label (^{3}H (^{14}C) and ^{14}C (^{3}H)) channels on a Packard 3380 liquid scintillation spectrometer. Two types of counting vials were used:

A. Capped 20cm^3 capacity polyethylene vials (Zinsser Analytic, supplied by A. & J. Beveridge Limited, Edinburgh, U.K., which contained 10cm^3 scintillant or,

B. Capped 6cm^3 capacity polyethylene vials ("inserts", Zinsser Analytic, supplied by A. & J. Beveridge Limited, Edinburgh, U.K.) which contained 5cm^3 scintillant. (These vials were placed within 20cm^3 capacity polyethylene vials for counting).

Four types of scintillant were used throughout the course of the work:

I. 4g 2,5-diphenyl oxazole (PPO), Koch-Light Limited, Haverhill, Suffolk, U.K., 1000cm^3 toluene (sulphur-free), 500cm^3 Triton X-100.

II. General- purpose emulsifying scintillant (liquid scintillator, Unisolve E), Koch-Light Limited, Haverhill, Suffolk, U.K.

III. General-purpose emulsifying scintillant (emulsifier scintillator 299), Packard-Becker, Chemical Operations, Groningen, Netherlands).

IV. 7g PPO, 400cm^3 sulphur-free toluene, 330cm^3 phenylethylamine (Koch-Light Limited, Haverhill, Suffolk, U.K.), 200cm^3 methanol, 50cm^3 water.

I, II and III were used for both aqueous and non-aqueous samples. IV was required for counting samples combusted using the sample oxidizer.

To assess the degree of quench that had occurred during counting (and hence permit estimation of the true count-rate issuing from the samples), two methods were adopted. The automatic external standard (AES) channels ratio method was used successfully for most samples by preparing calibration quench curves relating channels ratio values to the efficiency of counting for a series of standard vials containing the appropriate scintillant and sample. Construction of the calibration curves was achieved by counting samples on the appropriate channel(s), either (a) following

addition of up to 100mm³ of a methanol extract of alder leaves (to obtain a range of AES channels ratio values to vials already containing 5 or 10mm³ of standard ^{3}H or ^{14}C haxadecane and an appropriate non-radioactive sample or (b) following the addition of 5mm³ ^{14}C or ^{3}H hexadecane to single-labelled radioactive samples which had been counted once alone and were known to include a range of channels ratio values.

The efficiency of counting was calculated (following subtraction of the background counts (Bg) present in a vial containing scintillant only) from the relationship:-

$$\text{Efficiency of counting} = \frac{C - S}{A} \times 100$$

Equation 1.

Where: C = observed counts from the hexadecane and radioactive sample (when present)

S = observed counts from the sample alone

A = theoretical counts present in the hexadecane

Counting efficiencies and hence an estimate of the absolute radioactivity present in subsequent samples were then easily calculated from their AES ratios by reference to the appropriate calibration curve and the following relationships:-

$$\text{Radioactivity (counts) present in } ^{14}C \text{ sample} = \frac{\text{Counts observed in } ^{14}C \text{ channel} - Bg}{\text{Efficiency of counting } (^{14}C \text{ channel})}$$

Equation 2.

$$\text{Radioactivity (counts) present in } ^{3}H \text{ sample} = \frac{\text{Counts observed in } ^{3}H \text{ channel} - Bg}{\text{Efficiency of counting } (^{3}H \text{ channel})}$$

Equation 3.

Owing to the overlap of counts from the ^{14}C (^{3}H) channel into the ^{3}H (^{14}C) channel (but not vice versa) during dual-label counting, the formulae for ^{14}C (^{3}H) (i.e. ^{14}C counts in the presence of ^{3}H) and ^{3}H (^{14}C) (i.e. ^{3}H counts in the presence of ^{14}C),

samples are as follows:-

$$\text{Radioactivity (counts) present in } {}^{14}C\ ({}^{3}H) \text{ sample} = \frac{\text{Counts observed in } {}^{14}C\ ({}^{3}H) \text{ channel} - Bg}{{}^{14}C \text{ efficiency } ({}^{14}C({}^{3}H) \text{ channel})}$$

Equation 4.

$$\text{Radioactivity (counts) present in } {}^{3}H\ ({}^{14}C) \text{ sample} = \frac{\text{Counts observed in } {}^{3}H\ ({}^{14}C) \text{ channel} - \left[\text{OF.Radioactivity in } {}^{14}C\ ({}^{3}H) \text{ sample}\right]}{{}^{3}H \text{ efficiency } ({}^{3}H\ ({}^{14}C) \text{ channel})}$$

Equation 5.

The relationship between quenching and the overlap factor (OF) of ^{14}C counts (radioactivity in ^{14}C (^{3}H) sample) into the ^{3}H (^{14}C) channel, was determined experimentally in the absence of ^{3}H.

Whilst whole quench curves for ^{14}C samples were generally shaped as shown in Figure 6 , one or two linear equations (using at least five observations) were fitted by eye to cover the range of AES values expected for experimental samples, which was usually quite small. Quench equations evaluated by this method for several counting systems are presented in Table 7 .

The degree of quenching in individual ^{14}C samples for which it was not feasible to produce a quench curve, was assessed using an internal standard. Samples were counted once and recounted in the presence of $5mm^3$ ^{14}C hexadecane. Counting efficiencies were then determined using equation 1, and absolute counts using equation 2.

The counting time used normally was five minutes.When a batch of samples all contained less than 0.83Bq, however, the counting time was increased to ten minutes to reduce the standard error associated with the measurement.

5.2 Sample Oxidation

^{14}C labelled samples not greater than 0.5g in weight were placed in small polycarbonate capsules (Intertechnique, Plaisir, France) either directly following harvest, or after oven-drying at 60°C for at least 48h. The samples were then completely oxidized to $^{14}CO_2$ and H_2O in an 'Oxymat' sample oxidizer, model IN 4101 (Intertechnique, Plaisir, France), using the 'oxygen train' method

Figure 6 . Generalised 'quench' curve used in scintillation spectrometry.

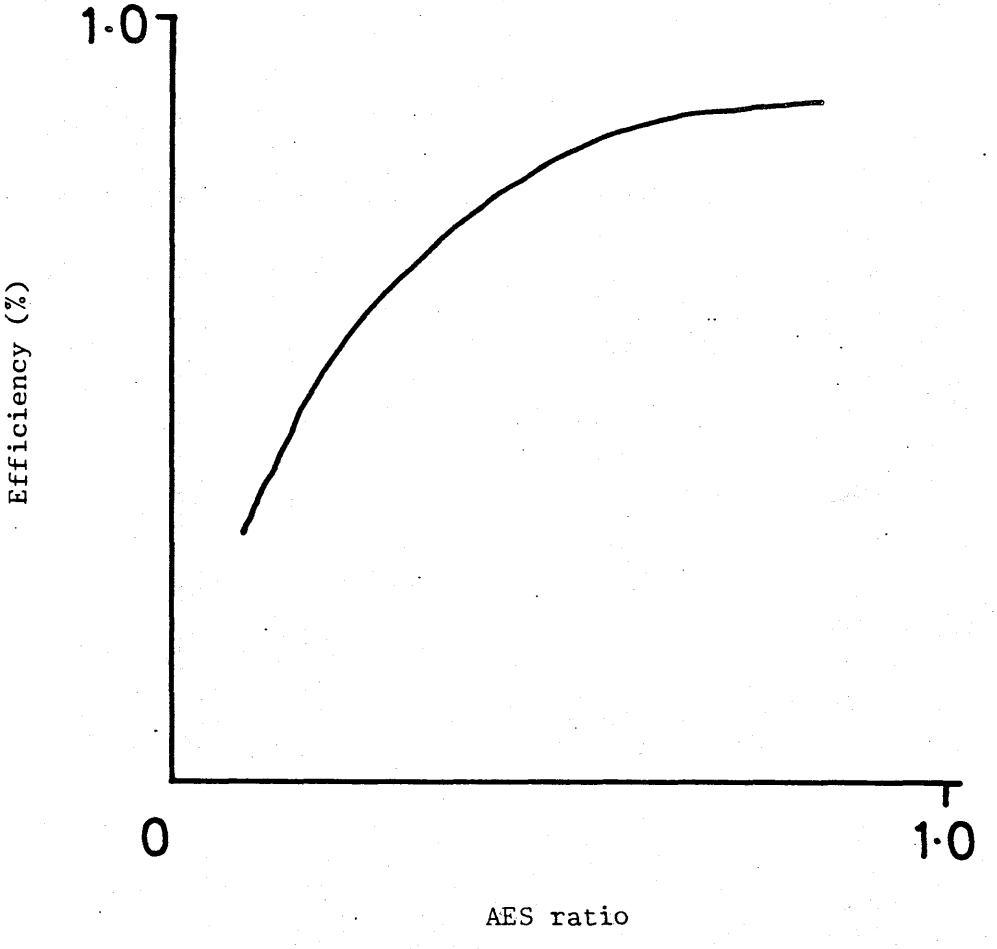

1·0
Efficiency (%)
0
1·0
AES ratio

Table 7. Quench equations for the Packard Scintillation Spectrometer constructed for several scintillation cocktails and sample types. See text for explanation of vial and scintillant types.

Nature of samples	Isotope(s)	Experiment type	Vial and scintillant type	Quench equations. AES = automatic external standard channels ratio. E = % counting efficiency
Oxidised dry	^{14}C	Assessment of sample radio-activity following the application of [2-^{14}C] ABA to plants	A IV	AES 0.1880 - 0.3200 E = 93.3xAES + 40.1 AES 0.3200 - 0.4300 E = 73.0xAES + 46.9
Oxidised fresh	^{14}C	As above	A IV	AES 0.3307 - 0.3579 E = 63.4
Dried following extraction in methanol	^{14}C	As above	B II	AES 0.1000 - 0.2400 E = 103.6xAES + 41.7 AES 0.2400 - 0.5200 E = 83.1xAES + 46.1 AES 0.5200 E = 86.90
Dissolved in methanol (200mm^3)	^{14}C	Recovery of [2-^{14}C] ABA internal standard following purification of extracts for endogenous ABA	B II	

1cm x 5cm x 200 μm silica-gel-coated plastic TLC strips	^{14}C	Metabolism of [2-^{14}C] ABA in plant parts	B II	AES 0.3000 - 0.6000 E = 55.0 AES + 54.0
1cm x 5cm x 200 μm silica-gel-coated plastic TLC strips	^{14}C, ^{3}H	As above	B II	$^{14}C(^{3}H)$ AES 0.3000 - 0.4600 E = 60.0^{x}AES - 18.0 AES 0.4600 - 0.6500 E = 152.0^{x}AES - 62.2 $^{3}H(^{14}C)$ AES 0.3000 - 0.6500 E = 56.0^{x}AES - 10.0 AES 0.3000 - 0.6500 OF = 0.53 - 0.59^{x}AES
1cm x 5cm x 250 μm silica-gel-coated TLC scrapings	^{14}C, ^{3}H	As above	B II	$^{14}C(^{3}H)$ AES 0.3000 - 0.4600 E = 72.0^{x}AES - 23.0 AES 0.4600 - 0.6500 E = 120.0^{x}AES - 47.0 $^{3}H(^{14}C)$ AES 0.3000 - 0.6500 E = 63.6^{x}AES - 13.8 AES 0.3000 - 0.6500 OF = 0.48 - 0.54^{x}AES

for sample combustion (Peng, 1977). Four samples expected to contain similar amounts of radioactivity were oxidized in succession followed by a blank. Batches of samples were oxidized in order of increasing radioactivity. Cylinder oxygen and nitrogen pressures were both maintained at 0.25 MPa. $^{14}CO_2$ gas produced was absorbed by the scintillant which contained phenylethylamine, and counted using the Packard 3380 liquid scintillation spectrometer. The efficiency of combusion for pure ^{14}C hexadecane was 88%. The efficiency of liquid scintillation counting was assessed by the AES channels ratio method.

5.3 The Acetylene Reduction Assay

Nitrogenase activity was assessed using the acetylene reduction assay in 2 -3 y old plants in Autumn, 1980 (prior to dormancy), Spring, 1981 (prior to bud-break and at bud-break) and Summer, 1981 (as the plants attained full foliage). Assays were conducted under natural photoperiod in the unheated greenhouse in which the plants were growing, usually for a period of 1h commencing between 10 - 12 a.m. Longer periods were used in assays made prior to dormancy or bud-break when nitrogenase activity was not quantified. Temperature and levels of Photosynthetically Active Radiation (PAR) were recorded.

Each plant was placed whilst still potted within a glass 'Quickfit' 1000cm^3 capacity culture vessel (actual volume 1,200cm^3, Gallenkamp & Co. Limited, London, U.K.). The rim of the jar was coated with a layer of vaseline to ensure a gas-tight fit before the two halves of a perspex cover were placed in position around the stem of the plant. A 'subaseal' stopper (number 47, Gallenkamp & Co. Limited, London, U.K.) was fitted in a hole cut in one half of the lid to enable gas samples to be added and removed. A ring of plasticine was used to seal the vessel at the point at which the stem entered the jar. Two control vessels were also prepared in an identical manner except that one did not contain a plant but was injected with acetylene (to assess the levels of C_2H_4 present in the acetylene) whilst the other contained a plant but was not injected with acetylene (to assess levels of ethylene produced by the plant, soil-organisms and also non-biologically).

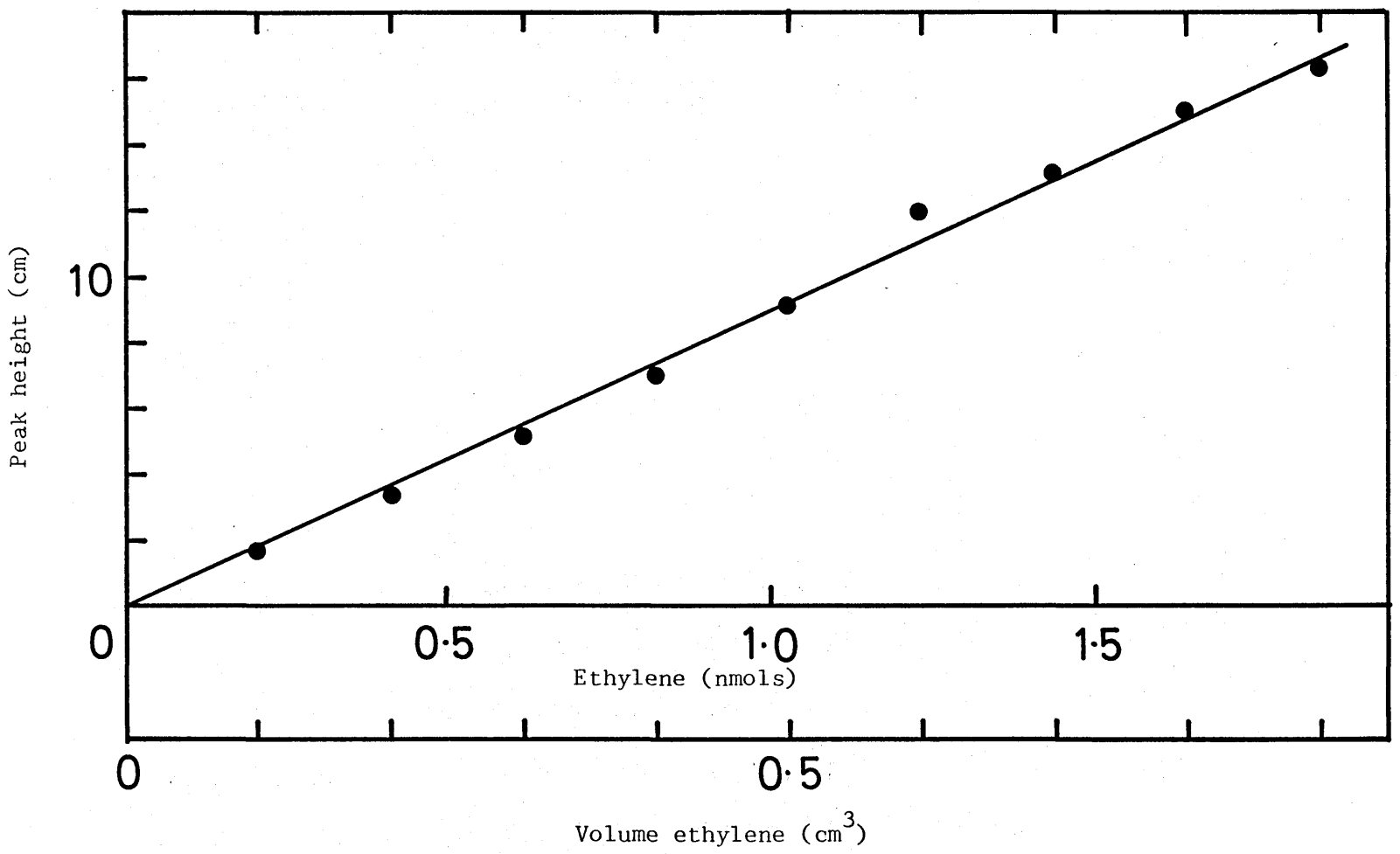
When all plants had been enclosed in the glass vessels, 50cm^3 acetylene was injected into each using a 50cm^3 capacity plastic syringe (B-D and Co. Limited, Ireland) fitted with a 1.5cm long disposable needle (B-D and Co. Limited, Ireland). After 1h, three 1cm^3 capacity gas samples were removed from the jar using 1cm^3 capacity plastic syringes also fitted with 1.5cm long disposable needles. The needles of each syringe were then inserted into rubber bungs to store the samples prior to analysis which took place not more than 2h following the assay. Once samples had been taken, the gas-space of each culture jar and potted root-system was determined by the volume of water which could be accommodated within the vessel. The nodules were then removed from the plants, washed, blotted dry and weighed. Nitrogenase activity was expressed as nmols ethylene produced per g.f.wt. of nodule material per plant*. The ethylene content of the gas samples was analyzed using a model 14 Pye 104 gas chromatograph (W.G. Pye Limited, Cambridge, England) fitted with a flame-ionisation detector. The sample was separated on a 1mX 4mm stainless steel column packed with Porapak R (Phase Separations, Clwyd, U.K.) and maintained at 40$\pm$5°C. The column-carrier gas was nitrogen at a flow-rate of 1cm^3 s^{-1}; air and hydrogen flow-rates were also maintained at 1cm^3 s^{-1}. The chart recorder (Servoscribe model RF541.20) was operated at 1mV and a chart speed of 600mm h^{-1}. The ethylene peak was symmetrical, eluted after 3 - 4 minutes retention time and preceded the acetylene peak. A graph (Figure 7) was constructed for each assay, relating detector response (peak height) to the amount of ethylene present in 0.1 to 0.9cm^3 of a 50 ppm ethylene calibration mix (BOC special gases), using the following relationship between volume and ethylene concentration:

$$E = \frac{K}{293} \times \frac{50}{10^6} \times \frac{1}{22.4 \times 10^3} \times V$$

where E = nmol ethylene present in volume Vcm^3 by 50ppm ethylene at temperature K degrees Kelvin. From this calibration, sample ethylene content was determined, and hence the nitrogenase activity of the plant assessed.

* The mean ($\pm$ SE) nitrogenase activity of 5 plants was assessed on each harvest date.

Figure 7. Typical standard curve showing the response of the FID (peak height) to 50ppm ethylene standards of the given volume and ethylene content at 22°C.


Peak height (cm)
10
0
0·5
1·0
1·5
Ethylene (nmols)
0
0·5
Volume ethylene (cm3)

5.4 Measurement of Plant Leaf Water Potential and Relative Water Content

Diurnal fluctuations in plant leaf water potential (leaf ψ) were measured on one occasion in plants harvested on a diurnal basis for endogenous leaf and nodule ABA content. The third leaf (which was fully expanded) from the apex was removed with a razor blade and fitted, with the petiole extruding, into a Scholander-type pressure chamber (Scholander et al., 1965). The positive pressure ('balance pressure') sufficient to force xylem sap back into and along the xylem conduits (so that it exuded from the cut surface) was recorded. This value normally balances exactly the negative tension of the water columns prior to excision and hence provides a measure of plant leaf water potential.

Leaf relative water content (RWC) was determined for the same plants in which leaf ψ was examined. The fourth leaf from the apex was excised with a razor blade and the petiole placed in a $20cm^3$ capacity glass vial containing distilled water. Vials were left in an 'equilibration box' (damp-paper lined polystyrene box) for a 12h period or longer before the leaf was re-weighed. Leaves were then dried for a period of not less than 24h in paper packets at c.70°C. Leaf RWC was calculated from the expression:

$$\text{Leaf RWC} = \frac{\text{Leaf f.wt. - leaf d.wt.}}{\text{Leaf turgid weight - leaf d.wt.}}$$

5.5 Measurement of Photosynthetically Active Radiation (PAR), Relative Humidity (RH) and Temperature

PAR (wavelengths 400 to 700nm) was recorded using a Licor Quantum sensor, Model LI-190S attached to a module integrator unit Model 190M. Temperature was monitored in the unheated greenhouse on a seasonal basis, with a Castell thermograph which was also used to record day and night-time fluctuations in growth-room temperature. Spot temperature and humidity measurements during the course of the diurnal experiments were made with a thermometer and hair hygrometer respectively.

--ooOoo--

6. Statistics

6.1 Computation of means and standard errors.

Replicate measurements made in experiments were usually averaged. The standard error (SE) was calculated from the following formula :

$$SE = \sqrt{\left(\Sigma Y^2 - \frac{(\Sigma Y)^2}{n} \right) \Big/ n(n-1)}$$

where Y are the variates and n is the number of observations. Standard errors of mean values are shown on graphs by vertical bars extending from the mean value to a point one SE above the mean and from the mean to one SE below the mean.

6.2 Comparisons made using a paired 't' test.

To make a paired comparison of data sets, a paired 't' test was conducted using the following formula to compute 't' :

$$t = \frac{\bar{D} - (\mu_1 - \mu_2)}{\sqrt{\left(\Sigma D^2 - (\Sigma D)^2 \Big/ b \right) \Big/ b-1}}$$

where $\bar{D}$ is the mean difference between the paired observations, D is the difference between individual paired observations, b is the number of pairs, and μ_1 and μ_2 are the population means of the two groups (the difference between these is assumed to be zero). Calculated values of 't' were then compared with the critical value of 't' with b-1 degrees of freedom, at a probability level of 5 % (P = 0.05) from 't' tables to assess whether there was a significant difference between the pairs.

6.3 Comparisons made using a 't' test.

Other comparisons between data sets were using the students 't' test. The formula for calculating 't' is given below :

$$t = \frac{\bar{Y}_1 - \bar{Y}_2 - (\mu_1 - \mu_2)}{\sqrt{\frac{1}{n}(s_1^2 + s_2^2)}}$$

where $\bar{Y}_1$ and $\bar{Y}_2$ are the means of groups 1 and 2, s_1 and s_2 are the standard deviations of the 2 data sets and μ_1 and μ_2 and n are defined as described in 6.1 and 6.2. Calculated values of 't' were compared with the critical value of 't' with 2(n-1) degrees of freedom as described in 6.2 to assess whether or not there was a significant difference between the groups.

6.4 The analysis of variance

To assess the relevance of sampling time and plant batches on diurnal leaf and nodule ABA content, the data from experiments 2 and 3 were analysed in a 2-way analysis of variance. This involved several preliminary computations before an analysis of variance table could be constructed, and conclusions reached. The data (ABA content (ng g^{-1} f.wt) were initially displayed as follows :

	Experiment (a = 2)	
	X	X
Sampling	X	X
time	X	X
(n = 5)	X	X
	X	X

The following calculations were then made (Sokal and Rohlf, 1969) :

1. Grand total : $\sum^{a} \sum^{b} Y$

2. Sum of the squared observations : $\sum^{a}\sum^{b} Y^2$

3. Sum of the squared column totals divided by the sample size of a column : $\dfrac{\sum^{a}(\sum^{b} Y)^2}{b}$

4. Sum of squared row totals divided by the sample size of a row : $\dfrac{\sum^{b}(\sum^{a} Y)^2}{a}$

5. Grand total squared and divided by the total sample size = correction term : $CT = \dfrac{(\sum^{a}\sum^{b} Y)^2}{ab}$

6. Sum of squares (total) : $SS_{total} = \sum^{a}\sum^{b} Y^2 - CT$
= (value computed in 2) - (value computed in 5).

7. Sum of squares (columns) : $SS_A = \dfrac{\sum^{a}(\sum^{b} Y)^2}{b} - CT$
= (value computed in 3) - (value computed in 5).

8. Sum of squares (rows) : $SS_B = \dfrac{\sum^{b}(\sum^{a} Y)^2}{a} - CT$
= (value computed in 4) - (value computed in 5).

9. Sum of squares (remainder) : $SS_{error} = SS_{total} - SS_A - SS_B$.
= (value computed in 6) - (value computed in 7) - (value computed in 8).

The results from these calculations were finally displayed in an analysis of variance table :

	df*	SS**	MS†	F_s ‡
Source of variation :				
A (columns : experiment)	X	X	X	X
B (rows : sampling times)	X	X	X	X
Error (remainder : discrepancy)	X	X	X	X
Total	X	X	X	X

* df = degrees of freedom ; df(A) = a-1

df(B) = b-1

df(error) = (a-1)(b-1)

**SS = sum of squares (from preliminary computations)

†MS = mean sum of squares = $\frac{SS}{df}$

‡F_s = Fischer statistic :

$$F_s(A) = \frac{MS_{(A)}}{MS_{(error)}}$$

$$F_s(B) = \frac{MS_{(B)}}{MS_{(error)}}$$

To assess whether or not sampling times or the plant batch (ie. experiment) had a significant effect on tissue ABA levels, calculated F_s values were compared with critical F_s levels (P = 5%) taken from statistical tables :

Critical $F_s(A) = F_{0.05}\,[df(A), df\ (error)]$

Critical $F_s(B) = F_{0.05}\,[df(B), df\ (error)]$

Values were computed for missing data where necessary. The method is described in Sokal and Rohlf, 1969.

RESULTS

RESULTS

Studies on the distribution of ABA in plants can be directed along two closely related lines of investigation;

(i) analyzing plant endogenous ABA levels
(ii) monitoring the transport of exogenously-applied ABA

In part 1 of the Results, analyses of endogenous ABA in Alnus are presented under two sections:

1.1 Technological aspects concerning the purification, identification and quantification of ABA in plant extracts;

1.2 Experiments involving the quantification of endogenous ABA in Alnus (including the investigation of seasonal changes in root and nodule ABA levels (1.2.1), the investigation of diurnal fluctuations in leaf and nodule ABA content (1.2.2) and the effect of root-applied 0.1mol m^{-3} synthetic ($\pm$) ABA on endogenous apex, leaf, root and nodule ABA levels (1.2.3)).

In part 2 of the Results, the transport of radioactivity and ABA following petiolar application of [2-^{14}C] ABA to plants growing under long and short photoperiods is described (sub-sections 2.1.1, 2.1.2). Mention is made of the metabolism of the applied [2-^{14}C] ABA (sub-section 2.1.3), and the effect of bark-ringing on the movement of ^{14}C between shoot and root (sub-section 2.1.4). The effects of photoperiod on nitrogenase activity and plant growth are recorded in sub-section 2.1.5. Finally, the translocation of ^{14}C and ethyl-acetate-soluble radioactivity (which was expected to contain all the 'free' [2-^{14}C] ABA plus other metabolites) following 0.1mol m^{-3} and 2.52 x 10^{-5} mol m^{-3} [2-^{14}C] ABA application to the roots of plants growing in water-culture under long days is described (section 2.2 and sub-sections therein).

1. Endogenous ABA in Alnus

1.1 Technological Aspects Concerning the Purification, Identification and Quantification of ABA in Plant Extracts.

1.1.1 The Purification of ABA

The analysis of PGS in woody plants is often beset with problems (Crozier, 1980). In the study of endogenous ABA in Alnus, considerable time and effort was spent in the development of a suitable extract purification procedure to enable successful quantification of ABA levels by physicochemical means. In this section, several techniques which were useful in achieving this goal are described.

1.1.1.1 Use of C18 SEP-PAK Cartridges

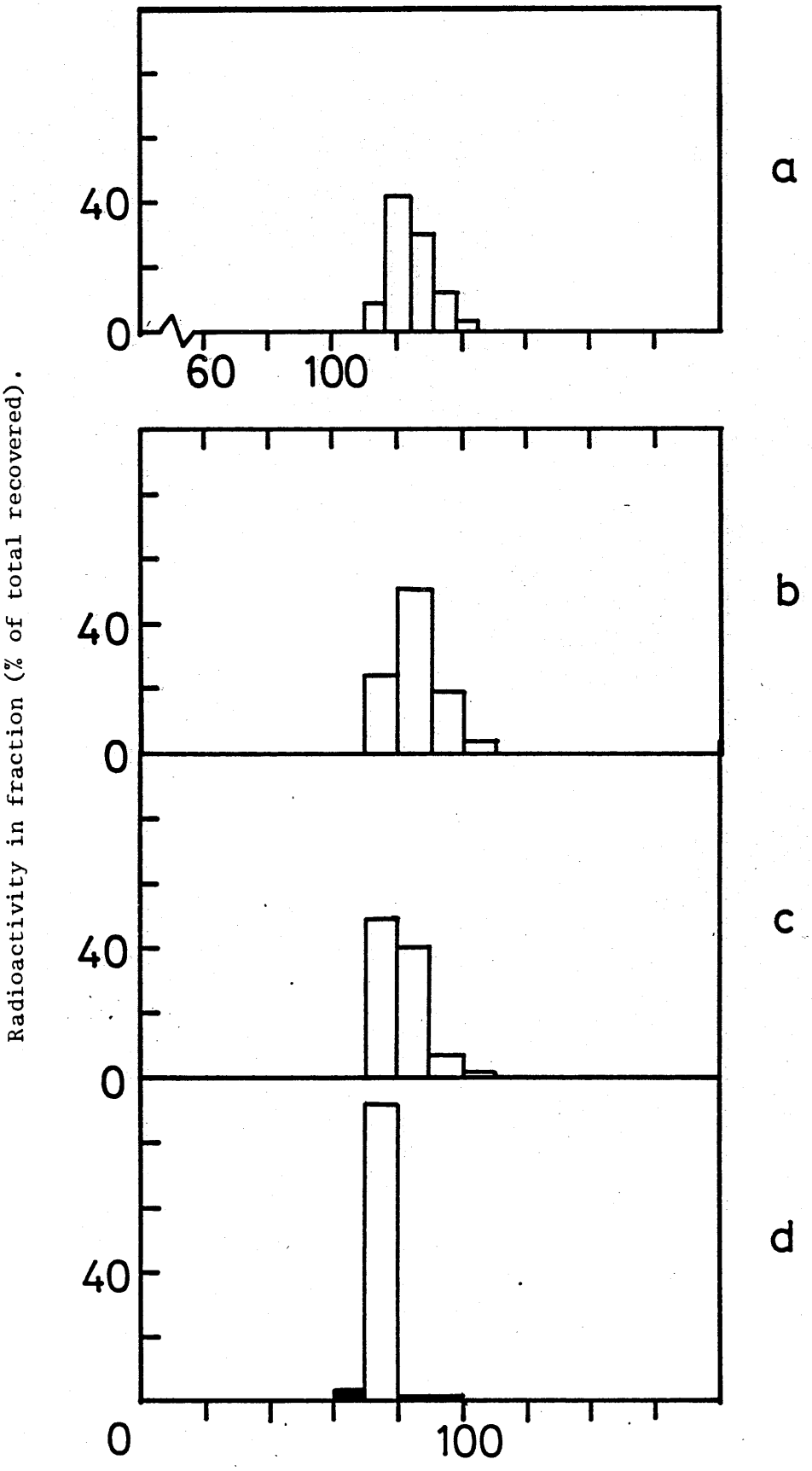
During the evolution of the purification schema for abscisic acid (Figure 3), experiments were carried out with C18 SEP-PAK cartridges (Waters Associates) to determine those conditions which would (a) reduce the sample volume prior to ether partitioning, (b) clean the sample, (c) retain the majority of the endogenous ABA.

Methanolic leaf and nodule extracts were spiked with [2-^{14}C] ABA and purified as normal, as far as the C18 SEP-PAK stage. Aliquots of 70cm^3 (in 100mol m^{-3} phosphate buffer adjusted to pH 3.0) were then applied to a C18 SEP-PAK, which had been prewashed with 2cm^3 methanol followed by 5cm^3 of distilled water. Samples were eluted with sufficient 5cm^3 aliquots of methanol in pH 8, 100mol m^{-3} phosphate buffer, to remove all of the radioactivity. (A range from 10% (10 parts per 100) to 50% (50 parts per 100) of methanol in phosphate buffer was used.) 15% Methanol in phosphate buffer fulfilled the above criteria most satisfactorily; profiles of radioactivity eluted from a leaf and nodule sample using this solvent are shown in Figure 8 .

Little radioactivity was eluted prior to the application of 15% methanol to leaf and nodule samples. The remainder of the total radioactivity recovered from

Figure 8. Profiles of radioactivity eluted by 15% methanol in 100mol m^{-3} phosphate buffer, pH 8.0 from C18 SEP-PAK cartridges following the application of: (a) authentic (±) cis trans [2-^{14}C] ABA in 110cm^3 phosphate buffer, (b) authentic (±) cis trans [2-^{14}C] ABA in 70cm^3 phosphate buffer, (c) leaf extract in 70cm^3 phosphate buffer, (d) nodule extract in 70cm^3 phosphate buffer.

■ = radioactivity eluted prior to the addition of 15 % methanol


a
b
c
d
40
0
60
100
Radioactivity in fraction (% of total recovered).
Total volume eluted (cm3)

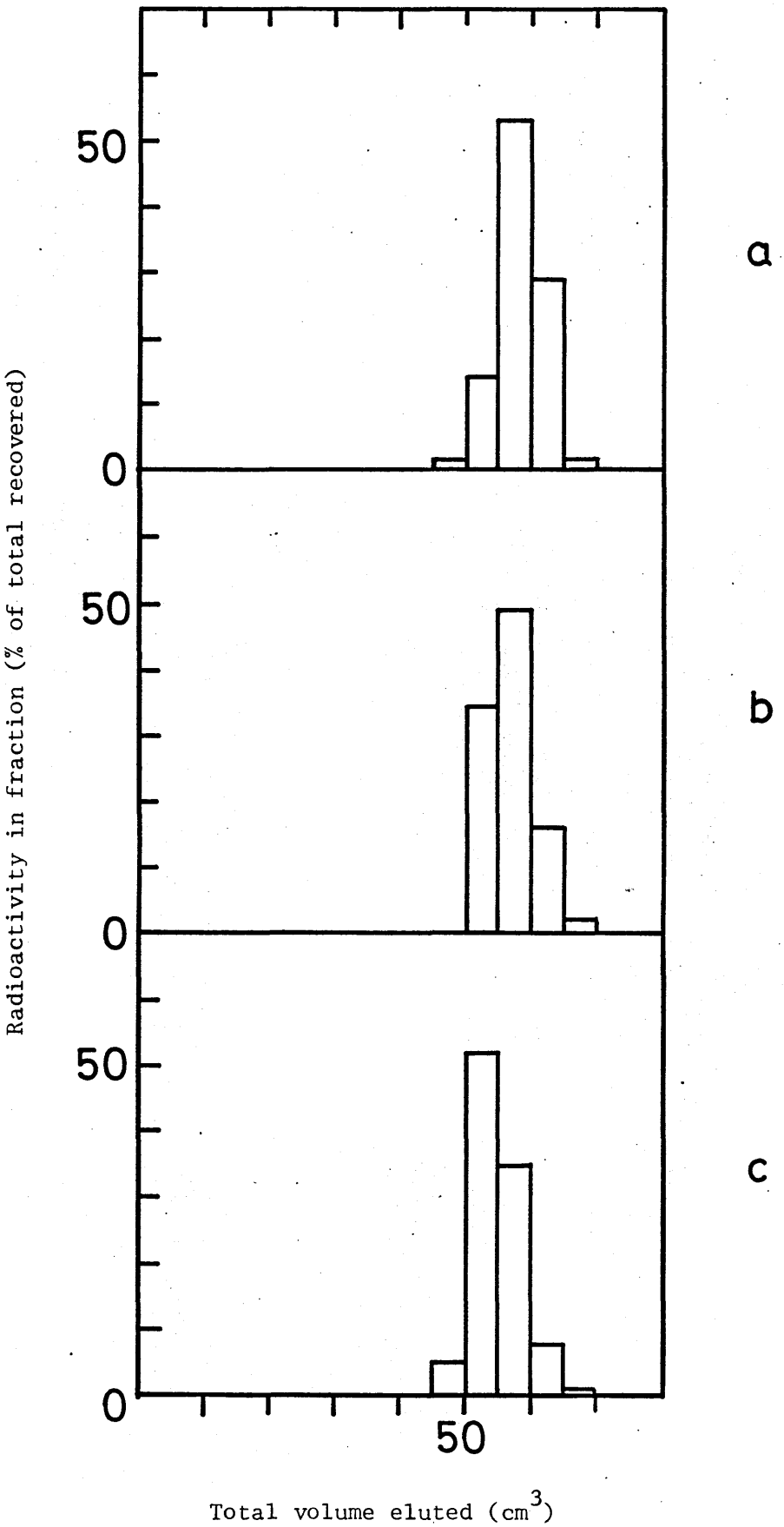
the column (which represented 97% of the total applied) was eluted in the first 40cm³ of 15% methanol applied. In the nodule samples, 92% of the total recovered was present in the first 10cm³ (Figure 8(d)), whilst this value was 49% in the leaf sample (Figure 8 (c)), and only 24% in [2-^{14}C] ABA applied in 70cm³ buffer (Figure 8 (b)). Assuming that the band of radioactivity in the sample represented [2-^{14}C] ABA, these results indicate that the elution of ABA was affected by other components of the extract.

Application of [2-^{14}C] ABA in 110cm³ of phosphate buffer rather than in 70cm³ did not alter the position of the radioactivity eluted by 15% methanol (Figure 8 (a)). This was also true for plant extracts applied in a slightly smaller or larger volume of phosphate buffer (not shown).

1.1.1.2 Use of Silica SEP-PAK Cartridges

Several trial runs were also conducted with silica SEP-PAK cartridges using the solvents described by Hubick and Reid (1980) to elute ABA. Once again, methanolic extracts were labelled with [2-^{14}C] ABA and purified according to the schema shown in Figure 3 . Following ether partitioning, extracts were redissolved in 1cm³ methylene chloride and applied to a silica SEP-PAK which had been prewashed with 2cm³ methylene chloride, and eluted with sequential 5cm³ aliquots of the solvents described in Figure 9 . In all cases, radioactivity was present in the methanol-containing fractions only. (This represented 97% of the total applied in standards and samples alike.) More radioactivity was eluted in the first two 5cm³ methanol-containing fractions following the application of a plant sample than pure [2-^{14}C] ABA (see Figure 9(a) and (b) for examples of profiles obtained with standard [2-^{14}C] ABA and a leaf extract respectively). The profile was further altered by the application of a plant sample containing more impurities; 57% of the total radioactivity recovered following the application of a leaf sample in which prior purification had not included the use of a C18 SEP-PAK cartridge, was

Figure 9. Profiles of radioactivity eluted from silica SEP-PAK cartridges following the application of the following in 1cm^3 methylene chloride: (a) authentic (±) cis trans [2-^{14}C] ABA, (b) leaf extract, (c) leaf extract (C18 SEP-PAK cartridge not used in prior purification steps). The following solvents were used sequentially to elute the sample: 30cm^3 methylene chloride, 5cm^3 of each of the following in methylene chloride: 5% ether, 5% ethyl acetate, 5% acetone, 4% methanol, 10% methanol, 20% methanol, 60% methanol, 5cm^3 absolute methanol.


50
0
a
50
0
b
50
0
c
50
Radioactivity in fraction (% of total recovered)
Total volume eluted (cm3)

eluted in the first two methanol-containing fractions (Figure 9 (c)). This figure was 34% in a leaf sample in which a C18 SEP-PAK cartridge had been used (Figure 9 (b)) and 15% in standard [2-^{14}C] ABA (Figure 9 (a)). Thus the elution of [2-^{14}C] ABA from silica SEP-PAK cartridges was affected by the nature or amount of other components of the applied extract, in a similar manner to the elution of [2-^{14}C] ABA from C18 SEP-PAK cartridges. Care was taken, therefore, to retain all the methanol-containing fractions in the purification of sample extracts.

1.1.2 The Identification of ABA in Plant Extracts

ABA was identified in a methylated extract from *Alnus* root nodules by full mass-spectral analysis and by GCMS/MIM. The presence of ABA in leaves, roots, nodules and apices of *Alnus* was investigated by GCECD and GCMS/SIM. Finally, proof was sought of the identity of the putative *cis* ABA Me peak following GCECD analysis, by UV isomerization.

1.1.2.1 Mass-spectral Analysis

Identification of *cis* ABA was by mass-spectral analysis in an extract prepared from over 100g f.wt. of nodules from greenhouse-grown plants. The relative abundances of the principle fragment ions produced by electron impact of an authentic methylated *cis* ABA standard and the methylated nodule extract are shown in Figures 10 (a)and 10 (b)respectively; the two spectra show strong similarities.

1.1.2.2 Analysis by GCMS/MIM

Further identification of *cis* ABA in a methylated nodule extract was carried out by multiple-ion monitoring. Ions with a m/z ratio of 125, 134, 162 and 190 were detected in the sample by co-chromatography with the same ions produced by the *cis* ABA present in a methylated synthetic ABA standard (see Figures 11 (a) and (b) respectively).

Figure 10. Relative abundance of the principle fragment ions with given m/z values obtained following mass-spectral analysis of (a) standard cis ABAMe, (b) a methylated nodule extract. See text for details of operating conditions.

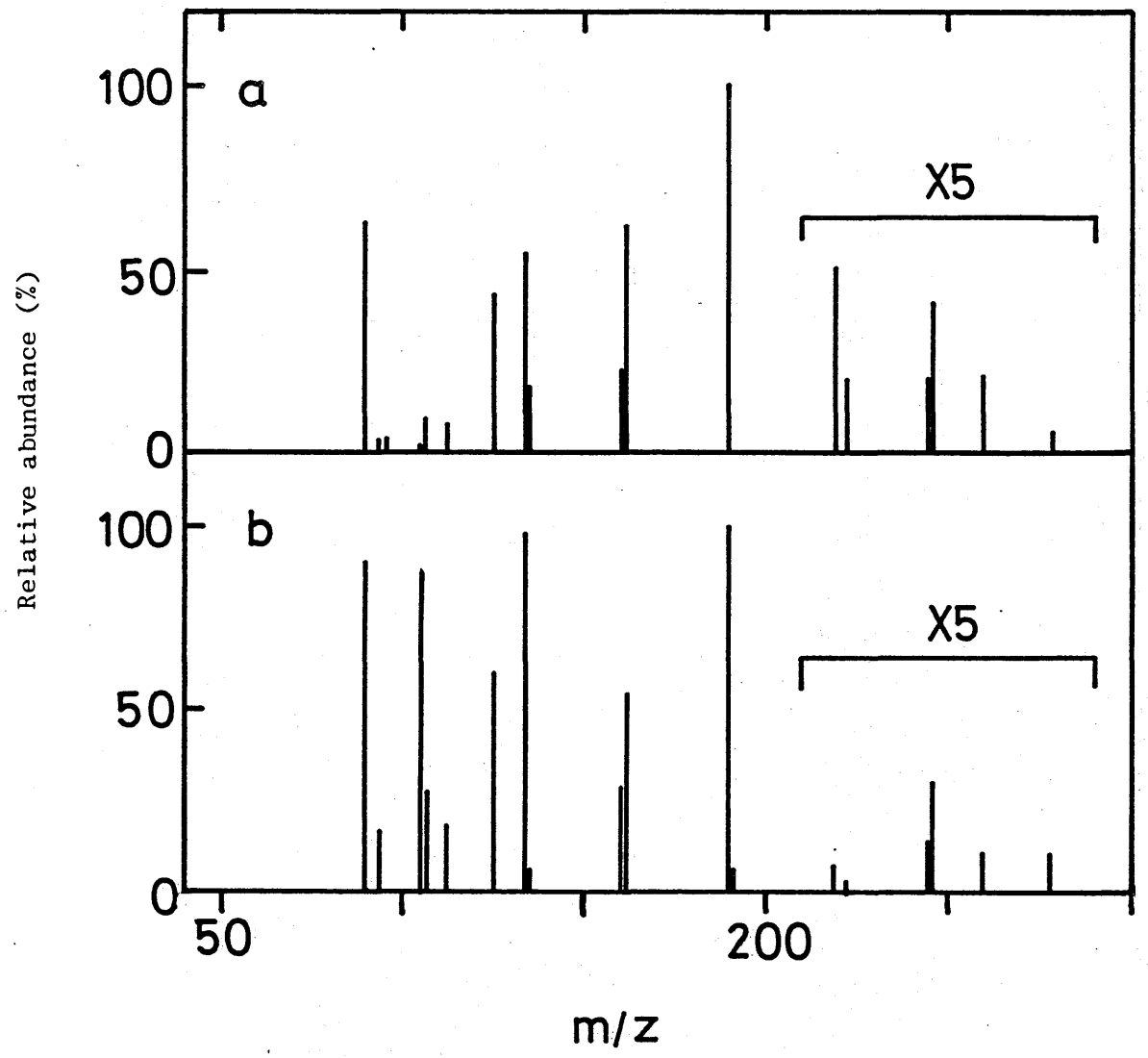
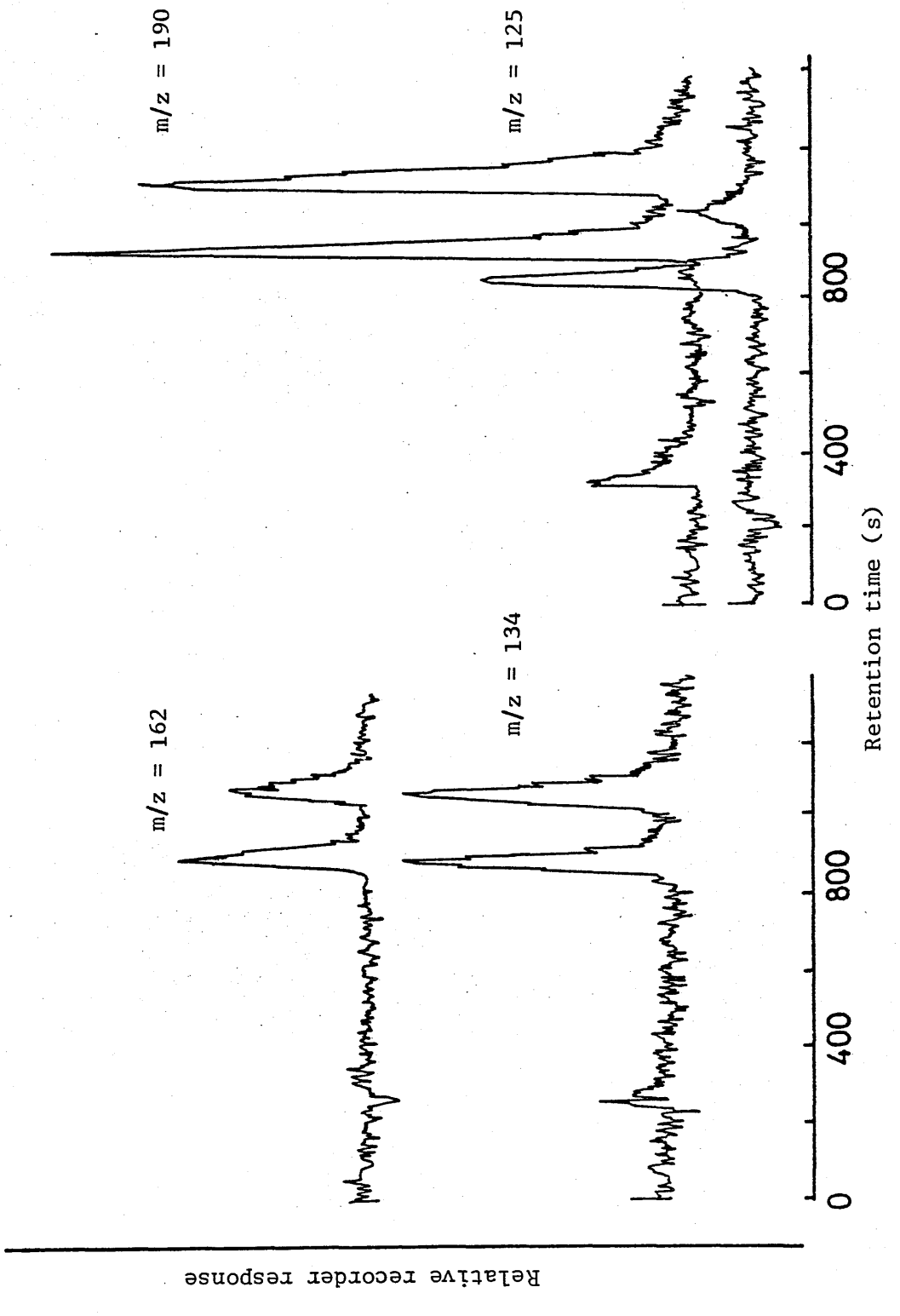

100
a
X5
50
0
100
b
X5
50
0
50
200
m/z
Relative abundance (%)

Figure 11. Multiple ion monitoring of (a) synthetic cis trans, trans trans ABAMe and (b) a nodule extract. The ionizing voltage was 24 V. m/z values monitored and attenuator settings were as follows:-

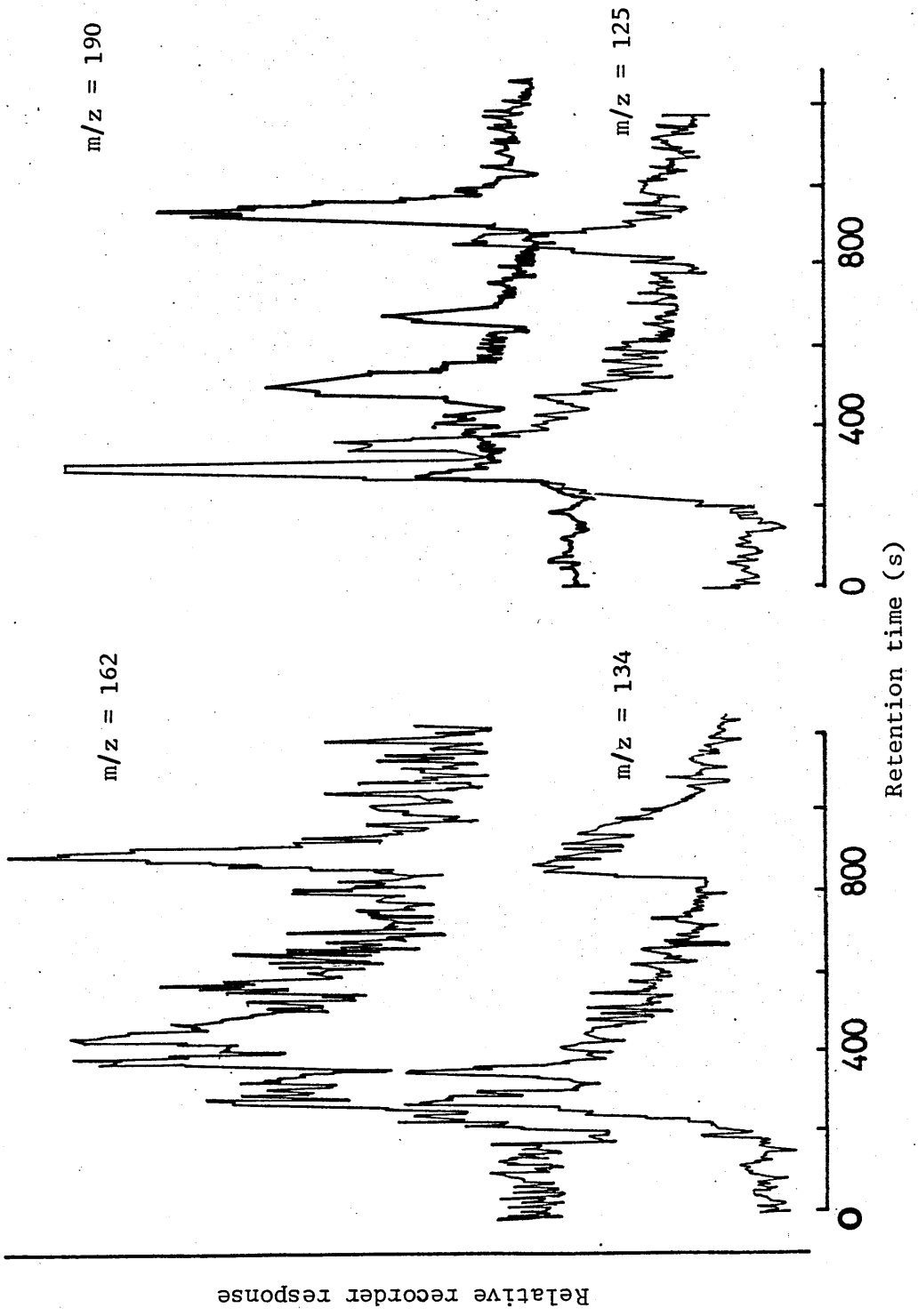
m/z		
	125	(x 10)
	134	(x 10)
	162	(x 3)
	190	(x 10)

The collector was operated at maximum sensitivity (x 7.2). See text for further details of operating conditions.

11(a)


m/z = 190
m/z = 125
m/z = 162
m/z = 134
0
400
800
Retention time (s)
Relative recorder response

11(b)


m/z = 190
m/z = 125
m/z = 162
m/z = 134
0
400
800
Retention time (s)
Relative recorder response

1.1.2.3 Analysis by GCECD and GCMS/SIM (m/z 190)

Other methods of identifying cis ABA were by GCECD and also GCMS using single-ion monitoring for the parent ion of ABA (m/z = 190). ABA was identified in extracts of all tissues examined (leaf, root, nodule and apex) by co-chromatography with synthetic ABA standards. Trans ABA was identified in all extracts purified for 'total' ABA by the same method, and was also present in some extracts purified for 'free' ABA.

GCECD and GCMS (m/z = 190) analysis of the same extract is shown for a sample purified for 'total' ABA from nodule material (Figure 12); chromatograms of authentic synthetic ABAMe are shown alongside each. Trace amounts only of trans ABA were detected in apex, leaf and nodule extracts purified for 'total' ABA; root extracts of plants grown in water culture, however, contained more trans than cis ABA.

1.1.2.4 Analysis by GCECD of a UV-treated Sample

The identity of the putative cis MeABA peak following GCECD analysis of a nodule extract was further confirmed by treatment of the extract for 5 minutes with UV light. This resulted in the enhancement of the trans ABA peak in the sample, and in the appearance of trans ABA in the cis ABA standard (Figures 13 (a) - (d)). A degree of ABA photolysis was observed, however, after the treatment (i.e. there was a reduction in the total height of the cis and trans ABA peaks following isomerization).

1.1.3 The Quantification of ABA in Plant Extracts

A number of steps were taken to ensure that as many of the vagaries as possible involved in the quantification of ABA levels in plant extracts were accounted for. Purification losses were quantified by using [2-^{14}C] ABA, and estimates of extract ABA content made following GCMS/SIM (m/z = 190) and GCECD analyses were compared. The purity of the putative ABA peak on GCECD chromatograms was investigated by the method of 'successive approximations', and by simultaneous GCECD and GCFID analysis. The variation in population root, leaf and nodule ABA levels was determined. Finally, a comparison was made between 'bound' ABA levels quantified using two methods.

Figure 12. GCECD (a) and GCMS /SIM (m/z = 190), (b) analysis of the same nodule extract and synthetic cis trans, trans trans ABAMe. During the ECD analysis, the zone temperature was 350°C, and the oven temperature was programmed to increase following an initial period at 175°C of 300s to 260°C at 0.65°C s^{-1} with a 240s period at 260°C. The column was a 10m flexible fused silicone capillary wall-coated with methyl silicone fluid and the carrier gas (H_2) flow rate was 58.8cm s^{-1}. See text for details of GCMS /SIM (m/z = 190) analysis.

The volumes of samples and standards injected, the attenuation settings used and the extract dilutions are tabulated below:-

	Volume Injected mm^3	Attenuation	Dilution of Extract (mm^3)	Standard Concentration (ng mm^{-3})
GCECD nodule extract	0.4	x 8	100	-
GCECD standard	0.4	x 8	-	4.0
GCMS /SIM m/Z = 190 nodule extract	2	x 3	50	-
GCMS /SIM m/z= 190 standard	1	x 3	-	40

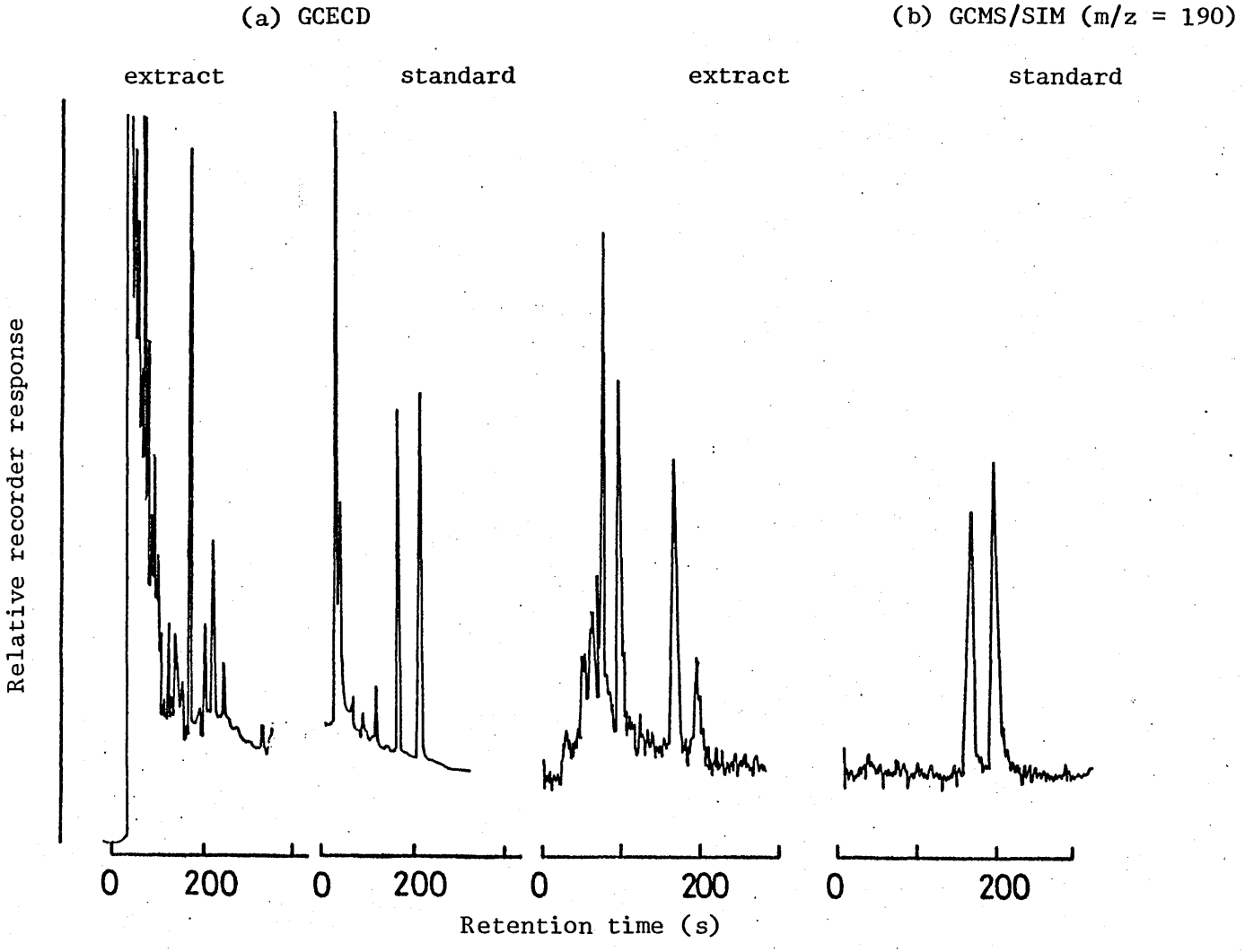
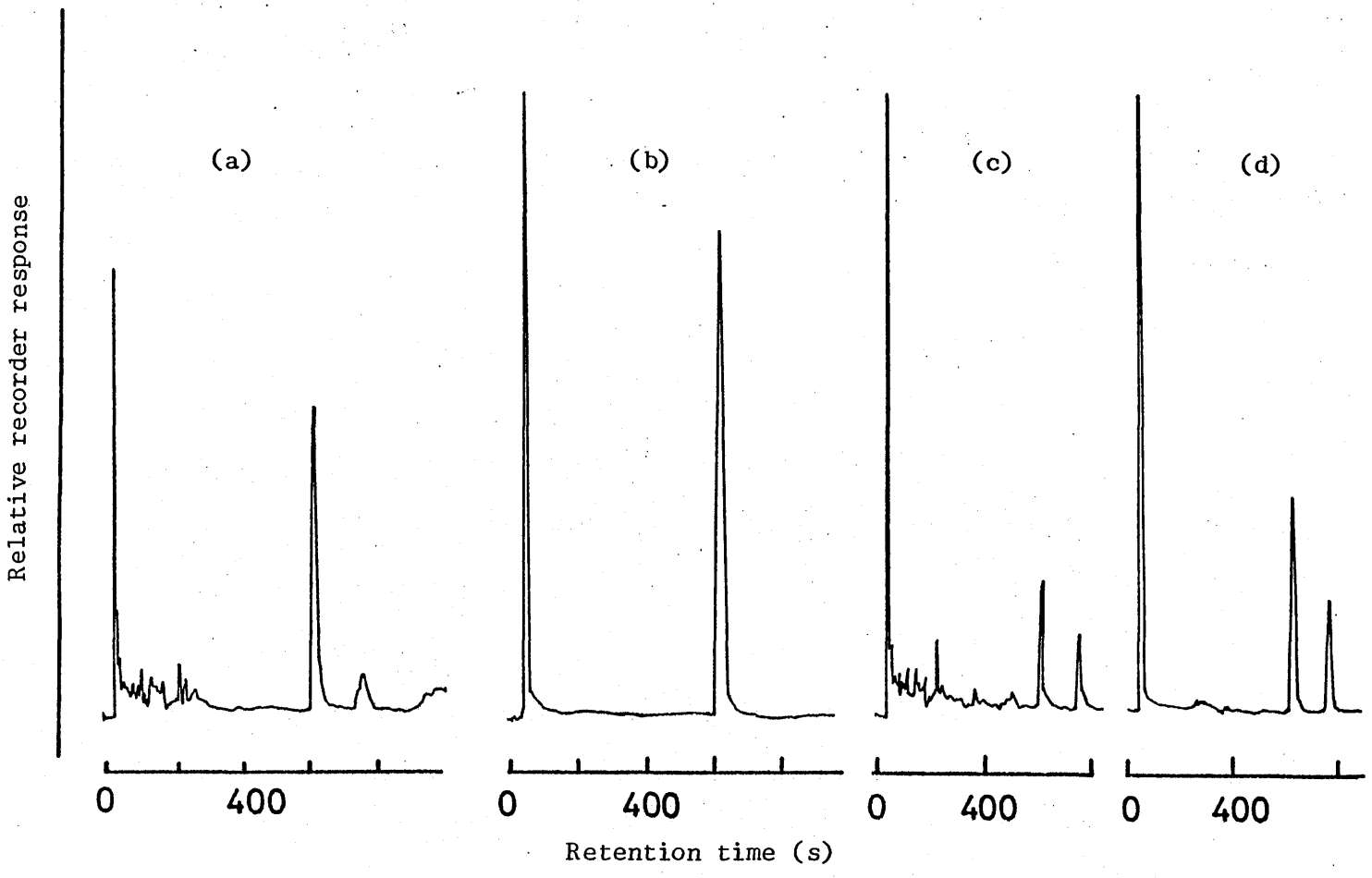

(a) GCECD
(b) GCMS/SIM (m/z = 190)
extract
standard
extract
standard
Relative recorder response
0
200
0
200
0
200
0
200
Retention time (s)

Figure 13. GCECD traces of a methylated nodule extract and authentic cis ABA standard prior to (traces (a) and (b)) and following (traces (c) and (d)) exposure to a UV source. The zone temperature was maintained at 300°C whilst the oven temperature was programmed to increase at 0.33°C s^{-1} to 240°C for 120s from an initial temperature of 220°C held constant for 840s. The column was a 20m long glass capillary, support-coated with SP2250. The ECD attenuation was X128 in all cases, and the sample volume introduced onto the column was 0.4mm^3. Trace (a) shows a methylated nodule extract, and trace (c) depicts the same extract following 300s irradiation from a UV source (UV 254nm). Traces (b) and (d) show methylated standard cis ABA (4ng mm^{-3}) prior to and following irradiation with UV light respectively.


Relative recorder response
(a)
(b)
(c)
(d)
0
400
0
400
0
400
0
400
Retention time (s)

1.1.3.1 The Recovery of [2-^{14}C] ABA added as an Internal Standard

[2-^{14}C] ABA was added to extracts following homogenization, to permit the quantification of ABA losses incurred during purification. An indication of the magnitude of these losses at several steps during this procedure is given in Table 8 . In the nodule extracts examined, losses were generally smaller during steps towards the end of the schema when the sample was cleaner. Final recovery of [2-^{14}C] ABA was generally between 25 to 60% during the purification of extracts for 'free' ABA, and between 15 to 50% during that for total ABA.

1.1.3.2 Tests of the Accuracy of Analysis

A paired 't' test demonstrated that there was no significant difference (at the 5% level) in ABA levels quantified by GCECD or GCMS/SIM (m/z=190) in all extracts of leaves, roots, nodules and apices analyzed by both techniques. Further verification of the accuracy of GCECD quantification of ABA (which was used for the analysis of all experimental extracts) was obtained using the technique of successive approximations. Extracts were purified, analyzed, purified further and re-analyzed, to verify that estimates of ABA levels did not alter significantly as a higher degree of sample purity was attained. This was found to be true for extracts separated on a capillary column, and detected by ECD. The quantification of extract ABA levels present in a leaf extract after two further purification steps following the initial analysis are shown in Figure 14 ; these values were very similar to the initial estimate, making it unlikely that ABA was co-chromatographing with another electrophilic compound. GCFID analysis carried out simultaneously with GCECD analysis confirmed that there were no detectable non-electrophilic compounds contributing to the GCECD ABAMe peak (Figures 15and 16).

1.1.3.3 The Variation in Plant Endogenous ABA Content

To assess the variation in the endogenous ABA content of potted plants, replicate batches of 20 plants each were harvested and the 'free' and 'total' ABA levels present in the root, nodule and leaf

Table 8. Range of recoveries of cis trans [2-^{14}C] ABA internal standard (83-400 Bq) at the given stages during the purification of 'free' ABA from extracts of 10-35g f.wt. nodules.

PURIFICATION STEPS	% RECOVERY AFTER COMPLETION OF STEP (s)	NUMBER OF EXTRACTS ON WHICH % RECOVERY IS BASED
Homogenisation Extraction in methanol Solvent removal *in vacuo* Redissolution in 100mol m^{-3} phosphate buffer, pH 8.0	60 - 100	4
PVP slurry pH 8.0	90 - 115	3
Filtration (pH 3.0) through paper floc. C18 SEP PAK cartridge	89	1
Ether partitioning	84 - 98	4
Silica SEP PAK cartridge	84	1
TLC of methylated sample	81 - 99	2

Figure 14. GCECD traces of a methylated nodule extract following successive 'purification' (traces (a),(b) and (c)). Trace (d) show the detector response to 7ng synthetic *cis trans*, *trans trans* ABAMe. The zone temperature was maintained at 350°C, whilst the oven temperature was programmed to increase at 0.133°C s^{-1} to 240°C from 160°C. The column was a 10m flexible fused silicone capillary wall-coated with methyl silicone fluid. The carrier gas was He at a flow rate of 24.4cms^{-1}. The ECD attenuation was X4 in trace (a) and X8 in traces (b), (c) and (d), and the final extract dilutions were 400mm^3 (trace (a)) and 200mm^3 (traces (b) and (c)). Part of the extract was purified following the schema shown in Figure 3 but omitting the use of a silica SEP-PAK, methylated and analyzed (trace (a)). Another part of the extract was purified exactly as outlined in Figure 3, methylated and analyzed (trace (b)), whilst the remainder was purified as detailed in Figure 3, but given an additional partitioning step against ethyl acetate (trace (c). Final extract *cis* ABA levels calculated from traces (a) - (c) were as follows :

Trace (a) : 311ng
Trace (b) : 320ng
Trace (c) : 314ng

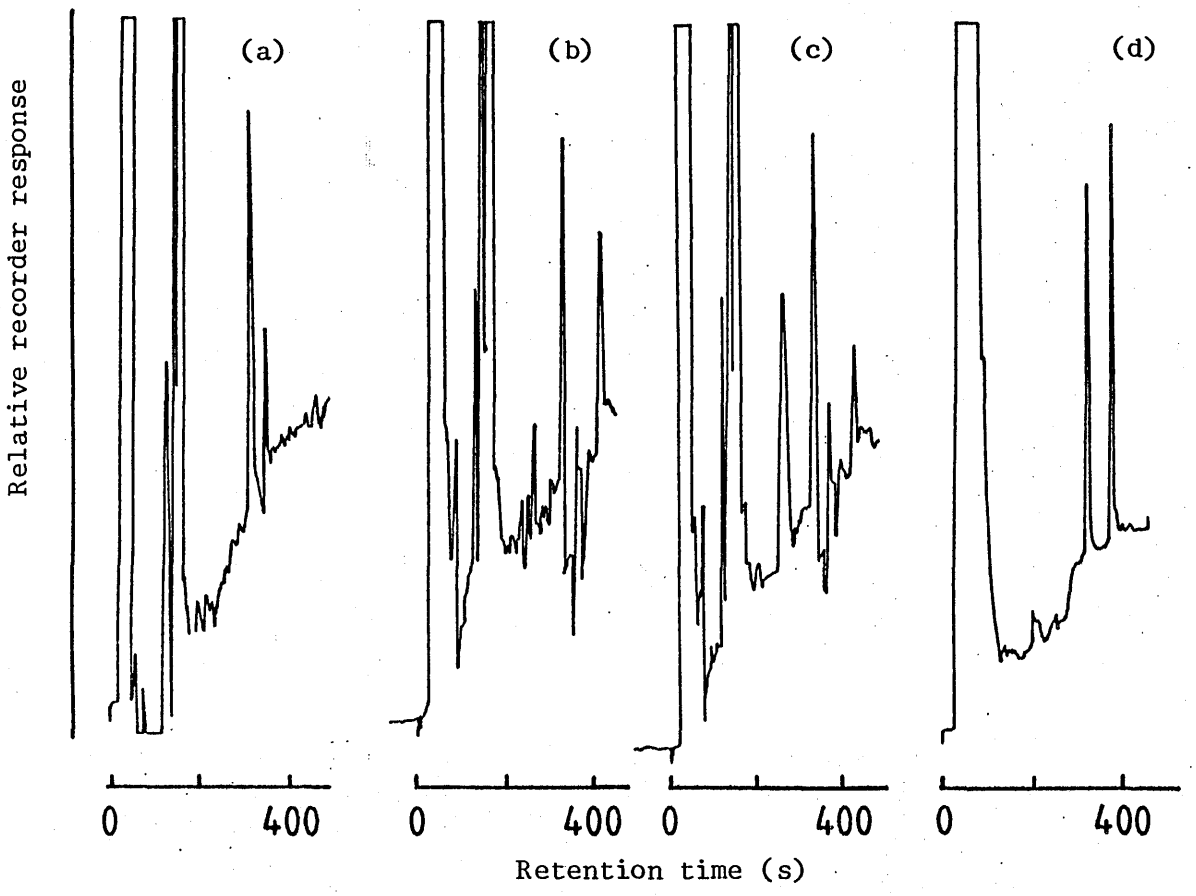

(a)
(b)
(c)
(d)
Relative recorder response
0
400
0
400
0
400
0
400
Retention time (s)

Figure 15. GCECD (a) and GCFID, (b) traces produced simultaneously of the same methylated nodule extract. Part (c) shows the GCECD response to a methylated synthetic cis trans ABA standard (representing 0.8ng cis ABAMe). The zone temperature was maintained at 350°C and the oven temperature was programmed to increase at a rate of 0.33°C s^{-1} to 280°C for 180s following an initial period of 600s at 190°C. Two 12.5m long WCOT flexible fused silicone columns were connected in parallel to permit detection by both flame ionization and electron capture detectors. The column carrier gas was H_2 flowing at a rate of 26.6cm^3 s^{-1}. Both the ECD and FID were operated at the most sensitive amplification range and at attenuations of X1. Volumes introduced onto the column were 0.4mm^3 in all cases.

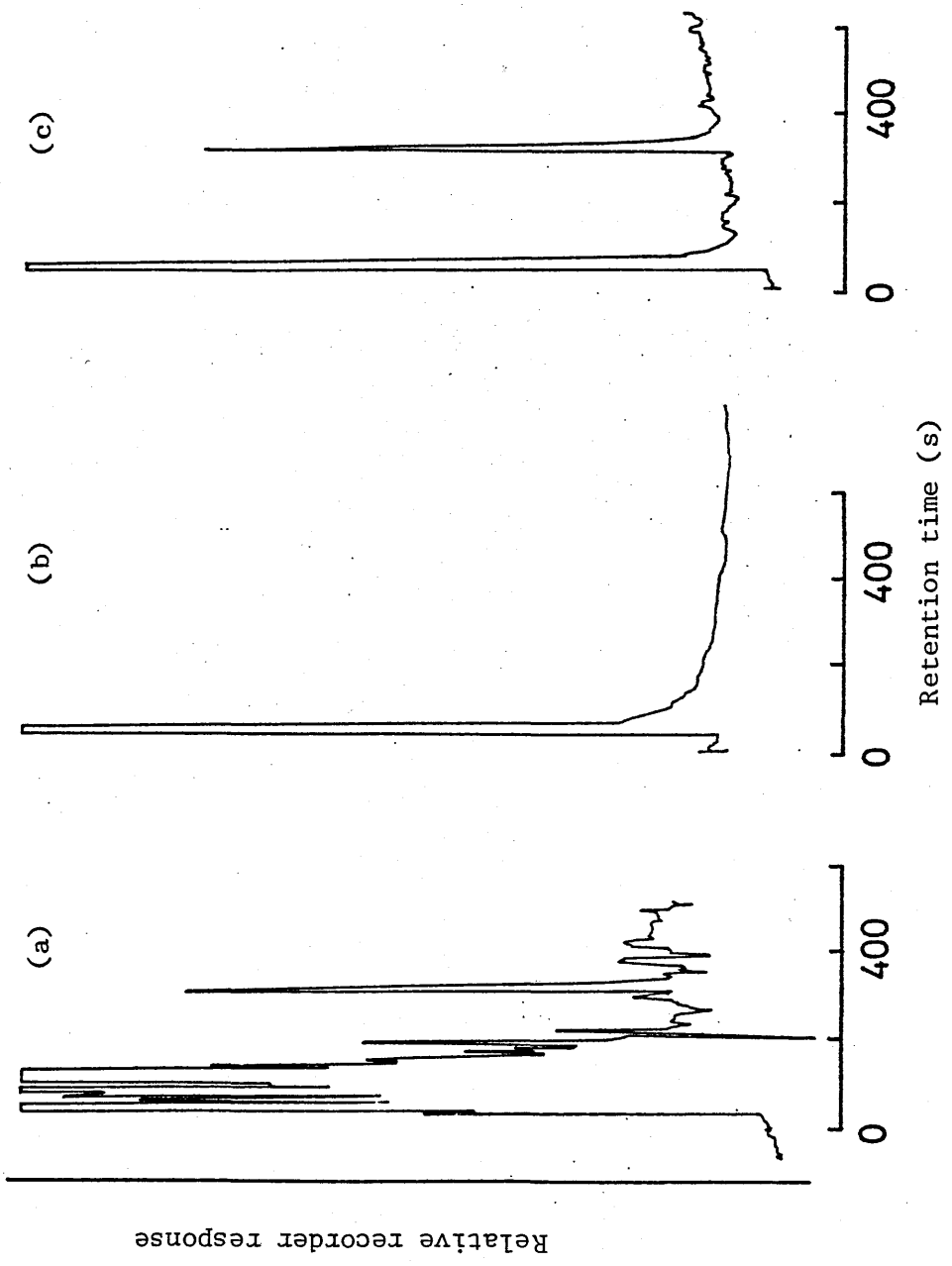
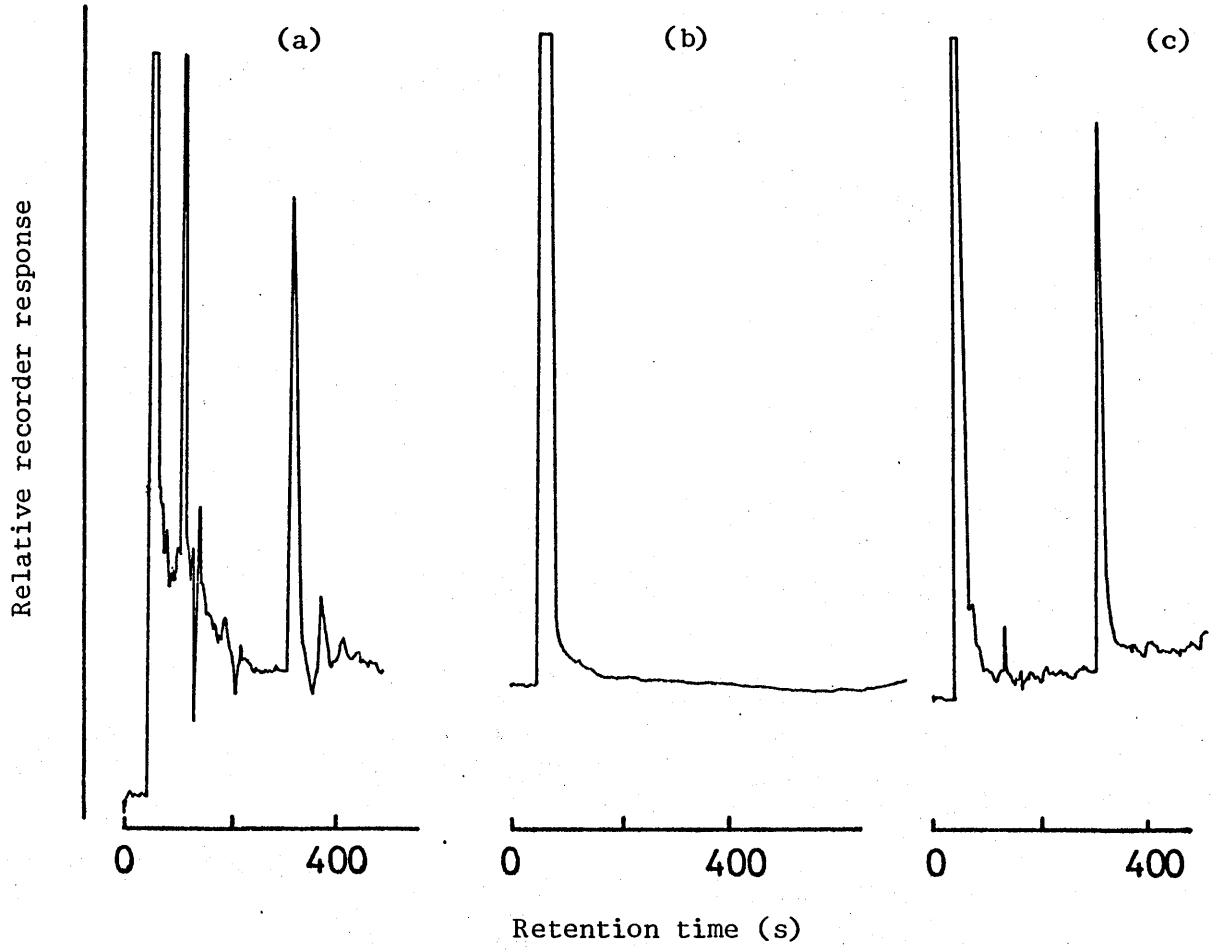

(a)
(b)
(c)
0
400
Retention time (s)
Relative recorder response

Figure 16. GCECD (a) and GCFID (b) traces produced simultaneously of a methylated leaf extract. Part (c) shows the GCECD response to a methylated synthetic cis trans ABA standard (representing 0.8ng cis ABAMe). See Figure 13 for remainder of details regarding the analysis.


(a)
(b)
(c)
Relative recorder response
0
400
0
400
0
400
Retention time (s)

material was assessed on a fresh weight basis. The data yielded by this experiment are presented in Table 9 ; the mean, standard error and coefficient of variation for 'free', 'total' and 'bound' ABA levels for each tissue are given. The largest coefficient of variation was present in the 'bound' ABA content of replicate leaf extracts. For the remaining extracts, however, this parameter fell within the range 1.85 (root 'total' ABA levels) to 30.18 (nodule 'total' ABA levels). Further statistical treatment of the complete data sets for one extract by 2-way analysis of variance demonstrated that there were no significant differences (at the 5% level) in the 'free', 'total' and 'bound' ABA content of replicate extractions. Additionally, there were no significant differences in the 'free' ABA content of the roots and nodules, although leaf 'free' ABA levels were significantly higher than those present in the root system. Differences between the 'total' ABA content of all three tissues were significant: Leaf 'total' ABA levels were significantly higher than nodule 'total' ABA levels, which in turn were significantly higher than root 'total' ABA levels.

Conjugates of ABA were more prevalent in the nodules than in the root tissue despite the fact that 'free' ABA levels were similar. Although 'total' leaf ABA levels were the highest of all, 'bound' ABA represented a smaller proportion of this figure than in nodule and root tissues.

1.1.3.4 A Comparison Between Two Methods of Quantifying 'Bound' ABA Levels

The quantification of 'bound' ABA levels is possible by the subtraction of 'free' from 'total' ABA levels, each independently assessed from two halves of a plant extract (Method I). Many authors, however, have divided extracts directly into 'free' and 'bound' ABA-containing fractions by appropriate use of a partitioning step (see Materials and Methods 3.4 for details). The two methods were compared in the quantification of 'free' and 'total' ABA levels in a root and nodule extract (Table 10). In the root extract, there was good agreement between extract 'free' and 'bound' ABA levles assessed using the two techniques. The correspondence was not as good for

Table 9. The endogenous ABA content (ng g^{-1} f.wt.) of leaves, roots and nodules from replicate batches of 20 plants each. 'Bound' ABA levels were assessed indirectly from 'free' and 'total' ABA measurements.

ABA LEVELS ng g^{-1} f.wt.

BATCH	NODULES			ROOTS			LEAVES		
	Free	Total	Bound	Free	Total	Bound	Free	Total	Bound
1		474.8		26.4	98.5	72.0	541.9	578.9	37
2	30.7	238.4	207.6	34.4	102.2	67.7	432.7	752.9	320.2
3	32.7	328.3	295.5	39.4	100.6	61.2	438.2	633.7	195.5
4	25.2	241.8	216.6	35.4				494.9	
5	18.4	308.4	289.9				538.6	509.7	0

Mean ng g^{-1} f.wt.	26.7	318.3	252.4	33.9	100.4	66.9	485.3	594.0	138.3
SE	3.20(4)	42.97(5)	23.37(4)	2.72(4)	1.07(3)	3.14(3)	28.96(4)	46.89(5)	74.02(4)
CV	23.95	30.18	18.52	16.07	1.85	8.13	11.93	17.65	107.12

Table 10. Comparison between measurements of 'free' and 'bound' ABA made directly from a single extract or indirectly from two extracts processed separately for 'free' and 'total' ABA.

ABA LEVELS (ng g^{-1} f.wt.)

	SINGLE EXTRACT		TWO EXTRACTS	
	'Free'	'Bound'	'Free'	'Bound'
Roots	33.3	35.3	39.7	26.2
Nodules	45.1	179.0	23.5	236.2

the nodule extract, however, in which estimates of 'free' ABA levels were smaller and 'bound' ABA levels larger when assessed by Method I. This most probably reflects incomplete partitioning of the extract during Method II; it seems unlikely that 'free' extract ABA levels were underestimated in Method I. In all experimental analyses, Method I was the chosen technique for the quantification of 'bound' ABA levels.

1.2 Experiments Involving the Quantification of Endogenous ABA in Alnus

1.2.1 The Influence of Seasonal Changes in the Environment on Nodule Nitrogenase Activity and Root and Nodule Endogenous ABA Levels

To establish whether or not seasonal changes in nitrogenase activity correlated with nodule 'free' abscisic acid content, both variables were studied in plants 3 - 4y old growing under natural photoperiods in an unheated greenhouse. Nitrogen fixation was monitored during 1980/1981, whilst nodule and root ABA levels were examined at intervals from 1980-1983. A record of fluctuations in PAR was also made throughout most of the occasions on which ABA harvests were made (Figure 17).

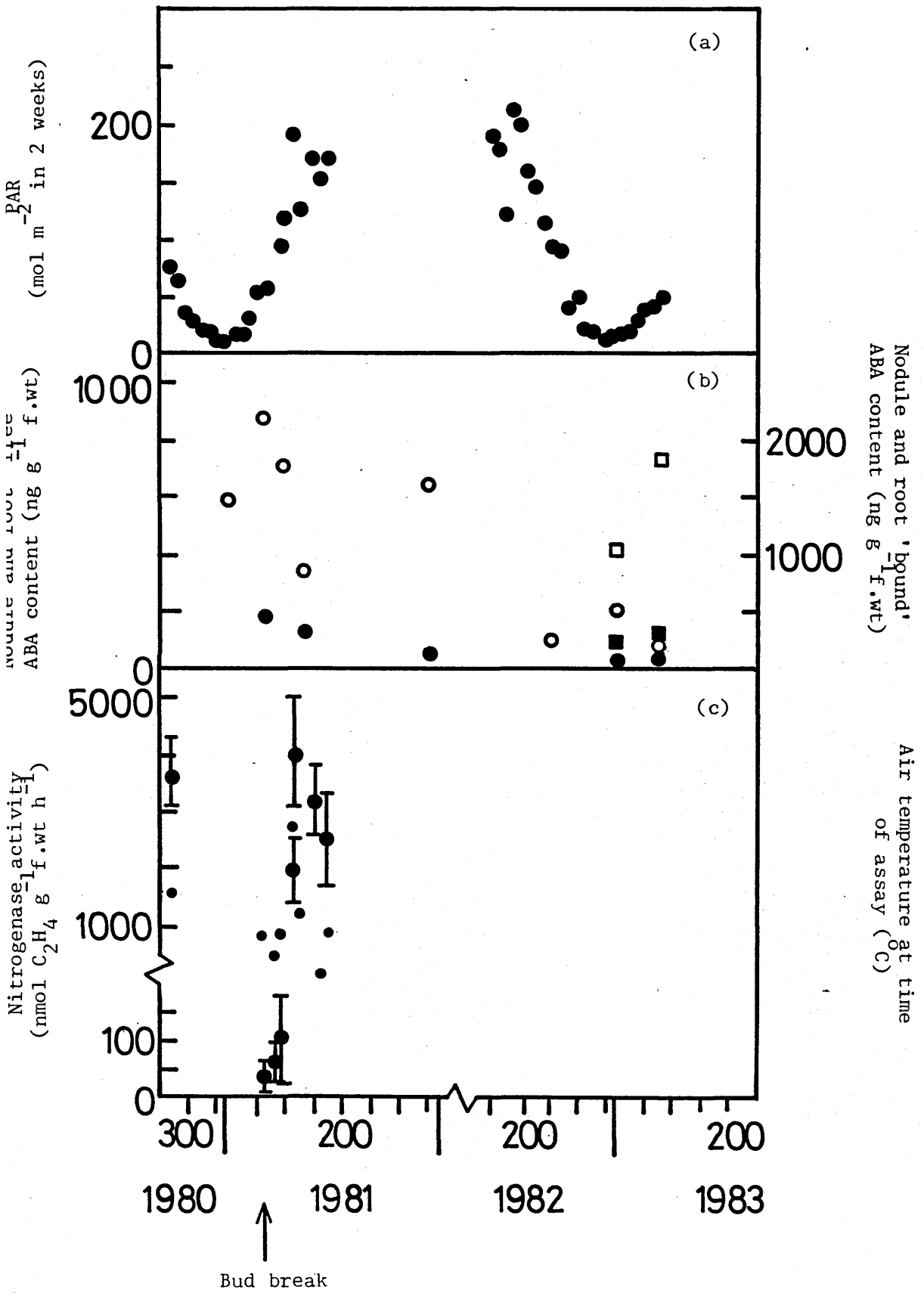
1.2.1.1 Seasonal Changes in PAR

The lowest levels of PAR were recorded during December and January (Figure 17); as little as 12.48 mol m^{-2} 2 weeks $^{-1}$ was recorded in December 1980. Values some 17 times greater were observed in mid June 1982, when levels reached 214.7 mol m^{-2} 2 weeks $^{-1}$.

1.2.1.2 Seasonal Changes in Nitrogenase Activity in Plants Growing Under Natural Photoperiod

Activity was first detected in March 1981 (7d prior to bud break which occurred on (day 77)), following the winter period in which no nitrogen fixation took place. The maximum value recorded in the assays made was 4078.9 $\pm$ 922.5 nmol $C_2H_4h^{-1}g^{-1}$ f.wt. nodules, which occurred in April. This followed PAR values of over 60 mol m^{-2} $week^{-1}$ in the preceding 4 weeks. Nitrogen fixation was also high towards the end of the

Figure 17. Seasonal fluctuations in (a) Photosynthetically Active Radiation (PAR) (mol m^{-2} $week^{-1}$) (b) nodule 'free' (symbol o) and 'bound' (symbol □) ABA levels (ng g^{-1} f.wt.); root 'free' (symbol ●) and 'bound' (symbol ■) ABA levels (ng g^{-1} f.wt.) and (c) nitrogenase activity $\pm$ SE (nmol C_2H_4 g^{-1} f.wt. h^{-1}) and air temperature (•) (°C) at time of assay.


(a)
200
0
PAR (mol m⁻² in 2 weeks)
(b)
1000
0
Nodule and root 'bound' ABA content (ng g⁻¹ f.wt)
2000
1000
(c)
5000
1000
100
0
Nitrogenase activity (nmol C2H4 g⁻¹f.wt h⁻¹)
300
200
200
200
1980
1981
1982
1983
Bud break
Air temperature at time of assay (°C)

1980 season, when an activity of 3732.2 ± 580 nmol $C_2H_4h^{-1}g^{-1}$ f.wt. nodules was observed; on this occasion, however, PAR values did not exceed 45 mol m^{-2} week $^{-1}$ in the preceding fortnight.

1.2.1.3 Seasonal Changes in Root and Nodule Endogenous ABA Levels in Plants Growing Under Natural Photoperiod

During the 1981 season in which total 'free' ABA levels only, were monitored, nodule ABA content increased from 595.2 ng g^{-1} f.wt. in mid January 1981, to the maximum level recorded of 879.0 ng g^{-1} in early March at a time when nitrogenase activity had not yet been detected (Figure 17). Values had only fallen by 169.8 ng g^{-1} f.wt. however, by mid March, when nitrogenase activity had recommenced. The smallest nodule 'free' ABA level recorded in this season occurred in May; by this time, nitrogenase activity had reached levels of over 3000 nmol C_2H_4 h^{-1} g^{-1} f.wt. in the assays made. By December 1981, nodule 'free' ABA content was almost double that observed in May.

To obtain a measure of cis and trans 'free' ABA levels, and, in addition, 'bound' nodule ABA content, nodules were harvested on 2 occasions during the early months of 1983. In this season, total 'free' ABA levels did not exceed 111.1 ng g^{-1} f.wt. and decreased between early January and mid March. (Bud-break occurred several days following the second harvest date). There was no consistent relationship in the 2 harvests made between nodule 'free' cis and trans ABA levels. Total 'bound' ABA levels were between 9.23 and 23.99 times greater than nodule total 'free' ABA levels. Additionally, 'bound' cis ABA levels were 3.07 - 3.12 times greater than nodule 'bound' trans levels.

Root total 'free' ABA levels were between 2.87 - 11.16 times smaller than nodule total 'free' ABA levels in the 1981 season and between 1.83 - 4.16 times smaller in 1983. The 'free' trans ABA content of the roots harvested in 1983 did not exceed 6 ng g^{-1} f.wt. . Relative changes in root total 'free' ABA levels did not

mirror those in the nodule tissue in either 1981 or 1983. Root total 'bound' ABA levels recorded in 1983 were 6.58 - 6.93 times greater than root total 'free' ABA levels. One again, the cis isomer was predominant: Root 'bound' cis ABA content was 1.37 - 1.44 times greater than the 'bound' trans ABA content (see Table 11).

1.2.2 Diurnal Variations in Leaf and Nodule ABA Levels

Following the observation that nodule ABA content was not static throughout the season, a study was undertaken to investigate the possibility of diurnal rhythms in nodule ABA levels. Accompanying measurements of leaf ABA content were also made. 'Free', 'bound' and 'total' (that is totalled cis and trans 'free' and 'bound') ABA levels in the nodule and leaf tissue of plants harvested on a diurnal basis are shown in Figures 18, 19 and 20. The experiment was performed three times of which the last two (experiments 2 and 3) were true replicates in that on both these occasions material growing under the controlled conditions of a growth chamber were harvested. (In the first experiment, plants were growing in a heated greenhouse.) Where absolute data concerning the replicate experiments are mentioned, those from the second experiment are presented first, and those from the third experiment are shown in brackets.

During the course of the experiment, a reduction in light levels was accompanied by an increase in relative humidity (Figures 19,(a),(b)and decrease in plant leaf water potential (Figures 20,(ii). In plants growing in a growth chamber, nodule 'total' ABA levels ranged between 703.1 - 1124.1 (504.9 - 1894.5) ng g^{-1} f.wt. whilst leaf 'total' ABA levels were between 1284.3 - 2129 (577.7 - 1428.3) ng g^{-1} f.wt. Leaf'total' ABA levels were generally greater than nodule 'total' ABA levels by between 1.34 - 2.42 (1.20 - 1.43) times, although one exception was observed (Figure 20 (ii)).

In all experiments, 'free' cis plus trans ABA levels were greater in the leaves than in the nodules (Figure 19,(i),(ii), (iv). However, the maximum 'free' cis plus trans ABA level recorded in the nodules of greenhouse-grown plants was 369.2 ng g^{-1} f.wt., whilst in plants growing in the growth

Table 11. 'Free' and 'bound' *cis* and *trans* ABA levels in the roots and nodules of *Alnus* grown in an unheated greenhouse under natural illumination. Total 'free' and total 'bound' ABA levels for these harvests are displayed in Figure 17, alongside results from the 1981 season.

DATE	TISSUE	'FREE' cis ABA	'FREE' trans ABA	'BOUND' cis ABA	'BOUND' trans ABA
8.1.83	Nodules	49.8	61.3	773.1	252
	Roots	23.3	3.4	103.8	71.9
17.3.83	Nodules	71.5	5.2	1394.0	446.0
	Roots	36.9	5.1	168.3	122.7

Figure 18. Diurnal fluctuations in leaf ⊢---⊣ and nodule ⊢——⊣ ABA levels (ng g^{-1} f.wt.):

(i) 'free' cis ABA levels
(ii) 'free' trans ABA levels
(iii) 'bound' cis ABA levels
(iv) 'bound' trans ABA levels
} Second Experiment

(a) 'free' cis ABA levels
(b) 'free' trans ABA levels
(c) 'bound' cis ABA levels
(d) 'bound' trans ABA levels
} Third Experiment

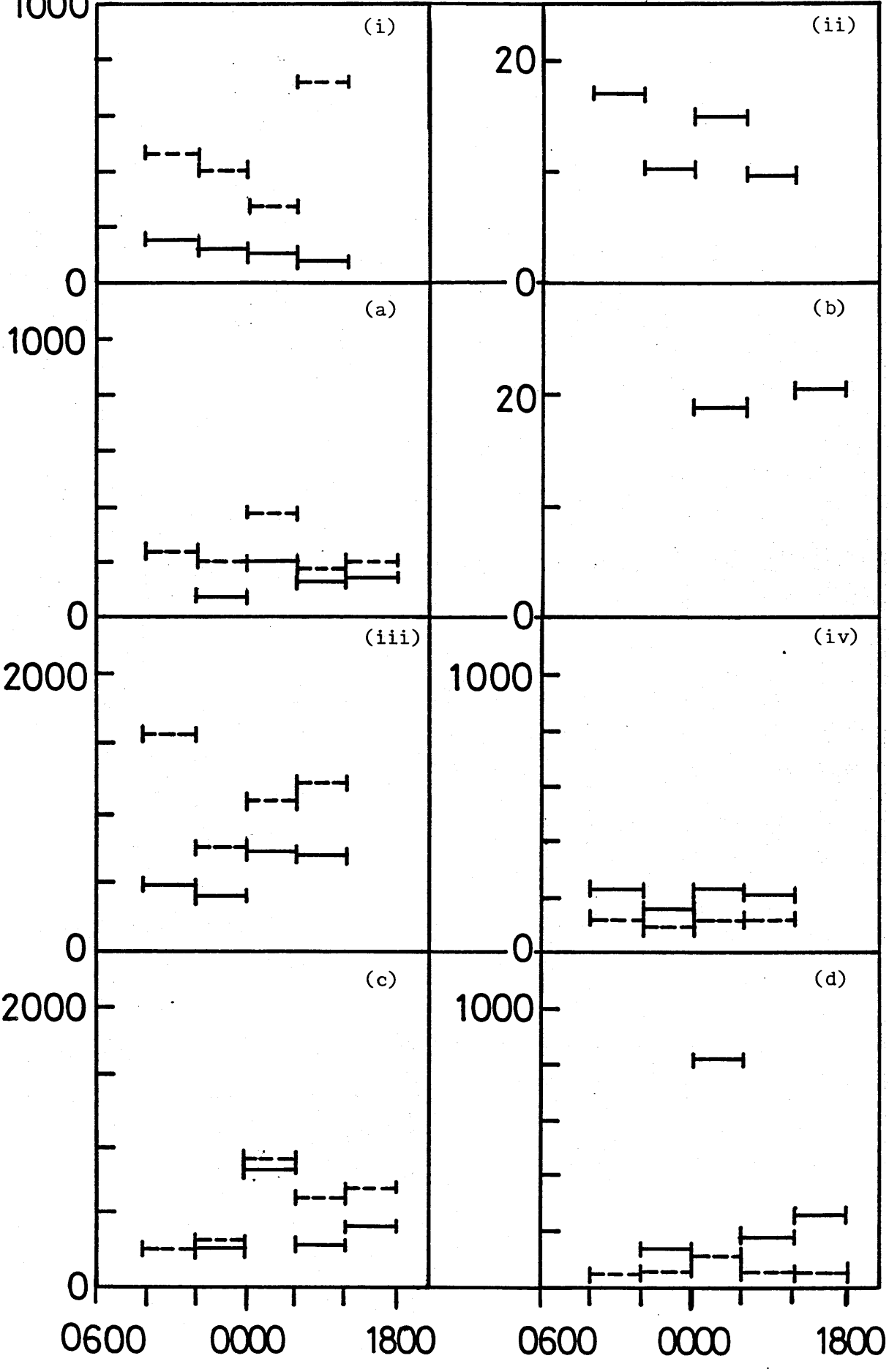

(i)
(ii)
(a)
(b)
(iii)
(iv)
(c)
(d)
1000
0
20
2000
0600
0000
1800
ABA levels (ng g⁻¹f.wt)
Time (24h clock)

Figure 19. Diurnal fluctuations in leaf ⊢---⊣ and nodule ⊢—⊣ ABA levels (ng g^{-1} f.wt.) with lighting and humidity measurements:-

A: Levels of photosynthetically active radiation (mol m^{-2} $2h^{-1}$) in experiments 1 and 2.

B: Relative humidity (%):-

Experiment 1 □
Experiment 2 ○
Experiment 3 ●

(i) 'free' cis plus trans ABA levels (First experiment)
(ii) 'free' cis plus trans ABA levels } Second experiment
(iii) 'bound' cis plus trans ABA levels }
(iv) 'free' cis plus trans ABA levels } Third experiment
(v) 'bound' cis plus trans ABA levels }

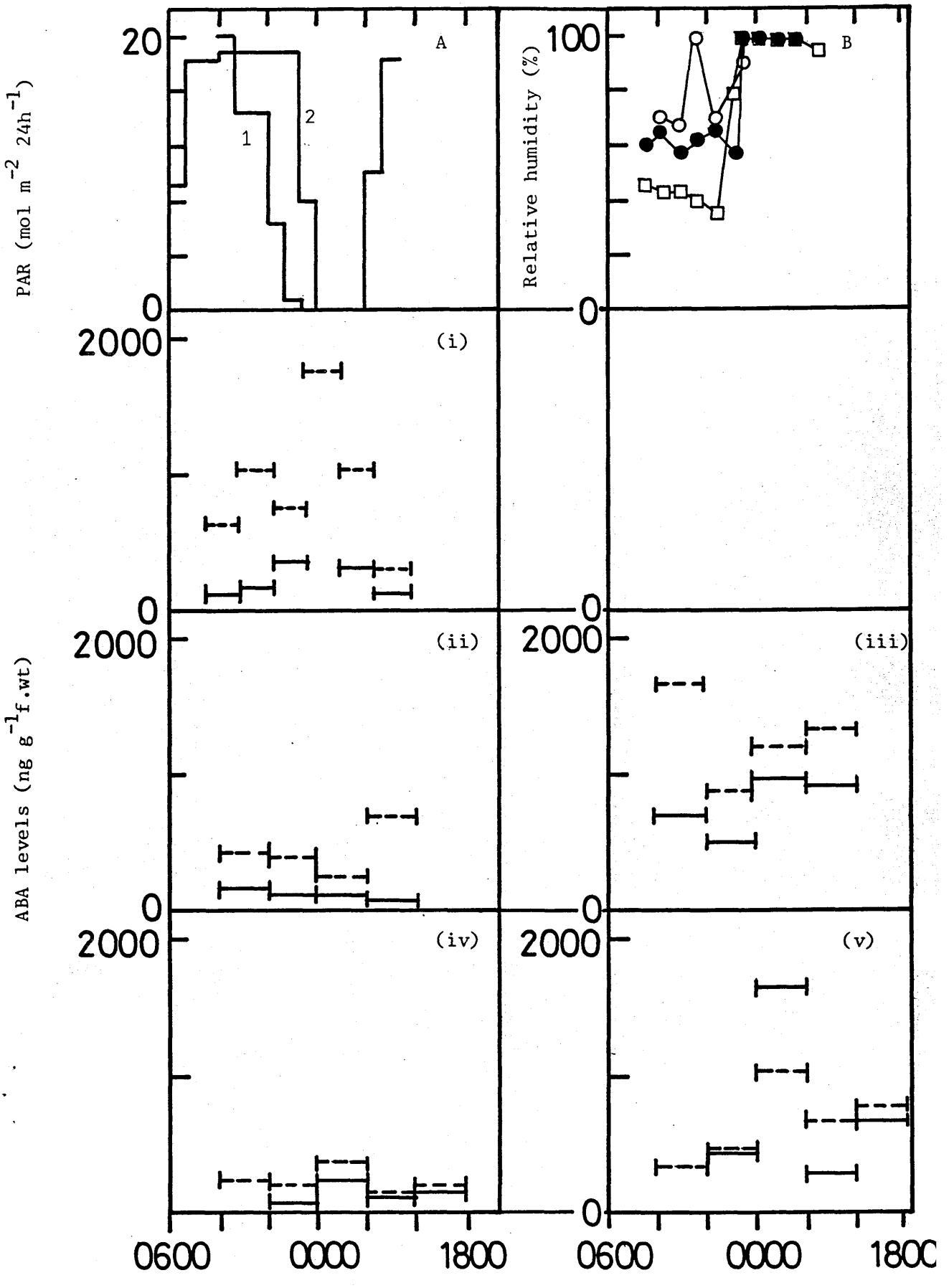
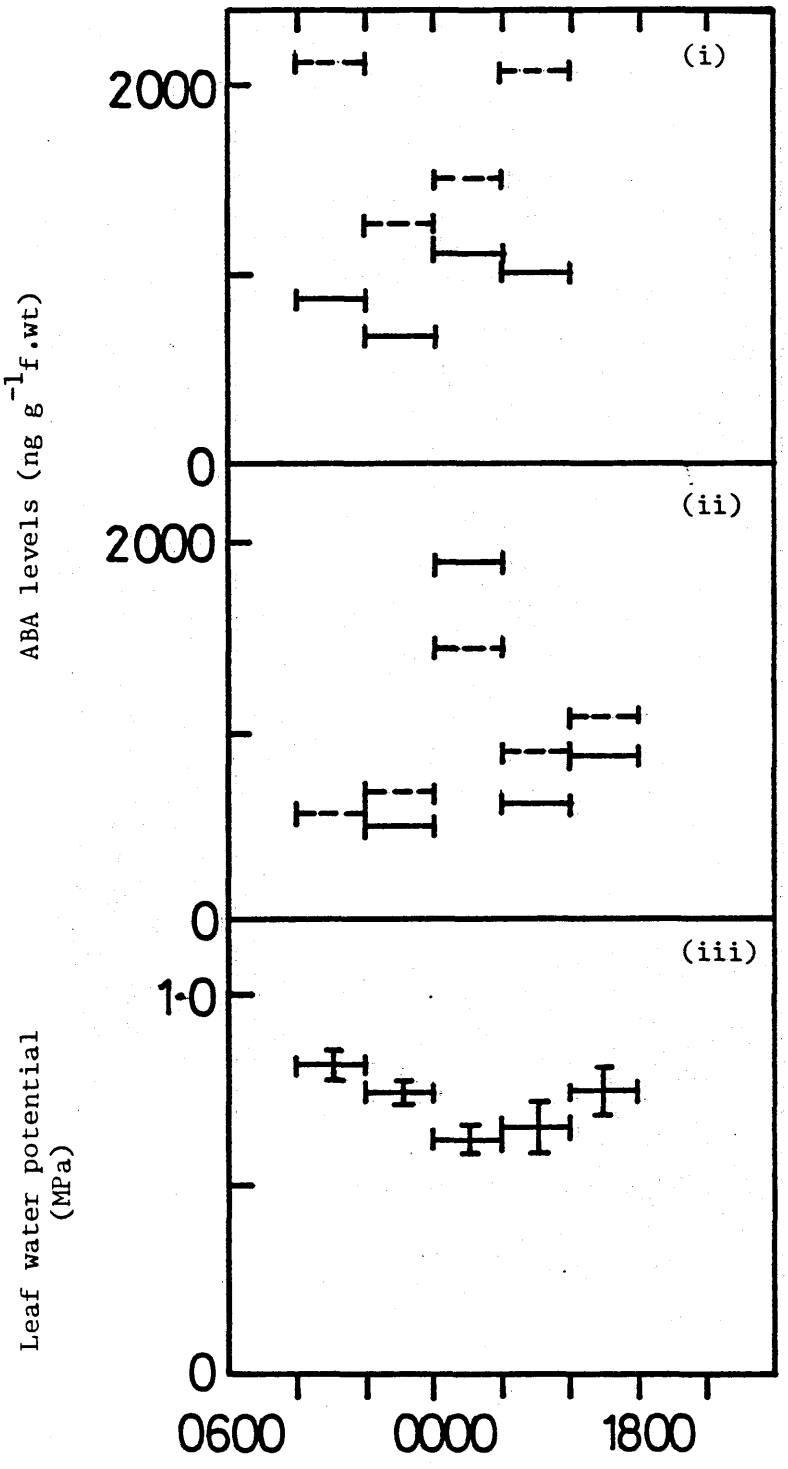

PAR (mol m^{-2} 24h^{-1})
20
0
A
1
2
Relative humidity (%)
100
0
B
ABA levels (ng g^{-1}f.wt)
2000
0
(i)
(ii)
(iii)
(iv)
(v)
0600
0000
1800
Time (24h clock)

Figure 20. Diurnal fluctuations in leaf ⊢---⊣ and nodule ⊢—⊣ ABA levels (ng g^{-1} f.wt.):-

(i) 'total' cis and trans ABA levels (Second experiment)

(ii) 'total' cis and trans ABA levels (Third experiment)

(iii) plant leaf water potential (MPa) measurements made during the third experiment on plants harvested for endogenous ABA content. Values are means of 5-13 measurements made on first fully expanded leaves (from the apex).


(i)
2000
0
(ii)
2000
0
(iii)
1·0
0
0600
0000
1800
ABA levels (ng g⁻¹f.wt)
Leaf water potential (MPa)
Time (24h clock)

chamber, this value was 234.4 ng g^{-1} f.wt. Higher leaf 'free' cis plus trans ABA levels were also present in the greenhouse-grown plants (in which a maximum of 1768 ng g^{-1} f.wt. was recorded) than in those growing in a controlled environment in which a maximum of 724.6 ng g^{-1} f.wt. was recorded (Figure 19,(i),(ii), (iv)).'Bound' cis plus trans ABA levels were higher than 'free' cis plus trans ABA levels by up to 9.75 (7.08) times in the nodules and by up to 4.43 (3.93) times in the leaves of plants growing in the growth chamber (Figure 19, (iii), (v)).

Levels of 'free' trans ABA recorded in the nodule material of plants growing under growth-room conditions were less than 21 ng g^{-1} f.wt., whilst no 'free' trans ABA at all was detected in leaf extracts (Figure 18 (b),(ii)). Amounts of 'free' cis ABA present in the leaves, however, (which ranged between 277.7 - 724.6 (182.7 - 375.6) ng g^{-1} f.wt.) were between 2.47 - 8.56 (1.50 - 2.79) times greater than those found in the nodules (Figure 18 (a), (i)). Additionally whilst leaf 'bound' cis ABA levels recorded in extracts throughout the course of the experiment (ranging from 778.6 - 1552.0 (286.0 - 926.6) ng g^{-1} f.wt.) always exceeded nodule 'bound' cis ABA levels by a factor of between 1.53 - 3.27 (1.09 - 2.05) (Figure 18 (c),(iii))the reverse was true for amounts of 'bound' trans ABA present in individual extracts from the two tissues (Figure 18 (d),(iv)). Thus whilst 0.30 - 0.49 (0.48 - 0.97) times as much trans as cis ABA was present as a conjugate in nodule extracts, this figure was only 0.08 - 0.13 (0.08 - 0.21) times as much in leaf extracts (Figure 18,(c), (d),(iii),(iv)), leading to absolute nodule 'bound' trans ABA levels which ranged from 160.7 - 265.0 (140.6 - 816.1) ng g^{-1} f.wt., some 1.57 - 2.32 (1.73 - 6.66) times greater than those measured in leaf tissue extracts (Figure 18,(d) (iv)).

Statistical analyses were carried out using the data from experiments 2 and 3 to investigate whether or not there were any significant differences in leaf and nodule 'free' and 'bound' ABA levels. A 2-way analysis of variance was conducted for each tissue to determine whether there were any differences in absolute ABA levels between experiments or between the harvests made throughout the course of any one experiment.

The analyses showed that there were no significant differences (at the 5% level) in leaf or nodule cis plus trans 'free' or 'bound' ABA levels (a) between replicate experiments or (b) on a diurnal basis.

1.2.3 The Effect of Root-applied 0.1mol m^{-3}(±) ABA on the Growth and Endogenous Leaf, Root, Nodule and Apex ABA Content of Plants Growing in Water Culture

The importance of ABA in the control of growth and bud dormancy in Alnus was investigated by exogenous application of a 0.1 mol m^{-3} ABA solution to the roots of plants growing in water culture, followed by an assessment of endogenous tissue ABA levels. This experiment was performed twice; where results are mentioned in the following text, those of the first experiment are presented first with those from the second experiment shown in brackets. 'Free', 'bound' and 'total' (that is totalled cis and trans 'free' and 'bound') leaf, root, nodule and apex ABA levels are presented in Figures 21,22,23 and 24.

1.2.3.1 Endogenous Leaf, Root, Nodule and Apex ABA Content of Plants Treated with 0.1mol m^{-3} ABA Via the Culture Solution

'Total' ABA levels present in all tissues examined were greater in plants that had been treated with ABA (Figure 24). In leaf, root, nodule and apex tissues, ABA treatment resulted in endogenous ABA levels 29.67 (217.71), 89.68 (611.42), 119.02 (290.13) and * (12.03) times greater respectively than those recorded in the same tissues of control plants. With only one exception (which occurred in the second experiment only), all the tissues examined of plants treated with 0.1mol m^{-3} ABA contained higher levels of both cis plus trans 'free' and cis plus trans 'bound' ABA than those of control plants (Figure 23). Levels of cis plus trans 'free' ABA in the leaf, root-nodule and apex tissues of ABA treated plants were 6.08 (49.39), 51.62 (230.69), 37.04 (19.76) and 6.57 (0.63) times greater than those present in control plants. Levels of cis plus trans 'bound' ABA in the leaf, root, nodule and apex tissues

* missing value

Figure 21. Levels of abscisic acid (ng g^{-1} f.wt.) in plants treated with 0.1mol m^{-3} (±) ABA via the root nutrient solution and in control plants also growing in water culture:

(i) 'free' *cis* ABA levels in control plants
(ii) 'free' *trans* ABA levels in control plants
(iii) 'free' *cis* ABA levels in ABA treated plants
(iv) 'free' *trans* ABA levels in ABA treated plants

} First experiment

(a) 'free' *cis* ABA levels in control plants
(b) 'free' *trans* ABA levels in control plants
(c) 'free' *cis* ABA levels in ABA treated plants
(d) 'free' *trans* ABA levels in ABA treated plants

} Second experiment

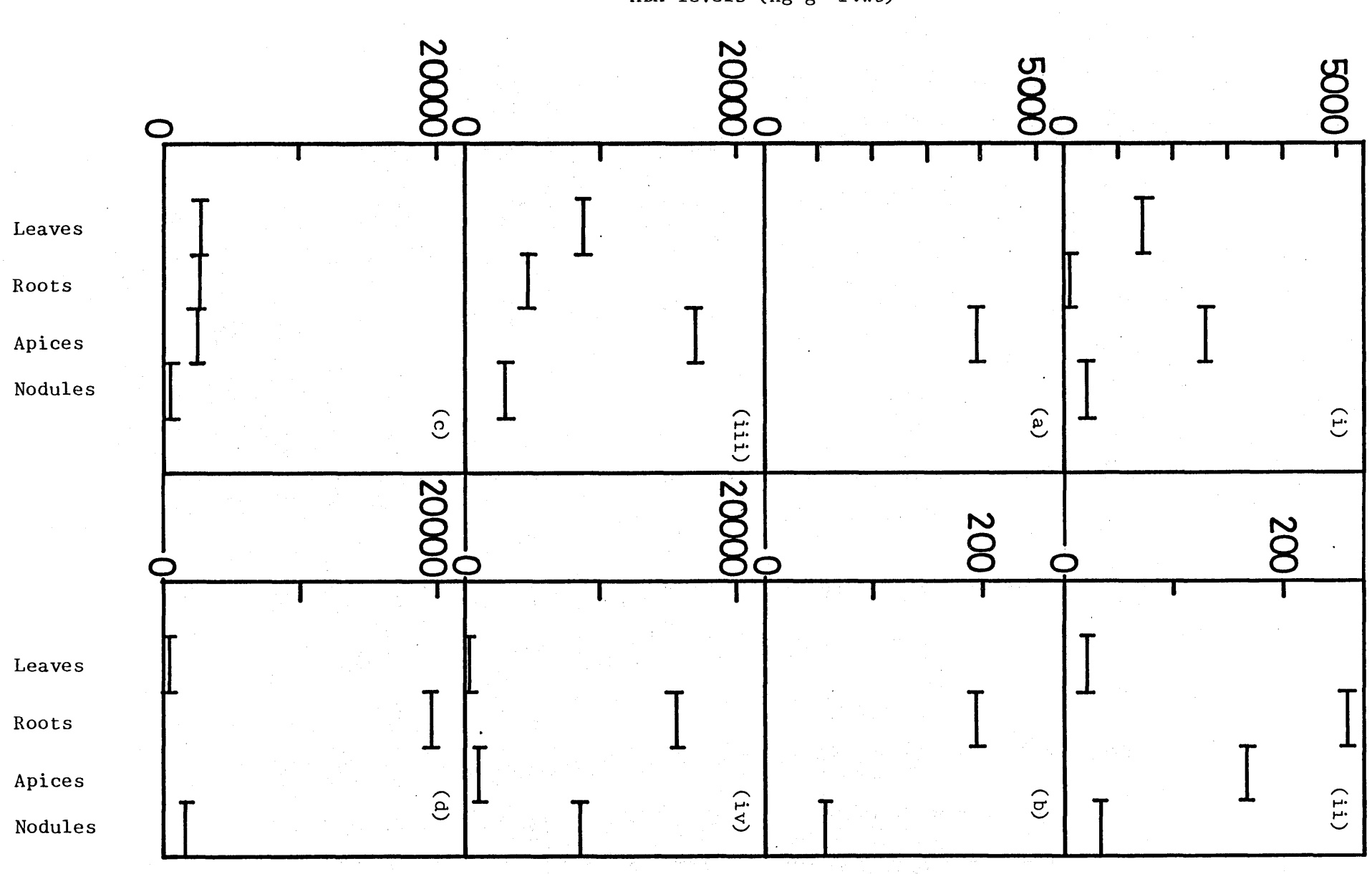

ABA levels (ng g^{-1}f.wt)
0
20000
0
20000
0
5000
Leaves
Roots
Apices
Nodules
(c)
(iii)
(a)
(i)
0
20000
0
20000
0
200
0
200
Leaves
Roots
Apices
Nodules
(d)
(iv)
(b)
(ii)

Figure 22. Levels of abscisic acid (ng g^{-1} f.wt.) in plants treated with 0.1mol m^{-3} ($\pm$) ABA via the root nutrient solution and in control plants also growing in water culture:

First experiment:
(i) 'bound' cis ABA levels in control plants
(ii) 'bound' trans ABA levels in control plants
(iii) 'bound' cis ABA levels in ABA treated plants
(iv) 'bound' trans ABA levels in ABA treated plants

Second experiment:
(a) 'bound' cis ABA levels in control plants
(b) 'bound' trans ABA levels in control plants
(c) 'bound' cis ABA levels in ABA treated plants
(d) 'bound' trans ABA levels in ABA treated plants

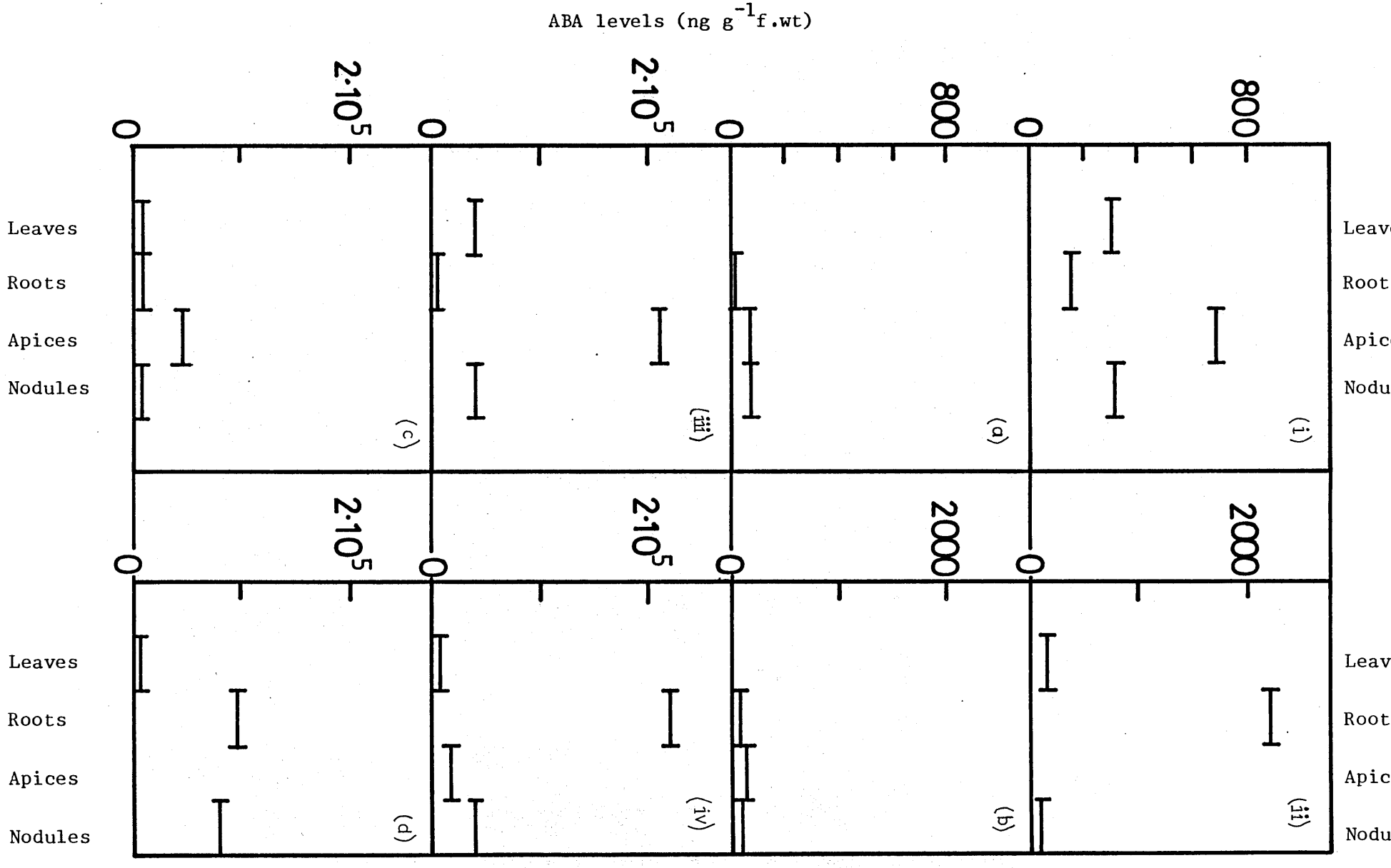

ABA levels (ng g^{-1}f.wt)
0
800
2000
2·10^5
Leaves
Roots
Apices
Nodules
(a)
(b)
(c)
(d)
(i)
(ii)
(iii)
(iv)

Figure 23. Levels of abscisic acid (ng g^{-1} f.wt.) in plants treated with 0.1mol m^{-3} ABA via the root nutrient solution and in control plants also growing in water culture:

(i) 'free' cis plus trans ABA levels in control plants (First experiment).
(ii) 'bound' cis plus trans ABA levels in control plants (First experiment).
(iii) 'free' cis plus trans ABA levels in ABA treated plants (First experiment).
(iv) 'bound' cis plus trans ABA levels in ABA treated plants (First experiment).

(a) 'free' cis plus trans ABA levels in control plants (Second experiment).
(b) 'bound' cis plus trans ABA levels in control plants (Second experiment).
(c) 'free' cis plus trans ABA levels in ABA treated plants (Second experiment).
(d) 'bound' cis plus trans ABA levels in ABA treated plants (Second experiment).

* = Missing result

ABA levels (ng g^{-1}f.wt)
(i)
(ii)
(a)
(b)
(iii)
(iv)
(c)
(d)
4000
2000
20000
2·10^5
0
Leaves
Roots
Apices
Nodules
*

Figure 24. Levels of abscisic acid (ng g^{-1} f.wt.) in plants treated with 0.1mol m^{-3} ($\pm$) ABA via the root nutrient solution and in control plants also growing in water culture:

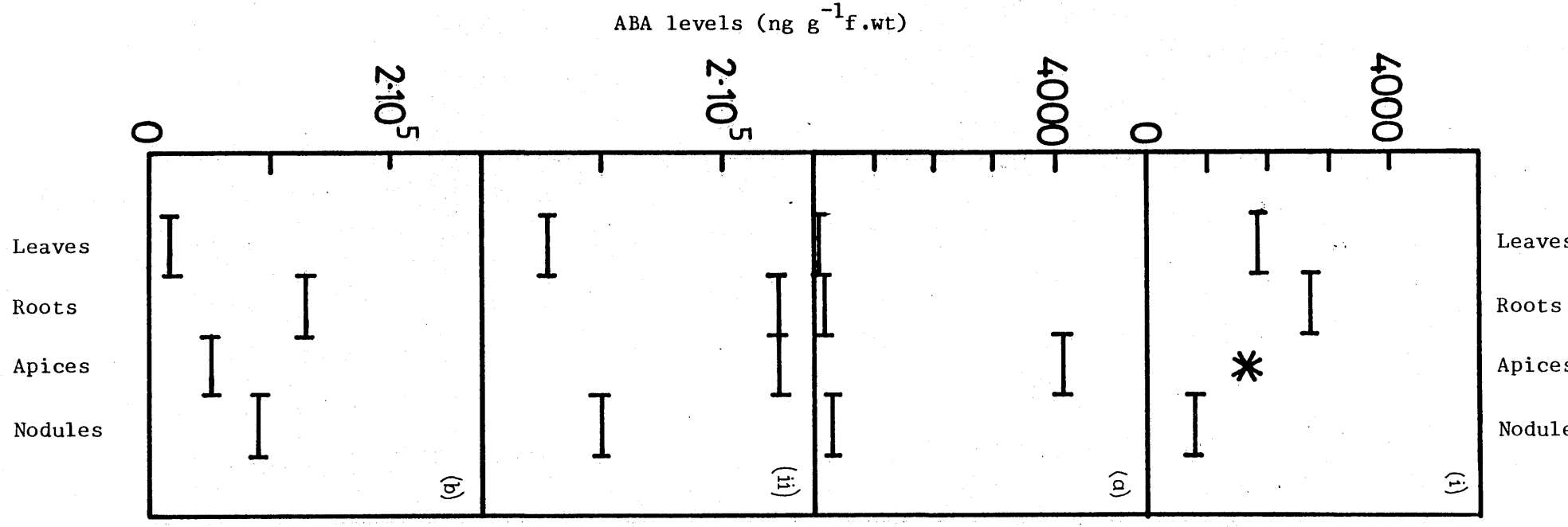
(i) total *cis* plus *trans* levels in control plants (First experiment).

(ii) total *cis* plus *trans* levels in ABA treated plants (First experiment).

(a) total *cis* plus *trans* levels in control plants (Second experiment).

(b) total *cis* plus *trans* levels in ABA treated plants (Second experiment).

* = Missing result


ABA levels (ng g^{-1}f.wt)
0
2·10^5
2·10^5
4000
0
4000
Leaves
Roots
Apices
Nodules
(b)
(ii)
(a)
(i)
Leaves
Roots
Apices
Nodule

of ABA treated plants were 111.82 (739.60), 96.05 (927.06), 199.65 (465.38) and ? (211.37) times greater than those recorded in control plants.

Except in root tissue (both experiments) and nodule tissue (second experiment only), 'free' trans ABA levels in control plants were considerably smaller than 'free' cis ABA levels (Figure 21 (a),(b),(i),(ii)). In the apices and leaves of control plants, 'free' trans ABA levels were at least 71.92 and 15.55 times smaller respectively than 'free' cis ABA levels. In the roots, however, control plant 'free' trans ABA levels were 1.94 (1.20) times greater than amounts of 'free' cis ABA, although the absolute values involved (134.5 (43.8) ng g^{-1} f.wt. cis ABA and 261 (52.7) ng g^{-1} f.wt. trans ABA) were quite small. Nodule 'free' trans ABA levels were 10.59 times smaller than 'free' cis ABA levels in the first experiment, but 1.05 times greater in the second experiment. In the leaves and apices of plants treated with 0.1 mol m^{-3} ABA, 'free' trans ABA levels remained 63.22 (9.71), and 15.03 (?) times smaller than 'free' cis ABA levels respectively, although the absolute trans amounts were at least 6.9 and 6.79 times greater respectively than those recorded in control tissues (Figure 21,(c),(d),(iii),(iv)).In the roots and nodules of ABA treated plants, however, 'free' trans ABA levels that were 3.24 (7.34) and 1.24 (2.57) times greater respectively than 'free' cis ABA levels were present. Absolute 'free' trans ABA levels recorded in these tissues were 59.78 (371.80) and 237.69 (27.72) times greater respectively than those in the control plant parts. In the leaves and apices of control plants, levels of 'bound' cis ABA were less than levels of 'free' cis ABA (Figures 21,22) by factors of 4.76 (68.56) and 3.81 (52.18) respectively. The relationship between cis 'free' and 'bound' ABA levels in the root and nodule tissue of the control plants apparently differed between experiments. In all of the control tissues, however, 'bound' trans ABA levels were greater than amounts of 'free' trans ABA by at least 8.46 (1.78) times. In

plants treated with 0.1 mol m^{-3} ABA, levels of 'bound' cis and trans ABA were far greater than those observed in control plants. 'Bound' cis ABA levels of 38,663 (11,718), 5,997 (10,496), 41,749 (9,793) and 211,224 (46,294) ng g^{-1} f.wt. were recorded in the leaf, root nodule and apex material respectively from these plants, whilst in the same tissues harvested from control plants, levels were only 309 (0.9), 154 (23), 311 (80) and 691 (75) ng g^{-1} f.wt. respectively. In ABA treated plants, 'bound' trans ABA levels of 8,188 (3,000), 220,964 (97,414), 43,007 (79,885) and 18,847 (1,306) ng g^{-1} f.wt. were present in the extracts from leaf, root, nodule and apex material, whilst those in the same tissues harvested from control plants were only 117 (19), 2,208 (94), 113 (112) and 149 ng g^{-1} f.wt.

In control plants, there were no consistent trends between experiments in the relative 'bound' cis and trans ABA levels present in each of the four tissues examined (Figures 22,(a),(b),(i),(ii)). However, in ABA treated plants, more clear-cut trends were observed. Whilst 'bound' cis ABA levels exceeded 'bound' trans ABA levels in the leaves and apices of these plants by a factor of 4.72 (3.90) and 11.21 (35.43) respectively in the root and nodule extracts, 'bound' trans ABA levels were greater than amounts of 'bound' cis ABA by 36.84 (9.26) and 1.03 (8.16) times respectively (Figure 22,(c),(d),(iii),(iv)).

1.2.3.2 Growth of Plants Treated with 0.1 mol m^{-3} ABA Via the Culture Solution

A 0.1 mol m^{-3} ABA treatment resulted in a reduction in plant height (Figure 25); treated plants were significantly smaller (at the 5% level) in height than controls by the 31st day after root-immersion in ABA. Other morphological changes observed in treated plants were as follows (Figures 26, 27, 28, 29) :

(a) necrosis of the leaves.

(b) the appearance of purple 'dormancy' buds in some specimens.

(c) stem thickening.

Figure 25. Height of control plants (●) and plants treated with 0.1mol m^{-3} ($\pm$) ABA via the root solution (o). Each point is a mean of measurements from 12 plants; the vertical line through each point represents two standard errors of the mean.

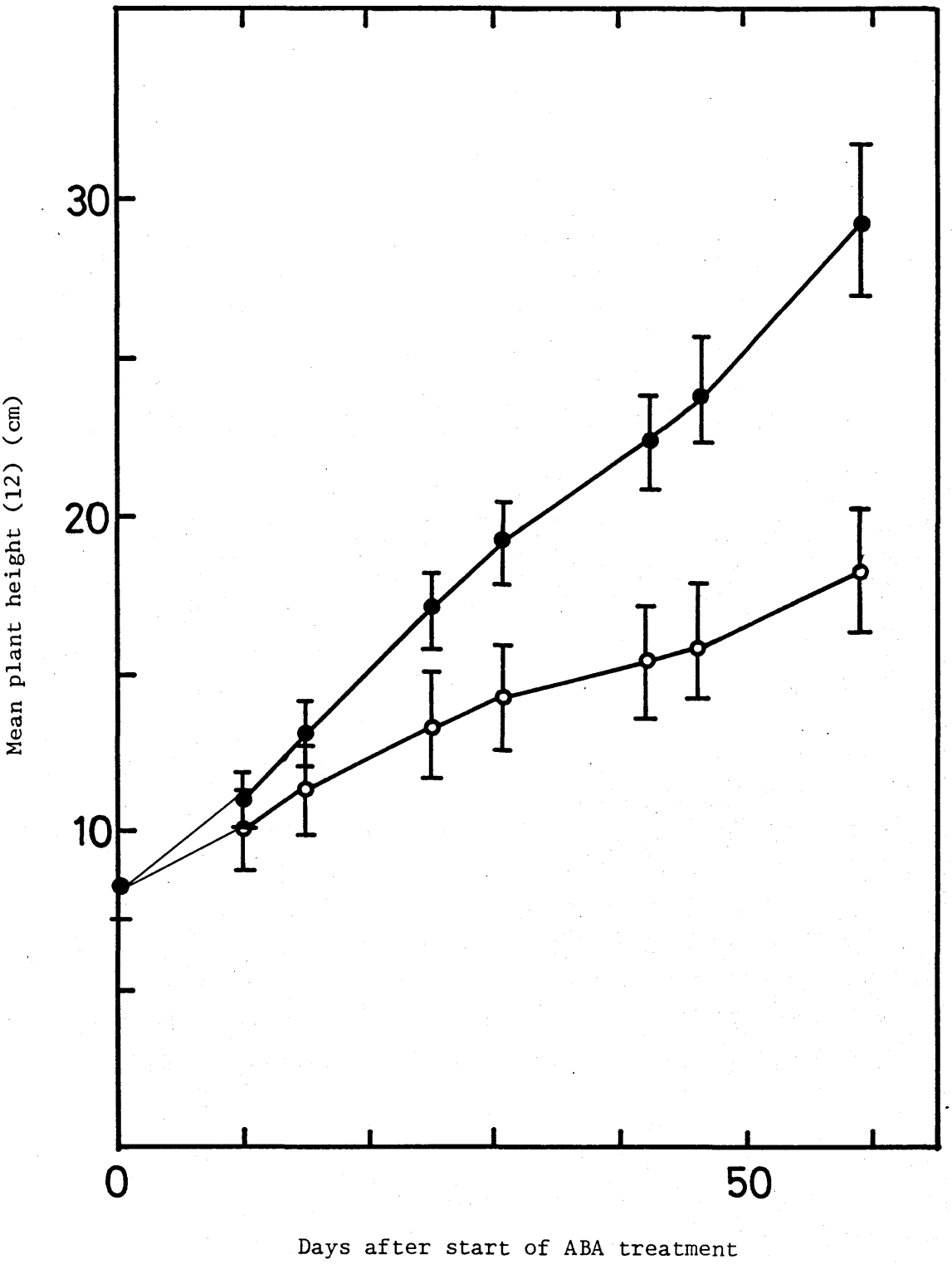

30
20
10
0
50
Mean plant height (12) (cm)
Days after start of ABA treatment

Figure 26. Control plants (on left of picture) and plants that had been in root-contact with 0.1mol m^{-3} ($\pm$) ABA for 59d (on right of picture).

Figure 27. Root and shoot systems of a control plant and a plant that had been in root contact with 0.1mol m^{-3} ($\pm$) ABA for 59d (on right of picture).

Figure 28. The root systems of a control plant and a plant that had been in root-contact with 0.1mol m^{-3} ($\pm$) ABA for 59d.

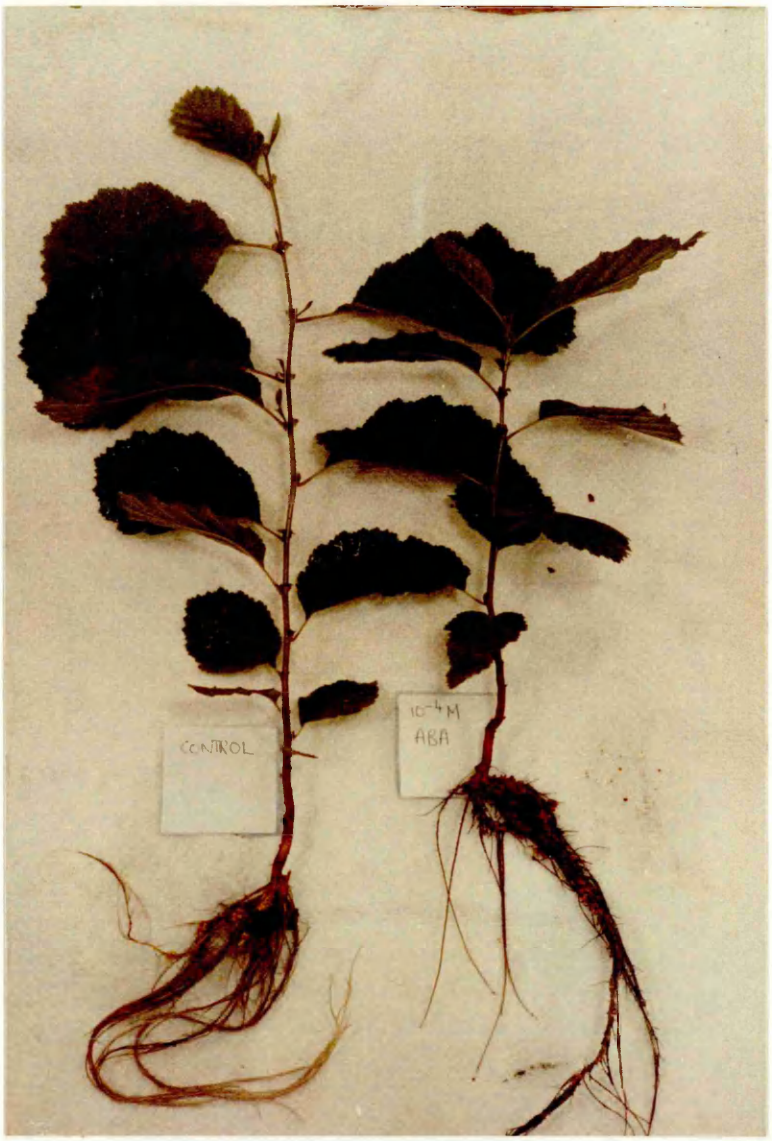


Figure 27

Figure 28

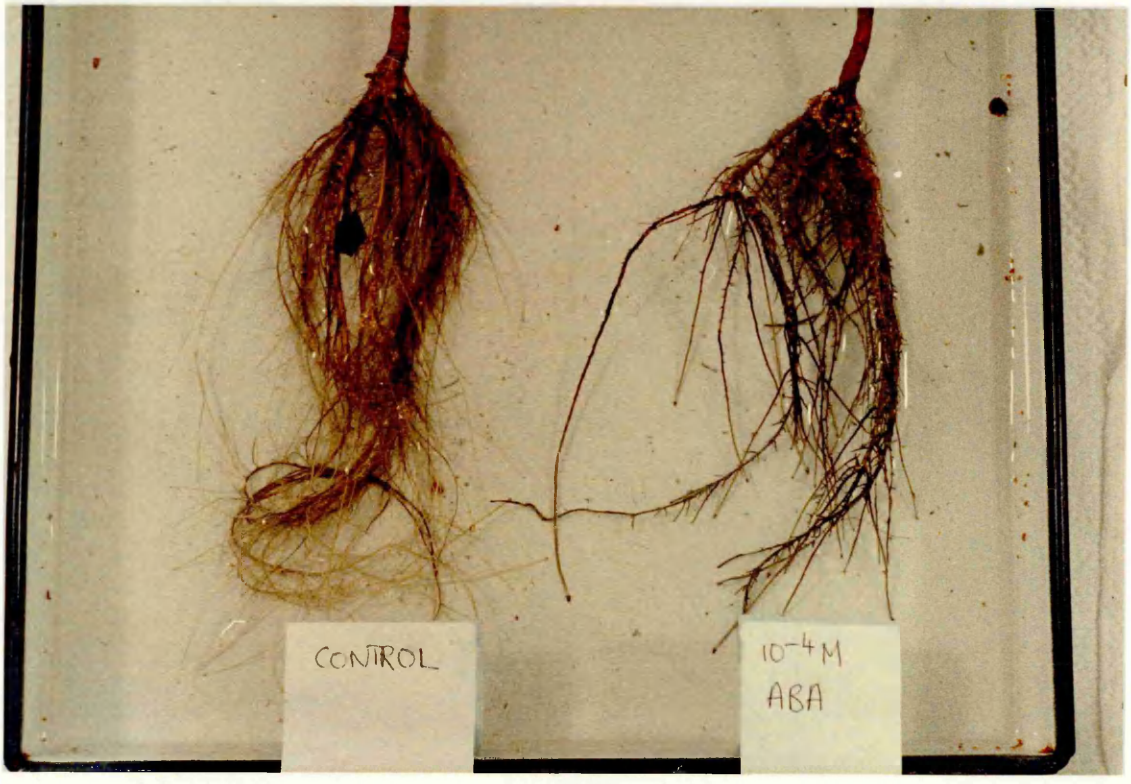


Figure 29. (a) Mature leaf from a control plant. (b) Mature leaf from a plant treated via the root solution with 0.1mol m^{-3} (±) ABA ×2.

29 (a)

29 (b)

(d) blackening of the roots.

(e) 'crumbly' nodules.

Differences in leaf, apex, bud, roots and nodule fresh and dry weights between control and ABA treated plants are shown in Figure 30. On both occasions on which the experiment was conducted, total shoot fresh and dry weights were reduced in ABA treated plants.

Leaf fresh and dry weight measurements were smaller following ABA treatment, reflecting possibly the effect of ABA on the stomata and hence the assimilation of carbon. ABA did little, however, to reduce root or nodule fresh and dry weights; in the second experiment, root growth (dry weight) was actually greater by 1.53 times in treated plants.

In addition to absolute measurements concerning the fresh and dry weights of the plants, comparison between the two treatments has been made by using the mean ratios of one observation to another for each plant (Table12). Using this method, trends in the following ratios between treated and control plants may be discussed:-

Leaf fresh to dry weights
Shoot fresh to dry weights
Root fresh to dry weights
Root dry weight to shoot dry weight
Root fresh weight to shoot fresh weight

Where absolute ratios are mentioned, those from the first experiment are described first, with those from the second experiment shown in brackets.

The shoot fresh to dry weight ratio was greater in control than in ABA treated plants; where shoot fresh weight was 2.946 ± 0.084 (3.801 ± 0.595) times greater than the dry weight in control plants, this value was only 2.603 ± 0.049 (3.487 ± 0.167) in ABA treated plants. The same trend was also observed in the root tissue in which the water content of treated plants was smaller than that of the controls. The magnitude of the fresh to dry weight ratios were greater in this tissue in both control and ABA treated plants than in the leaves or shoot, attaining values

Figure 30. Weight (g) of plants treated with 0.1mol m^{-3} ($\pm$) ABA via the root nutrient solution and control plants also growing in water culture:-

(i) fresh weight of control and ABA treated plants (plain and solid bars respectively). (First experiment)

(ii) dry weight of control and ABA treated plants (plain and solid bars respectively). (First experiment)

(a) fresh weight of control and ABA treated plants (plain and solid bars respectively). (Second experiment)

(b) dry weight of control and ABA treated plants (plain and solid bars respectively). (Second experiment)

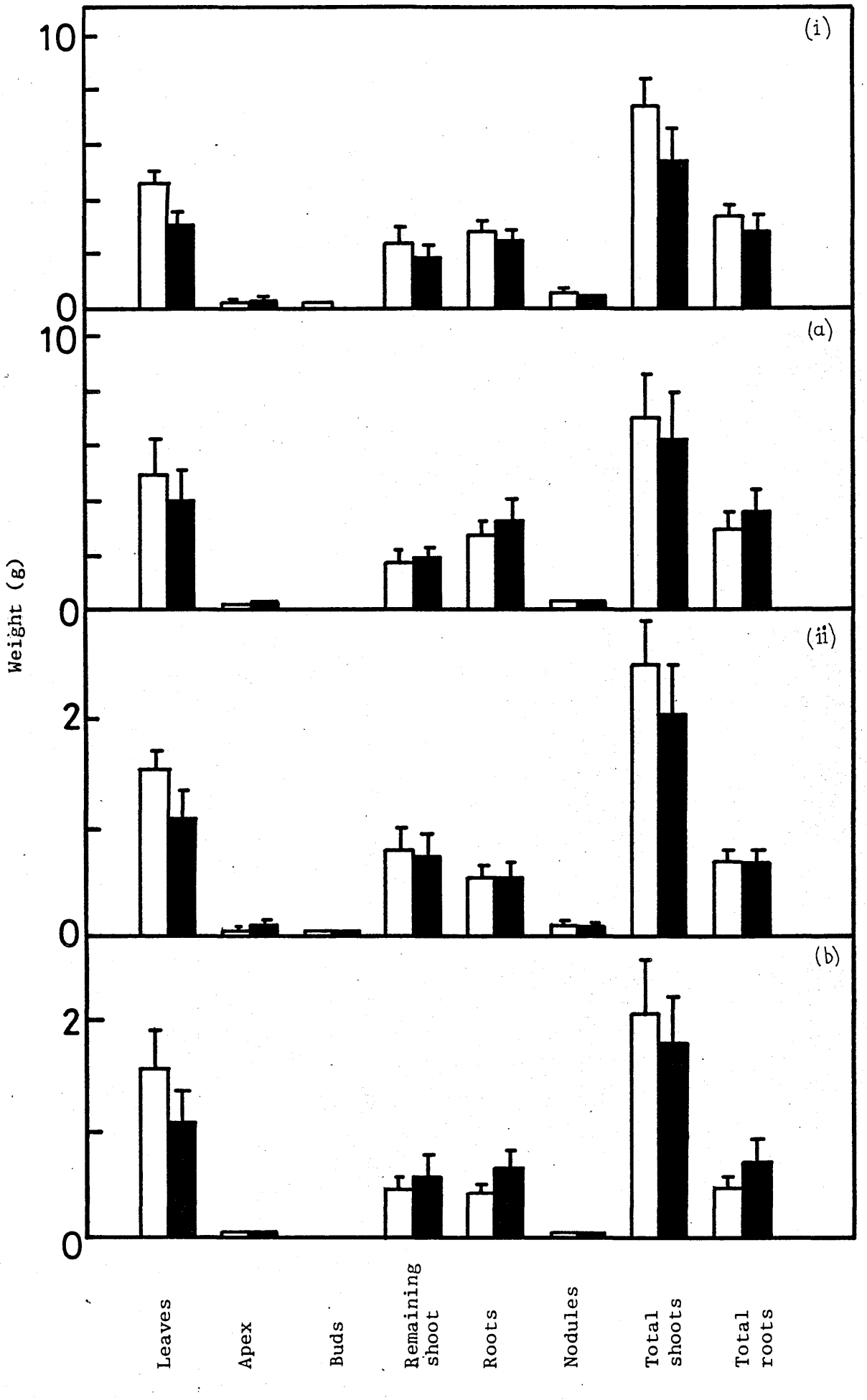

(i)
(a)
(ii)
(b)
10
0
2
Weight (g)
Leaves
Apex
Buds
Remaining shoot
Roots
Nodules
Total shoots
Total roots

Table 12. Fresh and dry weight measurements made on control plants and plants that had been in root contact with 0.1mol m^{-3} ($\pm$) ABA for 3 or 6 months (first and second experiments respectively).

	Ist experiment		2nd experiment	
	CONTROL	ABA	CONTROL	ABA
Plant fresh weight (g)	10.776 ± 1.551	8.283 ± 1.492	10.098 ± 2.139	9.841 ± 2.495
Plant dry weight (g)	3.228 ± 0.524	2.781 ± 0.552	2.555 ± 0.542	2.519 ± 0.635
Leaf fresh:dry weight	2.928 ± 0.074	2.732 ± 0.054	3.462 ± 0.555	3.755 ± 0.078
Shoot fresh:dry weight	2.946 ± 0.084	2.603 ± 0.049	3.801 ± 0.595	3.487 ± 0.167
Root fresh:dry weight	4.995 ± 0.354	4.350 ± 0.279	6.552 ± 0.078	5.190 ± 0.405
Root:shoot dry weight	0.274 ± 0.023	0.333 ± 0.022	0.242 ± 0.027	0.400 ± 0.032
Root:shoot fresh weight	0.456 ± 0.21	0.554 ± 0.039	0.464 ± 0.047	0.593 ± 0.053

of 4.995 ± 0.354 (6.522 ± 0.078) and 4.350 ± 0.279 (5.190 ± 0.405) respectively.

2. The Translocation of Exogenously Applied ABA in Alnus

2.1 The Effects of Long and Short Days on Plant Growth and the Translocation of Radioactivity and ABA Following Petiolar [2-^{14}C] ABA Application

2.1.1 The Translocation of ^{14}C in Plants Growing in Long and Short Photoperiods

The distribution of radioactivity in plants growing in long (16h) and short (8h) photoperiods 24h after [2-^{14}C] ABA application to the petiole of the third leaf from the apex, is shown in Table 13 and Figure 31 . Data from two independent experiments are presented. General trends in ^{14}C distribution in plants are described in the following text. Where absolute values are mentioned, results from the first experiment are quoted, and, where appropriate, those from the second are given in brackets.

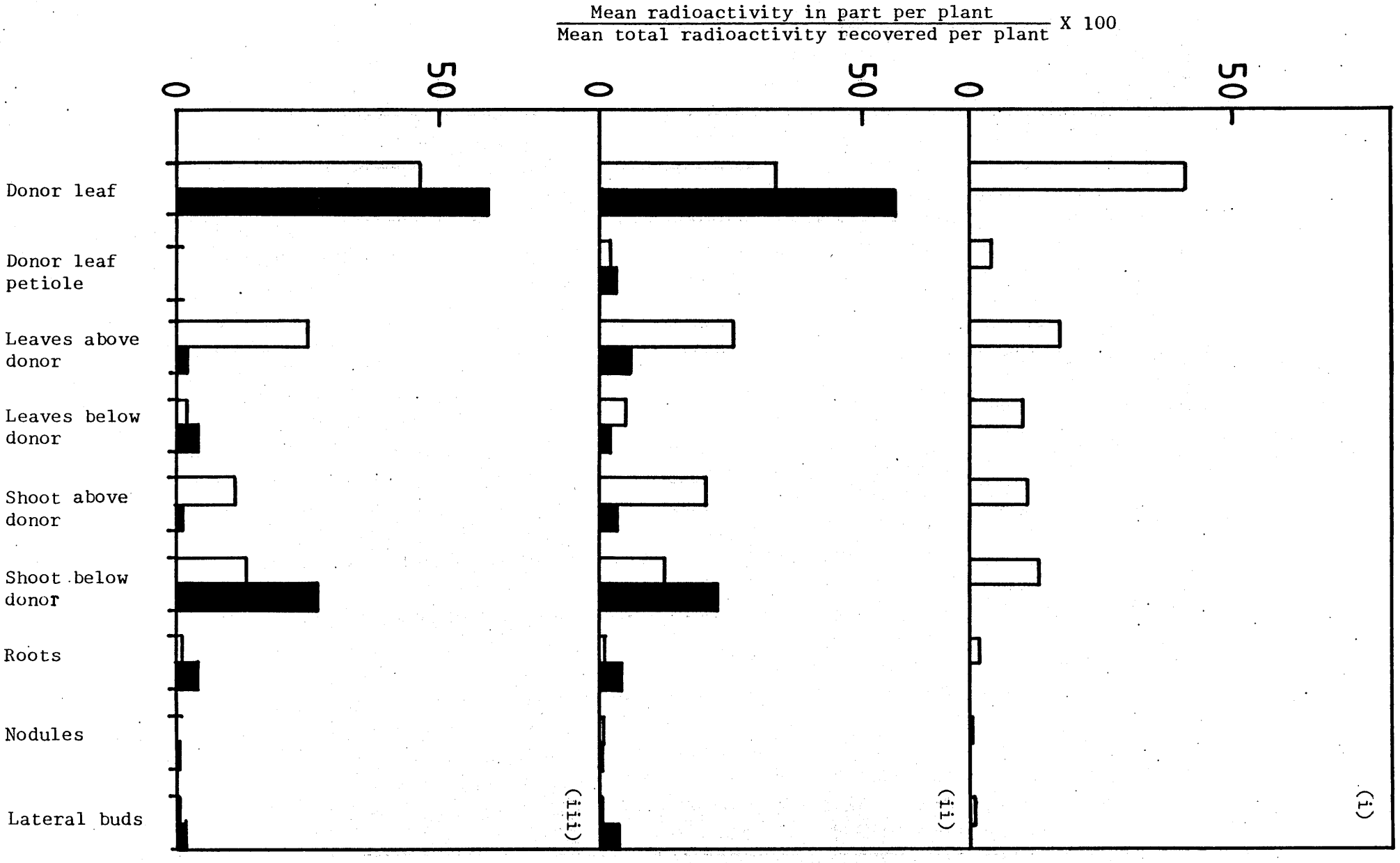
Radioactivity was recovered in all parts of the plant at the end of the translocation period in plants growing under both long and short photoperiods. In both cases, the majority was located in the donor leaf and petiole; this represented 35.5 (46.7) % of the total recovered (calculated from mean values per plant) in long-day plants, and 59.2 (59.8) % in short-day plants, at the end of the experiment. Only 0.3 - 0.6% of the mean total ^{14}C recovered was present in the root nodules. Translocation of ^{14}C from the donor petiole was predominantly acropetal in plants growing in long photoperiods; at the start of experiment 1, an average of 28.8% of the mean ^{14}C recovered in long-day plants was present in parts of the plant above the donor leaf, whilst 25.3 % was translocated to the lower part of the plant (Table 13). 47 (34) d from the start of the experiment (i.e. from the commencement of short days for short-day plants), in long-day plants, an average of 45.4 (36.2) % of the mean ^{14}C recovered had been translocated upwards, whilst only 18.2 (17.1) % was found in tissues below the site of application. After 47 (34) short-days, however, more ^{14}C was located in tissues beneath the donor leaf than above it following a 24h translocation period. Under these conditions, only 9.6 (2.9) % of the mean total radioactivity

Table 13. Distribution of radioactivity in plants following [2-^{14}C] ABA application via the petiole of the third foliage leaf from the apex. Plants were growing under long (16h) or short (8h) photoperiods. The translocation period was 24h from 0900 to 0900. Mean radioactivity $\bar{P}$ in each part is presented; this figure is also shown as a percentage of the mean radioactivity recovered per plant ($\bar{R}$). Data are means of 4 (first experiment) or 2 (second experiment) plants $\pm$ standard errors.

PLANTS	RADIO ACTIVITY FED TO PLANTS (Bq)	MEAN RADIOACTIVITY RECOVERED PER PLANT (Bq) = $\bar{R}$		DONOR LEAF AND PETIOLE	LEAVES ABOVE DONOR	LEAVES BELOW DONOR	SHOOT ABOVE DONOR	SHOOT BELOW DONOR	ROOTS	NODULES	LATERAL BUDS
Long-day plants (expt. 1) Start of experiment	1253.4	1234.4 ± 83.04	$\bar{P}$	566.5 ± 70.49	215.6 ± 13.93	119.9 ± 9.84	140.0 ± 26.35	167.3 ± 12.96	20.4 ± 3.81	4.8 ± 0.87	0
			$\frac{\bar{P}}{\bar{R}}$ x 100	45.9	17.5	9.7	11.3	13.6	1.6	0.4	
Long-day plants (expt. 1) End of experiment	1102.6	924.3 ± 3.17	$\bar{P}$	327.8 ± 27.51	233.9 ± 17.97	43.3 ± 7.99	185.6 ± 22.77	109.0 ± 0.09	12.4 ± 0.76	5.7 ± 1.69	6.4 ± 1.38
			$\frac{\bar{P}}{\bar{R}}$ x 100	35.5	25.3	4.7	20.1	11.8	1.3	0.6	0.7
Short-day plants (expt. 1)	1070.6	856.8 ± 18.89	$\bar{P}$	506.8 ± 36.93	52.2 ± 11.24	15.4 ± 4.18	30.1 ± 9.52	194.0 ± 16.10	31.5 ± 6.27	3.3 ± 0.73	23.5 ± 5.69
			$\frac{\bar{P}}{\bar{R}}$ x 100	59.2	6.1	1.8	3.5	22.6	3.7	0.4	2.7
Long-day plants (expt. 2) End of experiment	675.3	620.0 ± 4.58	$\bar{P}$	289.4 ± 1.13	156.9 ± 14.25	14.3 ± 0.19	67.6 ± 6.53	79.8 ± 0.76	7.6 ± 1.86	1.7 ± 0.29	2.7 ± 1.10
			$\frac{\bar{P}}{\bar{R}}$ x 100	46.7	25.3	2.3	10.9	12.9	1.2	0.3	0.4
Short-day plants (expt. 2)	1651.9	1241.6 ± 100.47	$\bar{P}$	742.0 ± 30.68	22.4 ± 4.91	52.3 ± 40.69	14.3 ± 1.54	332.6 ± 19.08	55.8 ± 5.43	4.32 ± 0.80	17.8 ± 8.19
			$\frac{\bar{P}}{\bar{R}}$ x 100	59.8	1.8	4.2	1.1	26.8	4.5	0.3	1.4

Figure 31. Distribution of radioactivity in given plant parts (expressed as (mean radioactivity in part per plant)/ (mean total radioactivity recovered per plant) x 100) following [2-^{14}C] ABA application to the petiole of the 3rd foliage leaf from the apex. Plants were growing under long (16h) or short (8h) photoperiods (plain and solid bars respectively). The presented data were calculated from profiles observed in four plants (first experiment) or two plants (second experiment):

(i) Plants at the commencement of the first experiment.
(ii) Plants at the end of the first experiment.
(iii) Plants at the end of the second experiment.


Mean radioactivity in part per plant / Mean total radioactivity recovered per plant X 100
0
50
Donor leaf
Donor leaf petiole
Leaves above donor
Leaves below donor
Shoot above donor
Shoot below donor
Roots
Nodules
Lateral buds
(iii)
(ii)
(i)

recovered was transported to the leaves and shoot above the donor leaf, whilst 28.5 (35.8) % was basipetally translocated. Levels of ^{14}C present in the shoot tissue beneath the donor increased by 1.9 (2.1) times under short-days and those recovered in the roots increased by a factor of 2.8 (3.7). There was also an increase from 0.7 (0.4) % to 2.7 (1.4) % (of the total recovered) in the radioactivity located in the lateral bud tissue of short-day plants.

2.1.2 The Translocation of [2-^{14}C] ABA in Plants Growing in Long and Short Photoperiods

Having established the pattern of ^{14}C translocation, the distribution of [2-^{14}C] ABA was investigated by TLC of extracts following ethyl acetate partitioning. Thin-layer chromatograms of plant extracts run in chloroform : methanol : water (75 : 22 : 3 v|v) following partitioning against ethyl acetate are presented in Figure 33. Chromatograms of [G-^{3}H] ABA developed in butan-1-ol : propan-1-ol : ammonia : water (2 : 6 : 1 : 2 v|v) and run in the presence or absence of a ^{14}C-labelled shoot extract, revealed that this solvent system was unreliable for the identification of ABA in older tissues by co-chromatography, when standard and sample were developed on separate plates (Figure 32). In this solvent, the [G-^{3}H] ABA standard run in the absence of a ^{14}C extract travelled further than that run as an internal standard (with the ^{14}C-labelled extract). This did not occur with the chloroform : methanol : water solvent, in which there was good correspondence in the Rfs of [G-^{3}H]. ABA run in the presence and absence of a ^{14}C-labelled extract, and which was used, therefore, throughout all experimental analyses. In both solvent systems, however, amounts of ^{14}C co-chromatographing with a [G-^{3}H] ABA internal standard (run with the shoot extract) were similar, enhancing the likelihood that the putative ABA peak was indeed [2-^{14}C] ABA.

Profiles of [2-^{14}C] ABA distribution in plants growing under long and short photoperiods are presented in Table 14. The data from one experiment only are shown. Where appropriate, however, results from the analysis of replicate extracts are shown in brackets.

Figure 32. Thin-layer chromatograms of ethyl acetate-soluble [^{14}C] radioactivity in a shoot extract following [2-^{14}C] ABA application to intact plants via the petiole of the third foliage leaf from the apex. The translocation period was 24h. The extract was prepared from 4 plants growing in long (16h) photoperiods. Extracts, spotted onto 20 x 5cm plastic TLC strips coated with 0.2mm silica gel GC_{254} were developed in chloroform:methanol: water (75:22:3 v/v) see graph (i), or butanol:ammonia: water:propanol (2:1:2:6 v/v) see graph (ii). The positions of authentic ^{3}H ABA run on the same TLC strip as the [^{14}C] extract and run on independent TLC strips under the same conditions are indicated by the markers ^{3}H ABA (I) and [^{3}H] ABA (s) respectivley.

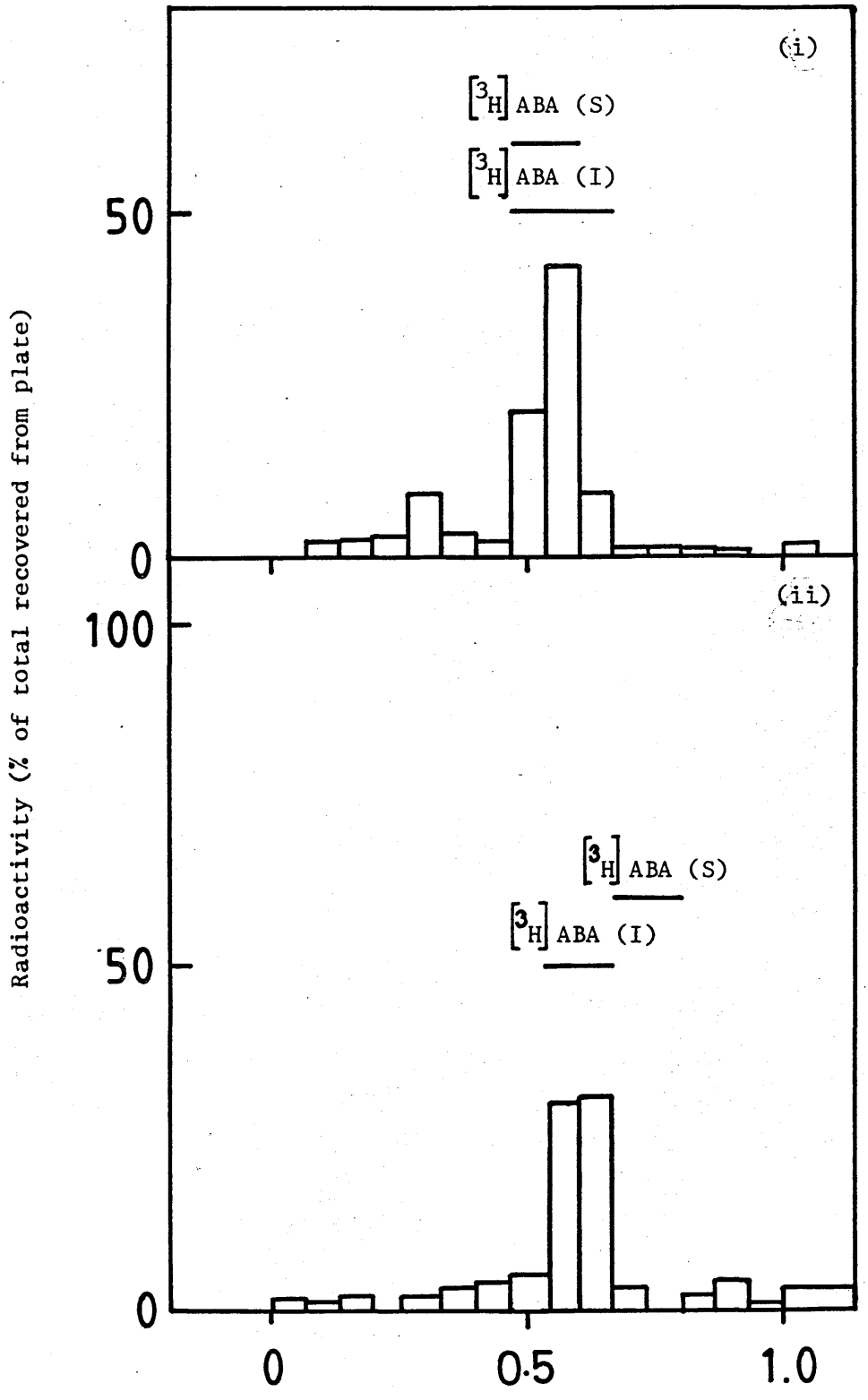
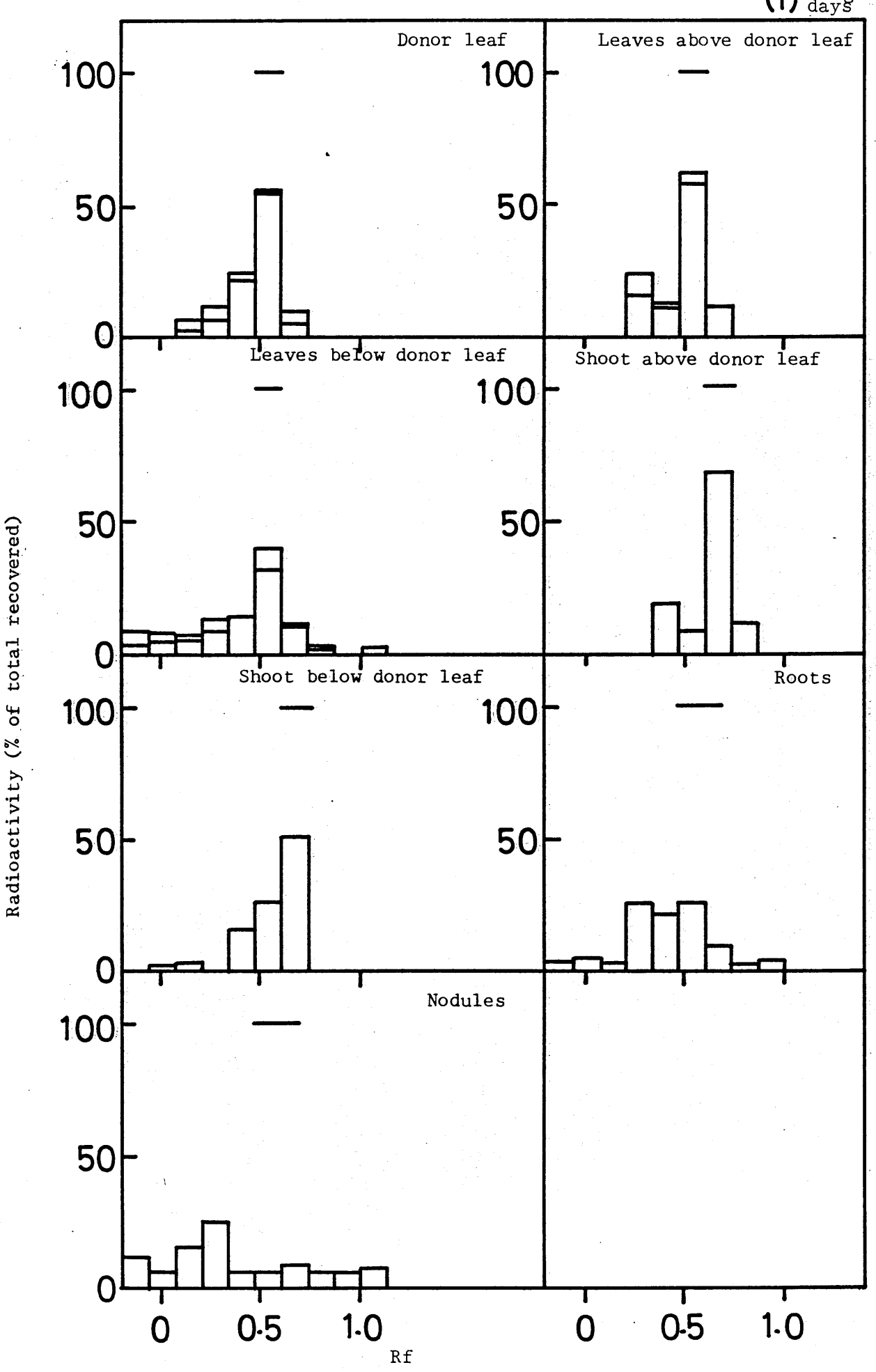

(i)
[3H] ABA (S)
[3H] ABA (I)
50
0
(ii)
100
[3H] ABA (S)
[3H] ABA (I)
50
0
0
0·5
1.0
Rf
Radioactivity (% of total recovered from plate)

Figure 33. Thin layer chromatograms of ethyl acetate-soluble radioactivity in given tissues following [2-^{14}C] ABA application to intact plants (via the petiole of the third foliage leaf from the apex). The translocation period was 24h. Plants were growing in long (16h, graph (i)) or short (8h, graph (ii)) photoperiods. Extracts were usually prepared from 4 plants. Results from replicate leaf extracts each from 2 long-day plants, however, are presented. Extracts, spotted onto 20 x 5cm plastic TLC strips precoated with 0.2mm silica gel GC_{254} were developed in chloroform:methanol:water (75:22:3 v/v). The position of authentic [2-^{14}C] ABA on independent TLC strips developed under the same conditions is indicated ——— .


(1) days
Donor leaf
Leaves above donor leaf
Leaves below donor leaf
Shoot above donor leaf
Shoot below donor leaf
Roots
Nodules
100
50
0
0
0.5
1.0
Rf
Radioactivity (% of total recovered)

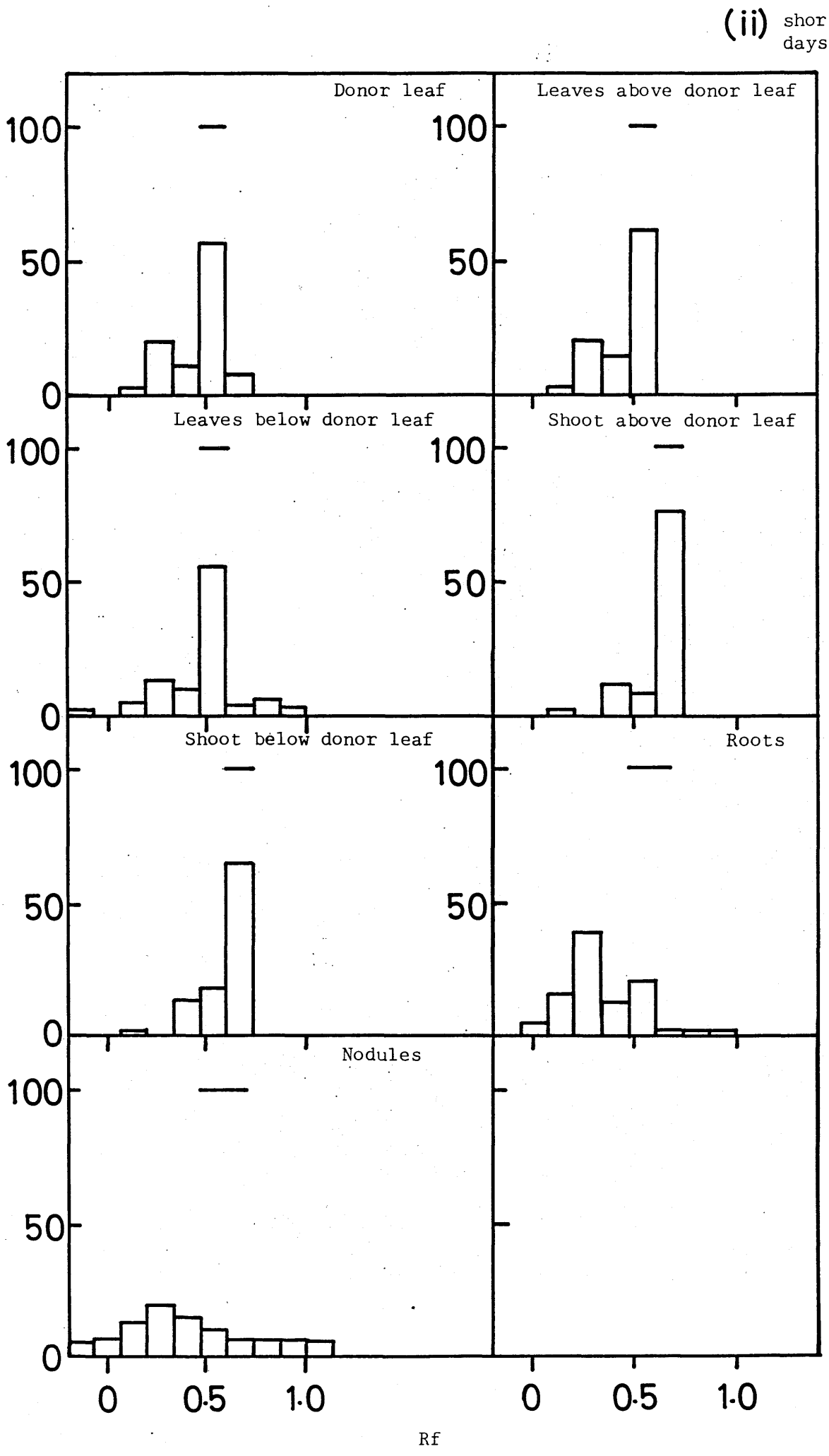

(ii) short days
Donor leaf
Leaves above donor leaf
Leaves below donor leaf
Shoot above donor leaf
Shoot below donor leaf
Roots
Nodules
100
50
0
0
0·5
1·0
Rf

Table 14. Distribution of [2-^{14}C] ABA in plants following [2-^{14}C] ABA application via the petiole of the third foliage leaf from the apex. Plants were growing under long (16h) or short (8h) photoperiods. The translocation period was 24h from 0900 to 0900. The results are presented as absolute [2-^{14}C] ABA values, (A); plant [2-^{14}C] ABA content is also expressed as a percentage of the mean total radioactivity translocated to that part ($\frac{A}{\bar{P}}$ x 100), as a percentage of the total ABA recovered per plant ($\frac{A}{\Sigma A}$ x 100) and as a percentage of the mean total radioactivity recovered per plant ($\frac{A}{\bar{R}}$ x 100). ^{14}C metabolites of [2-^{14}C] ABA plants growing in long and short days were investigated by ethyl acetate partitioning and TLC of tissue extracts, each usually prepared from 4 plants. Results from replicate leaf extracts each from 2 plants growing in long days, however, are shown in brackets. The recovery of radioactivity in plants (mean of 4 replicates) was assessed in a parallel experiment by sample oxidation.

Plants	Radio-activity fed (Bq)	Radio-activity recovered $\bar{R}$	Total ABA recovered		Donor leaf and petiole	Leaves above donor	Leaves below donor	Shoot above donor	Shoot below donor	Lateral buds
Long day at start	1253.4	1209.2	156.38 (158.80)	A	55.12 (55.29)	54.15 (57.78)	3.89 (2.51)	37.21	6.01	-
				$\bar{P}$	566.5$\pm$ 70.49	215.6$\pm$ 13.93	119.9$\pm$ 9.84	140.0$\pm$ 26.35	167.3$\pm$ 12.96	-
				$\frac{A}{\bar{P}}$ X100	9.74	25.96	2.67	26.58	3.59	-
				$\frac{A}{\Sigma A}$ X100	35.25 (34.82)	34.63 (36.38)	2.49 (1.58)	23.79 (23.43)	3.84 (3.78)	-
				$\frac{A}{\bar{R}}$ X100	4.56 (4.57)	4.48 (4.78)	0.32 (0.21)	3.08	0.50	-
Short day at end	1070.6	822.0	121.83	A	47.96	10.71	2.99	17.88	42.29	-
				$\bar{P}$	506.8$\pm$ 36.93	52.2$\pm$ 11.24	15.4$\pm$ 4.18	30.1$\pm$ 9.52	194.0$\pm$ 16.10	23.5$\pm$ 5.69
				$\frac{A}{\bar{P}}$ X100	9.46	20.52	19.41	59.40	21.79	-
				$\frac{A}{\Sigma A}$ X100	39.37	8.79	2.45	14.68	34.71	-
				$\frac{A}{\bar{R}}$ X100	5.83	1.30	0.36	2.17	5.14	-

[2-^{14}C] ABA distribution was similar to that of ^{14}C (Table 13, Figure 31), which is described above. The majority of the [2-^{14}C] ABA recovered was located in the donor leaf and petiole; this represented 34.24 (33.84) and 38.00 % of the mean total ABA recovered (4.46 (4.48) and 5.59 % of the mean total radioactivity recovered) in long and short-day plants respectively. Over one half (56.76 (58.13) %) of the total [2-^{14}C] ABA recovered in long-day plants was present in the leaves and shoot above the donor leaf whilst this value was less than one quarter (22.65%) in short-day plants. In plants growing under short photoperiods, 35.88% of the total ABA recovered (5.28% of the total radioactivity recovered) was present in those parts of the plant beneath the site of application; this strongly contrasted with plants growing under long photoperiods, in which this figure was 6.15 (4.22) % of the total ABA recovered (0.80 (0.69) % of the total radioactivity recovered). The largest difference in the [2-^{14}C] ABA content of the tissues below the donor leaf between the plants growing under different photoperiods occurred in the stem. In short-day plants, 33.51% of the total ABA recovered (4.93% of the mean total ^{14}C recovered) was present at this location, whilst in long-day plants, this figure was 3.73 (3.68) % (0.49% of the mean total ^{14}C recovered).

Finally, although chromatography of root and nodule extracts was attempted, it was not possible to draw any conclusions regarding the fate of the radioactivity entering the roots or root nodules owing to the low levels of ^{14}C involved.

2.1.3 The Metabolism of [2-^{14}C] ABA in Plants Growing in Long and Short Photoperiods

In addition to their use in determining the distribution of [2-^{14}C] ABA in long and short-day plants, the thin-layer chromatograms of the ethyl-acetate-soluble radioactivity of tissue extracts also provided some information concerning the metabolism of the applied ABA. Thin-layer chromatograms of leaf and stem tissue extracts from both long and short-day plants revealed that [2-^{14}C] ABA was the major radioactive compound present (Figure 33). There were no marked differences in the amount of [2-^{14}C] ABA present as a % of

the total radioactivity recovered per plate between extracts from long and short-day plants, although slightly more radioactivity was recovered as [2-^{14}C] ABA in extracts from leaf and shoot material from beneath the donor leaf in short-day plants.

Further information regarding the metabolism of [2-^{14}C] ABA in different tissues can be inferred from Table 14. In both long and short-day plants, the shoot tissue above the donor leaf contained the highest levels of [2-^{14}C] ABA expressed as a percentage of mean ^{14}C values recovered in the tissue ($\frac{A}{P}$ x 100). However, whilst [2-^{14}C] ABA levels reached 26.58% of the mean total radioactivity present in the shoot above the donor leaf in long-day plants, over twice this amount was recorded in short-day plants. The same trend was observed in the leaves and shoot tissues below the donor leaf of short-day plants, in which [2-^{14}C] ABA levels as a % of the mean radioactivity present in that sample were 7.3 (12.6) and 5.8 (5.9) times greater than in long-day plants. Within the tissues of plants growing under one photoperiod, similar amounts of radioactivity were recovered as [2-^{14}C] ABA in the leaves and shoot below the donor leaf, although the absolute percentages involved between plants of different photoperiods were different. Thus in long-day plants, whilst 2.67 and 3.59% of the radioactivity present in the leaves and stem below the donor leaf was attributed to [2-^{14}C] ABA, these values in short-day plants were 19.41% and 21.79% respectively. However, similar levels of radioactivity were recovered as [2-^{14}C] ABA in the donor leaves and leaves above the donor leaves of plants growing under both long and short photoperiods. (These were approximately 9.5% and 20% respectively.

2.1.4 The Mode of Transport of Petiole-applied [2-^{14}C] ABA in Plants Growing in Long (16h) Photoperiods

Two approaches were used to investigate the way in which ABA is translocated following petiolar application. In the first, plants were bark-ringed to assess the importance of the phloem to the basipetal movement of radioactivity. The second method involved examining root pressure sap for the presence of ^{14}C.

2.1.4.1 The Influence of a Phloem Block on the Translocation of ^{14}C

Only 0.416% of the total ^{14}C recovered (calculated from mean values per plant) 24h after the application of [2-^{14}C] ABA to the petiole of the 3rd leaf from the apex traversed a phloem block sited between the 4th and 5th leaves, to reach the lower stem and root system (Table 15). In control plants, the figure translocated to parts of the plant below the donor leaf was 15.6% of the mean total recovered. Levels of ^{14}C retained in the donor leaf of control and ringed plants (45.9 and 40.8 % respectively, calculated from mean values) showed little difference. However, less radioactivity was recovered in the leaves above the donor leaf in control plants than in ringed specimens (17.5% and 24.8% respectively). This effect was also observed in the shoot tissue above the donor, in which 11. 3% of the mean total ^{14}C recovered was present in control plants whilst this figure was 24.1% in ringed plants.

2.1.4.2 An Examination of Root Pressure Sap for Radioactivity

Radioactivity was detected in the bleeding sap issuing from plants growing in long photoperiods and decapitated 24h following the application of [2-^{14}C] ABA to the petiole of the third leaf from the apex. Levels of ^{14}C present in exudate collected over a 27h period are shown in Table 16. The mean volume of exudate collected per plant in the first 12h following decapitation was 119.2mm^3; this contained 33.9mBq mm^{-3} of radioactivity. During the period 12 to 24h following decapitation (which included the 8h dark period), 104.0 $\pm$ 33.61mm^3 of exudate was produced by the plants which contained slightly lower levels of radioactivity than before (22.2 $\pm$ 6.2 mBq mm^{-3}). Differences in both the radioactivity per unit volume of exudate and radioactivity per unit volume of exudate per unit time collected over the two 12h periods, were not significant at the 5% level, however. Investigation into the identity of the radioactivity present in the exudate by TLC revealed that a mean of 36.5% (4 replicates) co-chromatographed with authentic [2-^{14}C] ABA. These

Table 15. Distribution of radioactivity in control and bark-ringed plants following [2-^{14}C] ABA application to the petiole of the third foliage leaf from the apex. Plants were growing under long (16h) photoperiods. The translocation period was 24h from 0900 - 0900. Plants were bark-ringed using steam directed at the stem between the 4th and 5th leaves. Mean radioactivity ($\bar{P}$) in each part is presented; this figure is also shown as a percentage of the mean radioactivity recovered per plant ($\bar{R}$). Data are means of 4 (control) or 3 (bark-ringed) plants $\pm$ standard errors.

	RADIOACTIVITY FED TO PLANTS (Bq)	$\bar{R}$		DONOR LEAF AND PETIOLE	LEAVES ABOVE DONOR	LEAVES BELOW DONOR		STEM ABOVE DONOR	STEM BELOW DONOR		ROOTS	NODULES
						ABOVE RING	BELOW RING		ABOVE RING	BELOW RING		
Control plants (long days)	1253.4	1234.4 ± 83.04	$\bar{P}$	566.5 ± 70.49	215.6 ± 13.93	119.9 ± 9.84		140.0 ± 26.35	167.3 ± 12.96		20.4 ± 3.81	4.8 ± 0.87
			$\frac{\bar{P}}{\bar{R}} \times 100$	45.9	17.5	9.7		11.3	13.6		1.6	0.4
Bark-ringed plants (long days)	1192.4	958.2 ± 18.44	$\bar{P}$	390.7 ± 42.78	236.9 ± 17.05	7.3 ± 2.85	6.6 ± 3.25	230.3 ± 27.80	82.8 ± 14.56	3.5 ± 1.62	0.045 ± 0.045	0.11 ± 0.11
			$\frac{\bar{P}}{\bar{R}} \times 100$	40.8	24.8	0.7	0.6	24.1	8.6	0.4	0.005	0.011

Table 16. Radioactivity in the root exudate of individual plants decapitated 24h following the application of 1216.6 Bq of [2-^{14}C] ABA to intact plants via the petiole of the 3rd foliage leaf from the apex. Plants were growing in long (16h) photoperiods. Differences in both the radioactivity per unit volume of exudate and radioactivity per unit volume of exudate per unit time collected during the predominantly light (0900 - 2100) and predominantly dark (2100 - 0900) periods were not significant at the 5% level. Data are means of 6 replicates. Investigation into the identity of the radioactivity present in the exudate by TLC revealed that 36.5% (mean of 4 replicates) co-chromatographed with authentic [2-^{14}C] ABA.

TIME	MEAN VOLUME OF EXUDATE (mm^3)	MEAN RADIOACTIVITY PRESENT IN EXTRACT (mBq)	MEAN RADIOACTIVITY PER UNIT VOLUME OF EXUDATE ($mBq\ mm^{-3}$)	MEAN RADIOACTIVITY PER UNIT VOLUME OF EXUDATE PER UNIT TIME ($mBq\ mm^{-3}\ h^{-1}$)
0900 - 1500	74.4 ± 25.00	1975 ± 375.7	38.6 ± 12.70	6.4 ± 2.11
1500 - 2100	44.8 ± 16.41	1187 ± 286.1	29.3 ± 9.97	4.9 ± 1.65
2100 - 0900	104.0 ± 33.61	1509 ± 256.7	22.2 ± 6.20	1.8 ± 0.52
0900 - 1200	21.9 ± 8.69	120 ± 42.3	6.3 ± 2.96	2.1 ± 0.99

results indicate that phloem : xylem exchange of compounds may occur in the root system.

2.1.5 The Growth and Nitrogenase Activity of Plants Growing in Long and Short Photoperiods

To appreciate the effect of short photoperiods on plant growth, two variables, plant height and nitrogenase activity were monitored. Changes in the height of plants growing in long and short photoperiods are shown in Figure 34. Following as few as 6 short days, extension growth was arrested. After 36 short days, there were highly significant differences (at the 0.5% level) in the percentage increase in height of individual plants growing in the two photoperiods.*

The nitrogenase activity (acetylene reduction) of two plants growing in long and short photoperiods was assessed at 3 intervals following the onset of short days (Table 17). Plants growing in long-days in the heated greenhouse showed the most pronounced variations in mean nitrogenase activity per plant despite the fact that they were removed to the constant conditions of the short-day growth chamber prior to (for at least 1h) and during the assay. These fluctuations can probably be attributed to prevalent solar radiation and temperature fluctuations in the greenhouse several hours before the assay. From the results obtained with this small sample of plants, there was no indication that up to 41 short-days had any effect on nodule nitrogen-fixing capacity, which remained at between 13.2 ± 4.60 - 19.5 ± 4.00 nmol $C_2H_4 g^{-1}$ f.wt. h^{-1} when plants were grown in conditions of near-constant temperature. Nitrogenase activity of long-day plants assayed on the same occasions was between 9.8 ± 0.05 and 17.0 ± 2.00 nmol C_2H_4 g^{-1} f.wt. h^{-1}.

*Plants growing in short days were developing pigmented buds by this time (Figures 35 (a),(b) and 36).

2.2 The Translocation of Radioactivity and Ethyl-acetate-soluble Radioactivity Following Root [2-^{14}C] ABA Application to Plants Growing in Long (16h) Days

To complement the study of endogenous ABA levels in plants treated with unlabelled 0.1mol m^{-3} ($\pm$) ABA via the roots, an investigation was made with 2 - 3 month old plants, of the uptake and translocation of radioactivity and ethyl-acetate-soluble radioactivity ('free' ABA-containing) following the application of [2-^{14}C] ABA in a similar manner.

Figure 34. Heights of plants grown in long (o) or short (●) day conditions (16h or 8h photoperiods respectively). Values are means of 8 (8h photoperiod) or 7 (16h photoperiod) replicates; the vertical bars represent one standard error of the mean. By day 36, the percentage increase in height of individual plants was significantly smaller (at the 0.5% level) in the plants grown in 8h photoperiods.

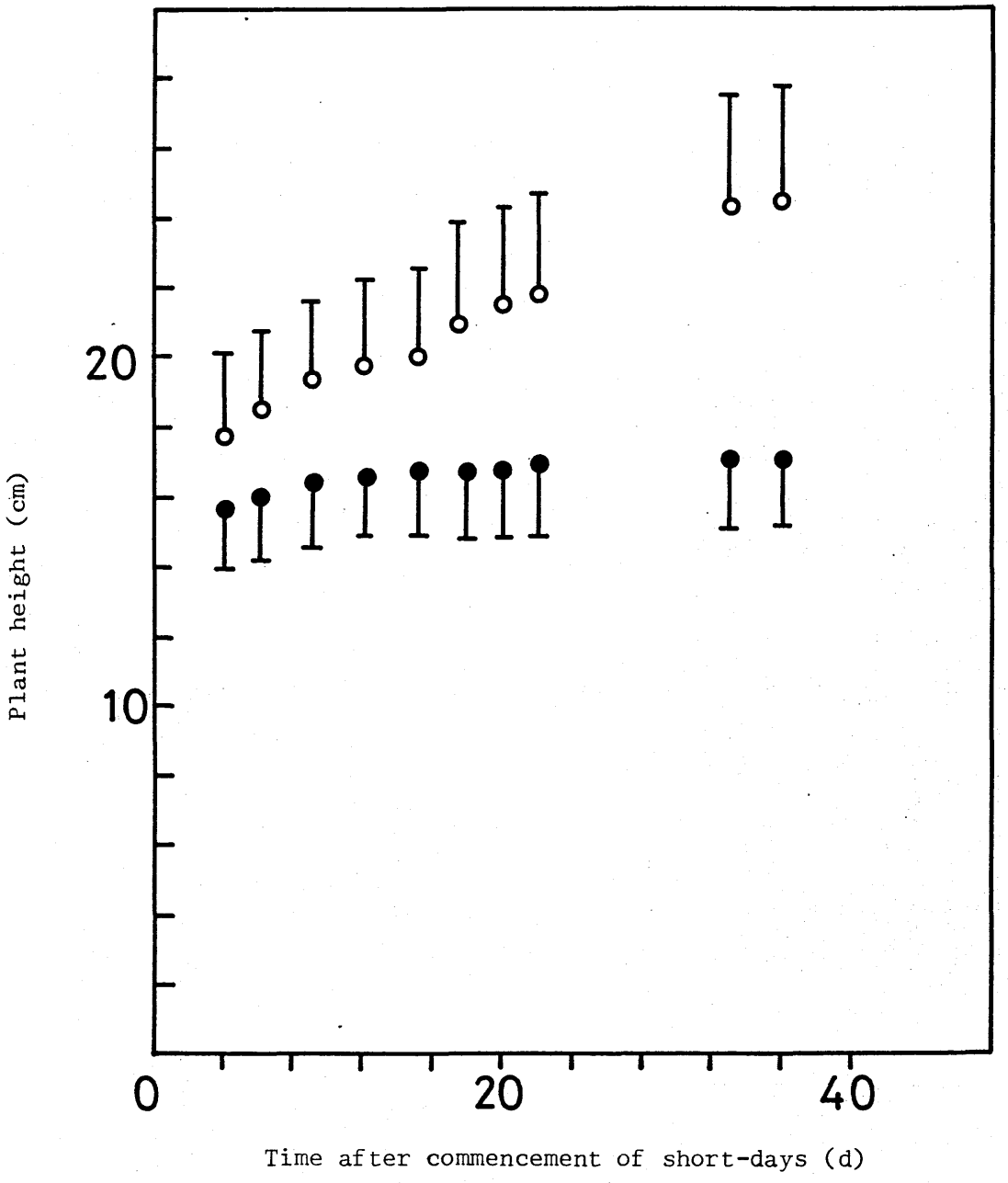

Plant height (cm)
20
10
0
20
40
Time after commencement of short-days (d)

Table 17. Nitrogenase activity (nmol $C_2H_4 g^{-1}$ nodule f.wt. h^{-1}) of plants growing in long (16h) and short (8h) photoperiods. Specimens were re-assayed on each successive occasion. The same batches of plants were used to study the translocation of petiole-applied [2-^{14}C] ABA in long and short day conditions.

	TIME AFTER COMMENCEMENT OF SHORT DAYS (d)		5	23	41
Nitrogenase activity (nmol C_2H_4 g^{-1} f.wt. h^{-1})	Plants growing in short days	Plant 1	14.4	23.5	17.8
		Plant 2	19.6	15.5	8.6
		Mean	17.0 ± 2.60	19.5 ± 4.00	13.2 ± 4.60
	Plants growing in long days	Plant 1	22.5	9.8	15.0
		Plant 2	9.6	9.9	19.0
		Mean	16.0 ± 6.45	9.8 ± 0.05	17.0 ± 2.00

Figure 35. (a) Apex of a plant approaching dormancy following growth under short photoperiods. (b) Actively growing apex of a plant growing under long photoperiods.

Figure 35 (a)

Figure 35 (b)

Figure 36. Appearance of a plant approaching dormancy following growth under short photoperiods for 36d.

2.2.1 The Translocation of Radioactivity Following Root Application of 0.1mol m^{-3} [2-^{14}C] ABA and 2.52 x 10^{-5} mol m^{-3} [2-^{14}C] ABA

^{14}C Translocation profiles obtained 5d following the application of 0.1 mol m^{-5} and 2.52 x 10^{-5} mol m^{-3} [2-^{14}C] ABA to the roots of plants growing in water culture under long days are shown in Table 18 . The complete experiment was carried out once only (experiment 2), although profiles of ^{14}C distribution in plants treated with 0.1mol m^{-3} [2-^{14}C] ABA were obtained with two batches of plants. Where comparisons between the two ABA treatments are made, values from the second experiment are given, with those from the first experiment shown, where appropriate, in brackets.

Assuming that all of the radioactivity recovered in the plant entered the root system as [2-^{14}C] ABA, in the presence of 0.1mol m^{-3} [2-^{14}C] ABA, only 2.325 (2.571) % of the total ABA available was taken-up by the plants. In the presence of 2.52 x 10^{-5} mol m^{-3} [2-^{14}C] ABA, 2.7 times more of the available [2-^{14}C] ABA entered the plants (representing 6.313% of that present in the culture solution).

In the presence of 0.1 mol m^{-3} [2-^{14}C] ABA, 48.02 (69.64) % of the radioactivity recovered (calculated from mean values per plant) was located in the root tissue, whilst smaller amounts were present in the stem (26.6 (16.27) %), leaves (21.41 (12.10) %) and nodules (3.89 (1.98) %). In plants treated with 2.52 x 10^{-5} mol m^{-3} [2-^{14}C] ABA, a total of 83.50% of the radioactivity recovered was found in the root system (roots and nodules), of which 9.10% was present in the nodules. Only 16.44% of the mean total ^{14}C recovered was present in the shoot system, and in these plants, 1.91 times as much radioactivity was located in the stem as in the leaf tissue.

2.2.2 The Translocation of Ethyl-acetate-soluble Radioactivity Following Root Application of 0.1 mol m^{-3} [2-^{14}C] ABA

Absolute levels of radioactivity present in extracts of plants harvested 5d following the application of 0.1 mol m^{-3} [2-^{14}C] ABA were too low to permit full analysis by TLC. Levels of radioactivity which partitioned into the organic phase following ethyl acetate partitioning were determined

Table 18. Distribution of radioactivity in plants following the application of 0.1mol m^{-3} or 2.52 x 10^{-5}mol m^{-3} [2-^{14}C] ABA to the roots. Plants were 2 months old and were growing in water culture under long (16h) photoperiods). The translocation of ^{14}C was examined after a 5d period. Mean radioactivity ($\bar{P}$) in each part is presented; this figure is also shown as a percentage of the mean radioactivity recovered per plant ($\bar{R}$). Data are means of 3-6 plants per experiment $\pm$ standard errors.

TREATMENT	RADIOACTIVITY FED TO PLANTS (Bq)	MEAN RADIOACTIVITY RECOVERED PER PLANT (Bq) = $\bar{R}$		LEAVES	STEM	ROOTS	NODULES
0.1 mol m^{-3} [2-^{14}C] ABA (specific activity 0.297 k Bq mg^{-1}) Expt. 1	196.3	5.04 ± 0.723	Mean radioactivity in part per plant (Bq) = $\bar{P}$	0.61 ± 0.105	0.82 ± 0.153	3.51 ± 0.432	0.10 ± 0.083
			$\frac{\bar{P}}{\bar{R}}$ x 100	12.10	16.27	69.64	1.98
0.1 mol m^{-3} [2-^{14}C] ABA (specific activity 0.904 kBq mg^{-1}) Expt. 2	596.9	13.87 ± 2.986	Mean radioactivity in part per plant (Bq) = $\bar{P}$	2.97 ± 0.839	3.69 ± 0.837	6.66 ± 1.418	0.54 ± 0.128
			$\frac{\bar{P}}{\bar{R}}$ x 100	21.41	26.60	48.02	3.89
2.52 x 10^{-5} mol m^{-3} [2-^{14}C] ABA (specific activity 3.59 MBq mg^{-1} Expt. 2	596.9	37.69 ± 3.883	Mean radioactivity in part per plant (Bq) = $\bar{P}$	2.13 ± 0.454	4.07 ± 0.883	28.05 ± 4.535	3.43± 0.498
			$\frac{\bar{P}}{\bar{R}}$ x 100	5.65	10.79	74.42	9.10

therefore, to gain some indication of the proportion of 'free' ABA present in the plants. The results are shown in Table 19. This investigation into the identity of the translocated radioactivity was carried out once during the first experiment. The majority of the ethyl-acetate-soluble radioactivity was located in the root tissue; this represented 8.01% of the mean total radioactivity per plant. In leaf, stem and nodule material, 6.62%, 5.31% and 0% of the total radioactivity recovered respectively, was ethyl-acetate-soluble.

2.2.3 The Metabolism of [2-^{14}C] ABA Following Root Application of 0.1mol m^{-3} [2-^{14}C] ABA

Although the contribution of [2-^{14}C] ABA to the total radioactivity present in the organic phase of extracts following ethyl acetate partitioning was not determined, it is possible to draw some conclusions regarding the fate of the [2-^{14}C] ABA which entered the plant.

In root tissue, only 11.51% of the mean radioactivity present was ethyl-acetate-soluble (Table 19); thus at least 88.49% of the ^{14}C present was not free ABA. The highest possible amounts of [2-^{14}C] ABA present in the tissues examined occurred in leaf extracts, where 54.75% of the ^{14}C present was ethyl-acetate-soluble; this high percentage may, however, represent an increase in the levels of ethyl-acetate-soluble metabolites. Maximum levels of radioactivity in the stem and nodule material that could be attributed to [2-^{14}C] ABA were 32.68 and 0% respectively.

--ooOoo--

Table 19. Distribution of ethyl acetate-soluble radioactivity 5d after the application of a 0.1mol m^{-3} [2-^{14}C] ABA solution (specific activity 0.297k $Bq.mg^{-1}$) to the roots. Plants were 2 months old and were growing in water culture under long (16h) photoperiods. Acidified aqueous extracts (each prepared from 4 plants) were partitioned against ethyl acetate to separate 'free' ABA and polar metabolites from ABA-GE and non-polar metabolites (the latter were retained in the aqueous phase). The results are presented as absolute ethyl acetate-soluble radioactivity (E); these values are also expressed as a percentage of the total radioactivity translocated to that part ($\bar{P}$) and as a percentage of the mean total radioactivity recovered per plant ($\frac{E}{\bar{R}}$ x 100). The recovery of radioactivity in plants (mean of 4 replicates) was assessed in a parallel experiment by sample oxidation.

TREATMENT	RADIOACTIVITY FED TO PLANTS (Bq)	RADIOACTIVITY RECOVERED (Bq) = $\bar{R}$		PLANT PARTS			
				NODULES	ROOTS	LEAVES	STEM
0.1 mol m^{-3} [2-^{14}C] ABA (specific activity 0.297k Bq mg^{-1}) Run 1	196.3	5.04 ± 0.723	Radioactivity in ethyl acetate phase (Bq) = E	0	0.404	0.334	0.268
			Mean radioactivity in part per plant (Bq) = $\bar{P}$	0.10 ± 0.083	3.51 ± 0.432	0.61 ± 0.105	0.82 ± 0.153
			$\frac{E}{\bar{P}} \times 100$	0	11.51	54.75	32.68
			$\frac{E}{\bar{R}} \times 100$	0	8.01	6.62	5.31

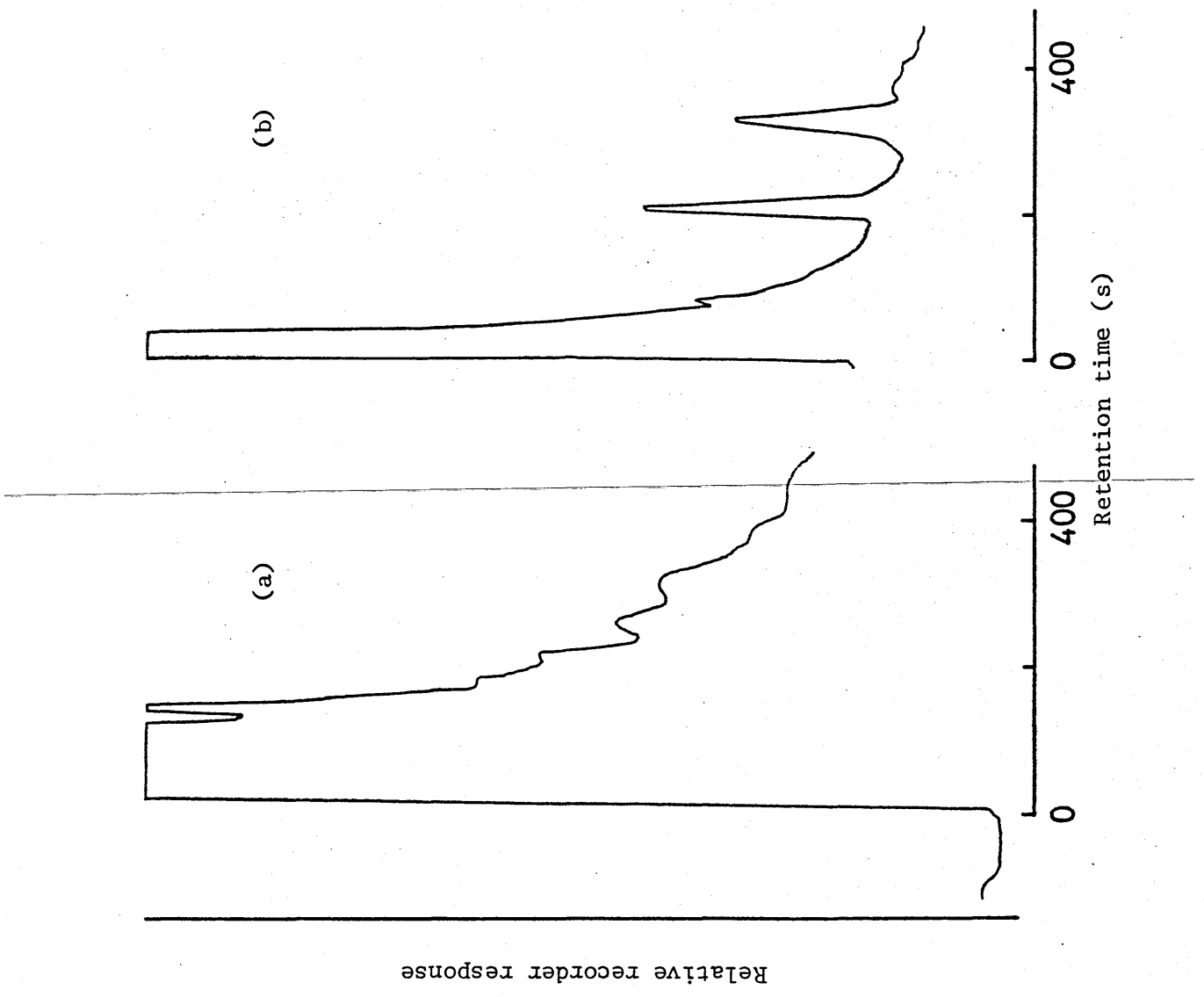
DISCUSSION

DISCUSSION

One method currently employed to investigate the mode of action of PGS rests on careful quantification of levels in tissue extracts (see Introduction). Whilst the absolute efficiency with which the organic solvent methanol removes ABA from different tissues remains unknown, steps can be taken to ensure that the subsequent quantification of the ABA extracted is possible.

In the work described, a purification schema suitable for removing contaminants in nodule, leaf and root extracts was developed, permitting ABA identification and quantification in tissue extracts. The techniques used were labour-intensive and time-consuming, limiting the diversity of experiments that could be attempted. However, considerable improvement in the purification and final analysis of ABA in root nodules was achieved over earlier methods in use at Glasgow, (compare Figure 37 (an early GC trace of a nodule extract made with a packed column) with Figure 12 (a recent GC trace of a nodule extract made with a capillary column). ABA was identified for the first time in extracts from Alnus nodules by mass spectral analysis (Figure 10(a) and (b) , GCMS/MIM (Figure 11(a) and (b) , GCMS/SIM (m/z = 190) and GCECD (Figure 12(a) and (b). Published work concerning the identification and quantification of ABA in the nodules of Glycine max did not use such rigorous procedures (Williams and Sicardi de Mallorca, 1982, 1984). Alnus root and leaf extracts were also shown to contain ABA using GCMS/SIM (m/z = 190) and GCECD. GCECD was the chosen method for the routine analysis and quantification of ABA in all extracts; the identity of the putative cis MeABA peak obtained was confirmed by exposing an extract to UV light which resulted in the appearance of trans ABA (which is

Figure 37. GCECD traces of a nodule extract (a) and 0.5 ng synthetic ABAMe (b), obtained using a packed column.


(a)
(b)
0
400
0
400
Retention time (s)
Relative recorder response

chromatographically distinct from the cis isomer).

In addition to monitoring purification losses by adding small amounts of [2-^{14}C] ABA to extracts, the accuracy of ABA quantification using GCECD analysis was checked. Quantification of extract ABA levels by GCMS/ SIM (m/z= 190) and GCECD yielded values which did not differ significantly.
Additionally, further purification of control extracts following GCECD analysis did not result in any significant alteration in the initial estimate of ABA content. Finally, it was verified by parallel extractions of leaf material in methanol and acetone, that no methanolysis of 'bound' ABA occurred with storage (yielding ABAMe , Neill et al., 1983).

Sandberg et al. (1982) point out that careful quantification of PGS should be accompanied by studies of the variation in PGS levels in a population of plants. Estimation of variation in the endogenous ABA content of plant parts harvested from seed-grown Alnus populations studied here is of particular importance because of the genetic variability inherent in such material. Due to the relatively large numbers of plants required for these analytical studies, insufficient material of greater genetic uniformity could be raised by vegatative propagation techniques (such as stem or leaf cuttings (Wheeler and McLaughlin, 1979)). Despite considerable variability in material, analysis of replicate batches of plants showed no significant difference (95% confidence limits) between replicate plant batches harvested at the same time, in 'free', 'total' or 'bound' ABA (Table 9). Nodule 'free' ABA content expressed on a nmol g^{-1} d.wt. basis was of the same order (0.18 nmol g^{-1} d.wt.) as that reported by Williams and Sicardi de Mallorca (1982) for active Glycine max nodules.

In greenhouse-grown Alnus plants there was no significant

difference between the 'free' ABA content of roots and nodules, although the leaves contained significantly higher amounts (Table 9 , Results 1.1.3.4). The nodules of plants both greenhouse and growth-room grown harvested on a diurnal basis also contained smaller amounts of 'free' ABA than the leaves (Figure 19 , Results 1.2.2). Assuming that 'free' ABA was equally 'extractable' from root, nodule and leaf material, these results suggest that the heterogeneous cell population comprising the leaf can sustain growth and functioning as a unit in the presence of higher amounts of 'free' ABA than the root system. This may be possible through ABA compartmentation. The distribution of ABA within plant cells is determined largely by pH gradients between subcellular compartments (Quarrie, 1984). ABA easily traverses membranes in its protonated form, but may become trapped as an anion in organelles in which the pH is high. Thus as much as 75-80% of leaf ABA may be located within the chloroplasts, having accumulated there as a result of the difference in pH between the cytoplasm and chloroplast stroma (Heilman et al., 1980).

The experiment examining the ABA content of replicate greenhouse-grown plant batches demonstrated that 'total' root ABA levels were significantly smaller than those in the nodules, which, in turn, were significantly smaller than those in the leaves. Similar results were observed in growth-room grown plants harvested over a 24h period. With only one exception, in these plants, 'total' nodule ABA levels were lower than those in the leaves. However, 'bound' ABA levels harvested during the course of the experiments did not follow exactly the same trends. In plants from the greenhouse, 'bound' ABA levels were consistently higher in the nodules and roots than 'free' ABA levels by up to 15.75 and 2.72 times respectively (Table 9). The leaves of these plants,

however, contained lower 'bound' ABA levels than 'free' ABA levels, (although the magnitude of this difference varied widely between replicate extracts). In growth-room grown plants harvested to examine diurnal leaf and nodule ABA levels, nodule 'bound' ABA content was also higher than the 'free' ABA content by up to 9.75 and 7.08 times in the second and third experiments respectively (Figure 19, Results 1.2.2). 'Bound' leaf ABA levels also exceeded those of 'free' ABA in these plants, by up to 4.43 (first experiment) and 3.93 (second experiment) times.

In the roots of Acer pseudoplatanus grown in water culture in a growth chamber under long days, 'free' ABA levels (20-26 ng g^{-1} f.wt.) were similar to those reported here for greenhouse-grown potted Alnus plants (Table 9) (Phillips et al., 1980). However, 'bound' ABA represented only 11.3 - 14.2% of the 'total' ABA pool present in the roots of Acer, whilst in Alnus, this figure was between 60.83 -73.10%. Leaf 'bound' ABA levels were smaller than amounts of the unconjugated form in hydroponically-grown Acer; the absolute levels involved (138.3 - 153.8 ng g^{-1} f.wt. 'free' ABA and 5.0 - 35.3 ng g^{-1} f.wt. 'bound' ABA) were smaller than those reported for Alnus grown in either the greenhouse or a growth chamber. Leaf 'free' ABA levels closer to those observed in Alnus were reported in turgid greenhouse-grown Xanthium plants by Zeevaart (1980) (340 - 380 ng g^{-1} f.w.t); in this study, leaf 'bound' ABA content (440 - 540 ng g^{-1} f.wt.) exceeded that of the unconjugated form.

Although it is clear that in some species the root system is capable of ABA biosynthesis (Walton, 1976; Hartung, 1980) some of the ABA present in the roots of Alnus may have originated in the leaves. Since conjugated ABA is not easily translocated, the roots and nodules of Alnus are probably capable of conjugating 'free' ABA.

Zeevaart (1983) has suggested that in the leaves of Xanthium, 'bound' ABA levels may represent a cumulative index of the water stress experienced by a particular leaf; this may account for the described difference in relative 'free' and 'bound' ABA levels between plant batches. Lighting and humidity which also differed between growth-room and greenhouse-grown plants may also have influenced the way in which leaf ABA was metabolized. Loveys (1979) observed differences in the metabolism of ± [2-^{14}C] ABA applied to the stems of Lycopersicon plants growing in tungsten or fluorescent light. (A far-red enriched light source enhanced the amount of [^{14}C] PA detected).

Having determined that there was little variation in the endogenous ABA content of a batch of plants growing under the same conditions, single batches (of at least 10 plants each) of 3-4 y old plants were harvested on a seasonal basis for root and nodule ABA analyses, (Figure 17). Parallel measurements were made with 2-3 y old plants of seasonal fluctuations in nitrogen fixation to provide an indication of nodule activity. Whilst many criticisms have been levelled recently at the acetylene-reduction method for absolute measurements of nodule nitrogenase activity (Dixon, 1983), the assay is accepted as satisfactory for the comparative measurements required in this study.

It is recognised that in Alnus (as in all legume and non-legume nitrogen-fixing symbioses), that the flow of photoassimilates to the nodules largely determines the rates of nitrogen fixation achieved (Wheeler, 1969; McNiel and Carpenter, 1979). During the 1981 season, the highest levels of nitrogen fixation occurred when the foliage was fully expanded, and following periods in which high solar radiation levels were recorded (Figure 17). However, nitrogenase activity was observed prior-to bud-burst as plants

emerged from dormancy (Figure 17). This effect was demonstrated earlier by Wheeler and McLaughlin, (1979) in 2y old Alnus glutinosa plants moved to long days following naturally-imposed dormancy. Intact xylem but not, apparently, phloem connections with the shoot were required, leading these authors to suggest that the removal of inhibitory substances from the nodules via the xylem may be a prerequisite to the initiation of nitrogen fixation in this species. One possibility was that this inhibitor might be ABA, and evidence for a lowering of ABA levels as nodules emerged from dormancy was sought, therefore.

Although nodule 'free' ABA levels observed during the 1981 season were higher during the winter dormant period than by late spring, a small decrease only occurred prior to the onset of nitrogen fixation. Thus, even though the most active period of nodule growth and nitrogen fixation correlated with ABA levels up to 2.5 times lower than during nodule dormancy, a decline in ABA levels alone does not seem to be responsible for 'triggering' renewed nodule growth and nitrogenase activity, if this compound acts in the way in which plant physiologists have conventionally proposed (see Introduction). However, this change may be of significance if, for example, it represents a decrease confined to the nodule meristem. It is also possible that significant changes in the ABA content of younger nodules may have been masked by higher levels in older tissue.

In the roots of Acer saccharum, the lowest endogenous ABA levels recorded correlated well with periods of vigorous root extension, leading Cohen et al., (1978) to suggest that in this species, ABA was involved in the control of root growth. (±) ABA was also shown to induce dormancy in the roots of Picea cuttings

growing in both long and short day conditions, when applied at a concentration of 0.1mol m^{-3} (Philipson and Coutts, 1979). The application of 0.1mol m^{-3} (±) ABA to the roots of young Alnus plants growing in water culture led to large scale changes in endogenous tissue ABA levels (Figures 21-24) and to the onset of shoot dormancy (Figures 26, 27 and 29).The lengthy time taken for visible signs of dormancy, however (30 to 60 d) suggests that factors in addition to changes in tissue ABA content were involved.

In Ribes nigrum (blackcurrant) buds, Wright (1975) suggested that the ratio of 'free' to 'bound' ABA levels was important in the control of dormancy. The highest 'free' to 'bound' ABA ratio of bud extracts occurred in the autumn, whilst the lowest was coincident with bud burst. A similar mechanism may also exist in Alnus nodules; two harvests made in 1983 demonstrated that as spring approached, 'free' nodule ABA levels declined whilst 'bound' ABA levels increased (Table 11). 'Bound' ABA was present in greater amounts than 'free' ABA in both nodules and roots; proportionately more was found as a conjugate in the nodules, however, in which 'bound' ABA levels were 9.23 - 23.99 times greater than 'free' ABA levels. In roots, levels of the conjugate exceeded those of the 'free' compound by only 6.58 - 6.92 times. In leaves, conjugation is thought to be irreversible (Milborrow, 1978) and conjugated ABA has been described as an end product of ABA metabolism (Neill et al., 1983). Conjugation of ABA in the nodules may represent the easiest method of removing the active form.

If it may be assumed that the trans ABA present in extracts was lost in the same quantities as endogenous cis ABA (i.e. that losses of the cis [2-^{14}C] ABA internal standard were also representative of losses of trans ABA), it is possible to make some statements regarding the measurements made in 1983, of 'free'

and 'bound' cis and trans ABA levels in the roots and nodules. Bangearth (1982) has suggested that certain plant parts can 'metabolize' ABA to trans ABA in the absence of light. This may represent the way in which trans ABA arose in the root system of Alnus. Both cis and trans ABA were present as conjugates in the roots and the nodules. When expressed as a proportion of 'free' ABA levels (cis or trans where appropriate), the conjugated trans ABA content of both the roots and nodules was greater than conjugated cis ABA levels. It has been reported that in rose leaves, trans ABA was esterified to trans ABAGE ten times faster than cis ABA (Milborrow, 1970; Loveys and Milborrow, 1984). It is possible that whilst cis ABA may be metabolized via other routes (even via trans ABA production!), the major pathway of trans metabolism may be via the formation of conjugates. Alternatively, bearing in mind reports that the methyl ester of ABA can undergo isomerization under UV light (Bangearth, 1982), it remains possible that the conjugated trans ABA present in root and nodule extracts was derived from conjugated cis ABA; the existence of an isomerase must be invoked, however.

Similar results were observed in nodule and leaf extracts from plants harvested on a diurnal basis (Figure 18). In this experiment in all cases, no 'free' trans ABA at all was detected in the leaf extracts. Any 'free' trans ABA formed by light-catalyzed isomerization in the leaves must be promptly removed, therefore, by conjugation or via an alternative metabolic route. Additionally, nodule extracts contained proportionately more conjugated trans ABA (of the total 'bound' ABA extracted) than leaf extracts.

No large scale changes in leaf or nodule ABA content on a diurnal basis could be detected in Alnus using the described

techniques (Results 1.2.2). This contrasts with the diurnal changes in leaf ABA levels reported for several species (see Introduction 2.5), for example in field-grown Pennisetum americanum (Henson et al., 1982), Prunus persica (Xiloyannis et al., 1980), Sorghum bicolor (Kannangara et al., 1982), Pisum (Kobriger et al., 1982). (Sandberg et al. (1982) also recorded diurnal changes in the IAA content of Pinus sylvestris seedlings grown under controlled conditions.) The oft-observed absence of a correlation between leaf ABA content and stomatal aperture has led to speculations that the sensitivity of stomata to ABA or the accessibility of the PGS to the stomata changes throughout the day (Henson et al., 1982).

In non water-stressed Alnus growing in a controlled environment, an alteration in whole leaf or whole nodule ABA levels is clearly unnecessary to the functioning of these organs. Additionally, if indeed the translocation of ABA occurs between organs in Alnus, careful regulation of tissue ABA levels must occur to prevent substantial deviation at any one time. These results also imply that the metabolism of 'free' cis ABA via a route other than conjugation must occur in the nodules if this isomer is imported to any extent from the leaves, since nodule 'bound' ABA levels do not alter on a diurnal basis.

Before the advent of techniques which made the quantification of ABA possible, many physiologists made exogenous ABA applications to plants to try to elucidate the role of this PGS. Early studies with applications of inhibitor β extracts and ABA to Ribes leaves led Wareing and his colleagues (El-Antably et al., 1967) to conclude that ABA was associated with the onset of bud dormancy in this species (Introduction) although later experiments by other workers did not support this conclusion. In the present work, the physiological effects of root-applied 0.1 mol m^{-3} ($\pm$) ABA on

water culture grown Alnus were observed, alongside measurements of endogenous leaf, root, nodule and apex ABA levels.

A significant reduction in plant height (over control plants) was observed in plants which had been in root-contact with 0.1 mol m^{-3} ABA for 31 d (Figure 25). 30 - 60 days following root-immersion in ABA, many plants were showing other symptoms of impending bud dormancy (Figures 26-29 Results 1.2.3.2). These included leaf necrosis, the appearance of purple 'dormancy' buds in the leaf axils and at the apex, stem-thickening, and a reduction in stem internode distance. Whole plant fresh and dry weights were smaller in treated plants than control specimens; this is most likely to be due to a reduction in the assimilation of carbon caused by ABA-mediated stomatal closure. However, ABA treatment increased the root:shoot ratio of plants (when expressed on a fresh or dry weight basis), an effect which has been observed in Capsicum, Zea (Watts et al., 1981) and Brassica oleracea (Biddington and Dearman, 1982). In Brassica, whilst root-applied racemic 0.01 mol m^{-3}ABA increased root dry weight (this was attributed to a direct effect on root growth), root length was not altered significantly. In other experiments, however (Watts et al., 1981; Yamaguchi and Street, 1977), root length was increased following ABA treatment. Water stress has a similar effect as applied ABA on root growth increasing the root area for water absorption (Watts et al., 1981).

Philipson and Coutts (1979) reported that 0.1 mol m^{-3}(±) ABA induced root dormancy in both long and short day conditions in Picea cuttings from 12y old plants growing in culture solution, although this effect did not occur in juvenile 1y old seedlings. Lateral roots were observed to emerge from the dormant main axes in the presence of ABA.

ABA treatment also reduced the fresh to dry weight ratios of the shoots and roots in Alnus, (Table 12). In decapitated Glycine max grown in water culture and exposed to a 3 bar hydrostatic pressure difference the addition of synthetic (±) ABA to the nutrient solution (to give a final ABA concentration of 0.05 - 0.1 mol m^{-3}) resulted in a decrease in xylem exudation rate (Markhart, 1982). This was attributed to a decrease in the hydraulic conductance of the roots. Although this effect does not appear to be universal in experimental plants (e.g. Tal and Imber, 1971; Glinka, 1980), if, indeed ABA reduced water uptake in Alnus this may have caused, in part, the observed reduction in the fresh to dry weight ratios of treated plants (Table 12). However, since plants remained in contact with the ABA for a lengthy period of time, it remains possible that this was a secondary effect associated with the onset of bud dormancy.

As well as the effects of root-applied ABA on dormancy, an indication of the fate of the applied ABA was sought in a parallel experiment with younger (2 to 3 months old) Alnus plants. After 5d of root contact with 0.1 mol m^{-3} or 2.52 x 10^{-5}mol m^{-3} [2-^{14}C] ABA, radioactivity was present in all parts of the plants examined (roots, nodules, shoots and apices; Table 18). In all except the nodule tissue, at least some radioactivity was ethyl acetate soluble (Table 19) and hence may have included free [2-^{14}C] ABA. Following both treatments, the majority of radioactivity recovered was present in the root system. However, whilst 48.02% (experiment 2) to 69.64% (experiment 1) of the total radioactivity recovered was present in the roots of plants in contact with 0.1 mol m^{-3} ± ABA, 74.42% was present in the roots of plants treated with 2.52 x 10^{-5} mol m^{-3} (±) ABA. Additionally, in the presence of only 2.52 x 10^{-5} mol m^{-3}(±) ABA, 2.7 times more of the

radioactivity available in the culture solution entered the plant than when the roots were immersed in a 0.1 mol m^{-3} (±) ABA solution. Assuming that all of the radioactivity present in the plant initially entered as ABA, this result suggests that over a 5d period in the presence of 0.1 mol m^{-3} (±) ABA, the root system became saturated with ABA limiting further uptake. The existence of either specific or non-specific saturable binding sites for ABA or the initiation of systems capable of ABA metabolism were hypotheses suggested by Markhart (1982) to explain the gradual decline in xylem exudate ABA levels in *Glycine max* roots treated with 0.05 - 0.1 mol m^{-3} ABA to a steady-state value over 6h. In *Alnus* plants treated with 0.1 mol m^{-3} (±) ABA the transport of radioactivity from the roots to the shoots was increased (over those treated with only 2.52 x 10^{-5} mol m^{-3} ± ABA) and there was evidence (from an examination of ethyl-acetate-soluble radioactivity, Table 19) to suggest that much of the ABA which remained in the roots was conjugated. By comparison, much higher percentages of ethyl-acetate-soluble radioactivity were present in the leaves of treated plants. If the radioactivity which reached the leaves was 'free' [2-^{14}C] ABA, this observation suggests either that 'free' ABA may be accumulated (possibly sequestered?) by these organs, or that the major pathways of ABA metabolism in the leaves does not involve the formation of conjugates.

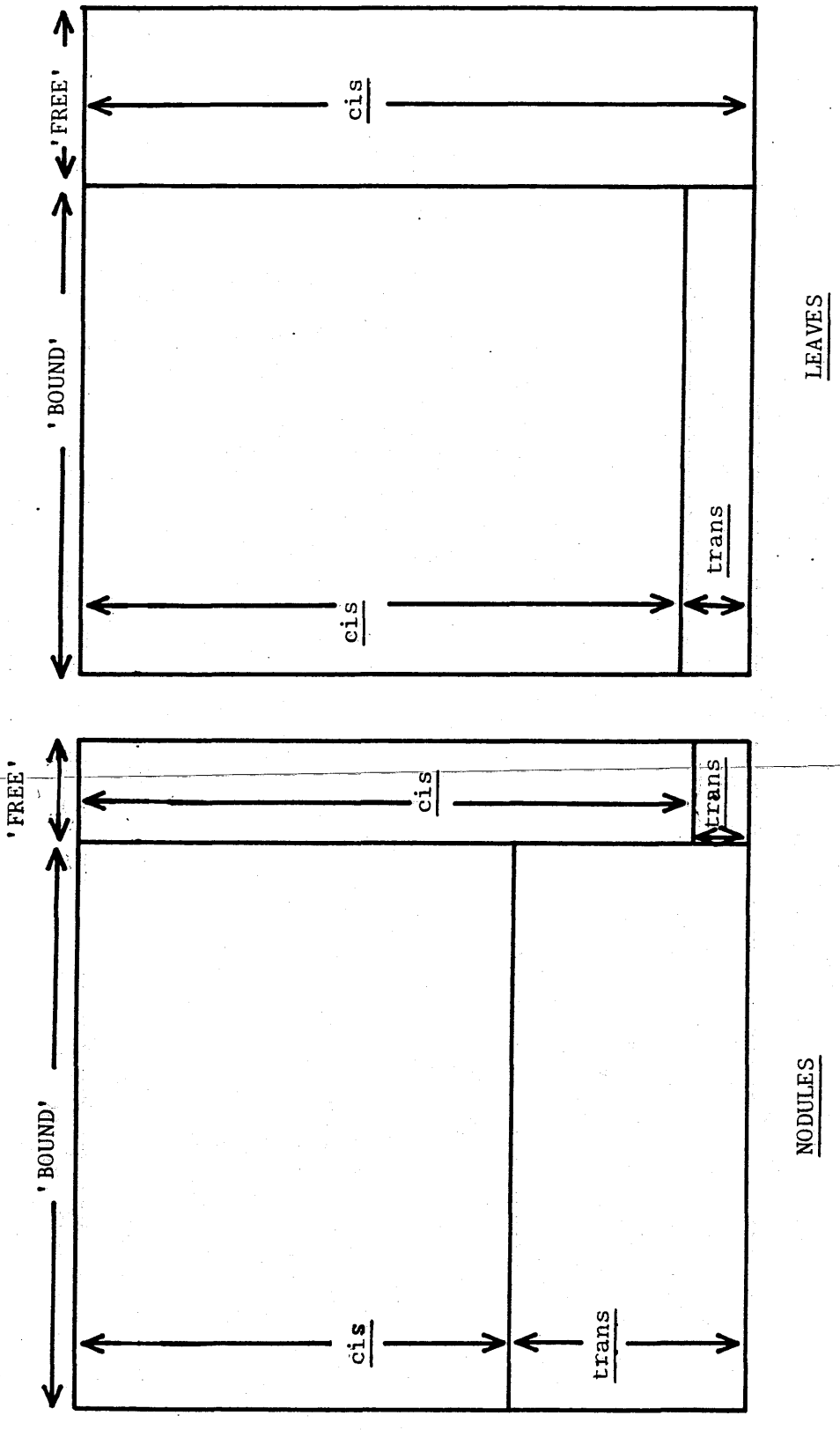
The translocation of ABA in *Glycine* and *Phaseolus* following root application occurs primarily via the transpirational water column (Fiscus *et al.*, 1982; Markhart, 1982); a similar mechanism probably exists in *Alnus*. In the shoots of *Alnus*, however, steam girdling experiments by Hocking (1973) indicated that phloem translocation of ABA to other parts is the major pathway of ABA transport. In the experiments conducted during the course of the present work, very little radioactivity traversed a phloem block

situated between the fourth and fifth leaves from the apex following petiolar [2-^{14}C] ABA application to the third leaf (Table 15). In the shoots of most plants, ABA is phloem translocated (Walton, 1980). [2-^{14}C] ABA was identified in the root pressure sap from plants decapitated 24h following leaf petiole [2-^{14}C] ABA application, however (Table 16), indicating that phloem:xylem exchange of ABA may occur in the root system of Alnus.

The endogenous ABA content of plants which had been in root contact with 0.1mol m^{-3} (±) ABA for 30 to 60 d and were showing signs of shoot dormancy was also examined in an attempt to study further the way in which plants can deal with large amounts of ABA. Plant propagation in water culture for use in experiments examining ABA levels is particularly useful since it largely removes the possibility of water stress induced variations in tissue endogenous ABA content (this remains one of the largest problems in the investigation of ABA levels in non-water-stressed tissue). Phillips et al., 1980 have demonstrated that in seedlings of Acer pseudoplatanus water saturation deficits (%) per plant ranged from 1.79 - 11.68 (mean 7.06 ± 0.97 SE) in soil, but from only 6.34 - 7.00 (mean 6.65 ± 0.08 SE) in plants in liquid nutrient medium.

Control plants grown in water culture had lower levels of 'bound' cis ABA than the 'free' isomer. In pot-grown plants, the reverse was true, however (Figure 38). ('Bound' trans ABA levels exceeded 'free' trans ABA levels in all tissues examined in both pot and water culture grown plants). These results suggest that the metabolism of 'free' cis ABA by conjugation in the leaves of plants growing in water culture is not as important as in plants that are pot-grown. This may be because the leaves of plants growing in culture solution never experience water stress; as a result, 'bound' ABA levels (the 'index' of water stress, Zeevaart,

Figure 38. Relative proportions of 'free' and 'bound' *cis* and *trans* ABA levels of the total present, in nodule and leaf extracts from plants harvested on a diurnal basis. Mean ABA levels per extract were first calculated from 8 or 9 observations using the data from two experiments (experiments 2 and 3). The diagram was drawn using values calculated from these mean figures. The total ABA content of nodule extracts ranged from 504.9 - 1894.5ng g^{-1} f.wt., whilst that of the leaves ranged from 577.7 - 1071.8ng g^{-1} f.wt.


'FREE'
cis
'BOUND'
cis
trans
LEAVES
'FREE'
cis
trans
'BOUND'
cis
trans
NODULES

1983) did not accumulate to the same extent as in pot-grown specimens.

In all tissues extracted, root 0.1mol m^{-3} ABA treatment resulted in an increase in total ('free'plus 'bound') ABA levels (Results 1.2.3.1, Figure24). The largest increase in tissue 'total' ABA levels occurred in the roots and nodules which were in contact with the ABA solution. In all tissues, 'bound' ABA content increased proportionately more than 'free' ABA levels in treated plants. The results of the root [2-^{14}C] (±) ABA feeding experiments described above in addition to the observations of Markhart (1982) and Fiscus (1982) that both *cis* and *trans* ABA are taken up by the roots of *Glycine max* and *Phaseolus vulgaris* make it likely that the observed increases in endogenous ABA levels in all parts of the plant were due at least in part to ABA taken up from the culture solution. It remains possible, however, that *de novo* synthesis caused by root stress contributed to the shoot ABA levels. The increase in 'bound' ABA levels of treated plants probably represents an attempt to keep 'free' ABA levels down, to permit normal cell functioning.

'Free' and 'bound' *trans* ABA levels exceeded the respective 'free' and 'bound' *cis* ABA levels of both root and nodule extracts from treated plants although a similar trend was observed in all but one case in the same tissues of control plants, the magnitude of the difference between the levels of the two isomers was much smaller in these plants. These results indicate that in the root system of *Alnus* growing in water culture, it is more important to reduce 'free' *cis* ABA levels than 'free' *trans* ABA; this fits the current hypothesis that *trans* ABA has little or no biological activity (Loveys and Milborrow, 1984). In the aerial parts, however, 'free' and 'bound' *cis* ABA levels exceeded those of the

trans isomer. This may be because very little 'free' trans ABA at all was translocated from the roots to the shoots; most may have been conjugated in the root system. (In decapitated Glycine max root-treated with (±) ABA via the culture solution, xylem exudate concentration of the cis isomer averaged only 0.5 ± 0.05 of the ambient cis ABA concentration, whilst that of the trans isomer averaged 0.16 ± 0.04 (Markhart, 1982). Alternatively, in Alnus, the shoot system may be able to remove 'free' trans ABA more successfully than the roots. In the roots, leaves and apices of both control and treated plants, proportionately more 'free' trans than 'free' cis ABA was conjugated; this suggests that in these tissues, conjugation represents the major pathway of trans metabolism. (The results for nodule tissue did not follow the same trends in both experiments).

Dormancy in alders can be induced readily by short-day treatment (Wheeler and McLaughlin, 1979). To explore further the possible involvement of ABA in this event, the translocation of foliar-applied (±) [2-^{14}C] ABA was studied in plants growing under long or short-day conditions. 24h following leaf petiolar (±) [2-^{14}C] ABA application, radioactivity was present in all parts of young Alnus plants growing in long photoperiods (Table 13 ; Hocking, 1973).

In addition to the expected influence of short days on the height of young Alnus plants (Figure 34), the translocation of radioactivity and ABA 24h after petiolar [2-^{14}C] ABA application was shown to be affected by the photoperiod under which the plants were growing. After 34 to 47 short days, less radioactivity was translocated away from the donor leaf (3rd leaf from the apex) than in plants maintained in long days (Table 13). Additionally, whilst under long photoperiods an average of 28.8% (experiment 1)

of the mean ^{14}C recovered was transported to parts of the plant above the donor leaf, this figure reached only 2.9%(2nd experiment) to 9.6%(1st experiment) in short-day plants. In contrast to long-day plants, the translocation of radioactivity from the site of application was predominantly basipetal in plants grown under short photoperiods. Everat-Bourbouloux (1982) demonstrated that following the application of [2-^{14}C] ABA to a mature leaf of Vicia faba, ^{14}C was initially translocated to tissues underlying the donor leaf. If the donor leaf was then removed, a redistribution of radioactivity was observed: 6h later, two thirds of the ^{14}C exported was translocated to the upper part of the axis. It seems highly likely, therefore, that some redistribution of ^{14}C occurred in Alnus over the course of the 24h translocation period, although it is not possible to speculate on any differences which may have occurred between long and short day plants.

In Alnus, [2-^{14}C] ABA distribution (levels of [2-^{14}C] ABA present in a given tissue expressed as a percentage of mean [2-^{14}C] ABA recovered per plant) 24h following petiolar application were similar to those of ^{14}C (Table 14).

Tissue [2-^{14}C] ABA levels (expressed as a percentage of the mean radioactivity present in a given tissue (Table 14)) indicated that there were certain differences in the metabolism of ABA in plants grown in long and short photoperiods. Much more of the radioactivity located in the leaves and stem below the donor leaf and in the stem above the donor leaf of short day plants was present as [2-^{14}C] ABA than in plants grown in long photoperiods. Similar proportions of radioactivity were identified as ABA in the leaves above the donor leaf, and in the donor leaf of plants from both treatments. Owing to the small amounts of radioactivity involved, it was not possible to conclude whether or not there

were differences in the ABA content of the roots and nodules of plants grown under the two photoperiods. Assuming that the absolute levels of radioactivity reaching the tissues did not alter the metabolism of [2-^{14}C] ABA, these results indicate either that the rate of ABA metabolism in the leaves and stem below the donor leaf, and shoot above the donor leaf was retarded in short-day plants, or that owing to the altered pattern of transported radioactivity in these plants, more unmetabolized ABA reached these tissues than in long day plants. Either way, it is possible that an increase in the 'free' ABA content of these tissues of short-day plants was at least partially responsible for the onset of the dormant condition.

Although feeding of (±) ABA via the root system can in time induce shoot dormancy, there is very little evidence from the present study to suggest that changes in whole tissue ABA levels alone control nodule or bud dormancy in Alnus. Alnus nodules can sustain growth and functioning in the presence of high levels of ABA compared with the rest of the root system; the role which ABA may play in nodule physiology remains to be determined. Whilst the translocation studies carried out during the course of this work indicate that the nodules probably receive ABA from the shoot system with photosynthate, it is possible that the nodules themselves synthesize ABA.

It is clear that in future work concerning the role and function of PGS, plant physiologists must rid themsleves of the straight-jacket approach of single PGS research; additionally, future studies must deal with PGS action sites, receptor sites and subcellular location if progress is to be made. This author considers it likely that future routine quantification of ABA in tissue extracts will be achieved largely by the use of HPLC and radioimmunoassay techniques (see Introduction 2.2), although there

will always remain a case for identifying this compound by GCMS techniques.

Finally, further study of the metabolism and transport of PGS in non-nodulated herbaceous and woody plant species is probably required before the role of PGS in the development and function of the more complex root nodule system can be resolved.

LITERATURE CITED

*denotes article was not read in detail

Abou-Mandour, A.A., Hartung, W., 1980. The effects of abscisic acid on growth and development of intact seedlings, root and callus cultures and stem and root segments of Phaseolus coccineus Z. Pflanzenphysiol. 100: 25-33.

Ackerson, R.C., 1980. Stomatal response of cotton to water stress and abscisic acid as affected by water stress history. Plant Physiol. 65: 455-459.

Addicott, F.T., Lyon, J.L., 1969. Physiology of abscisic acid and related substances. Ann. Rev. Plant Physiol. 20: 139-164.

Akkermans, A.D.L., Roelofsen, W., 1980. Symbiotic nitrogen fixation by actinomycetes in Alnus-type root nodules. In : Nitrogen fixation. Eds. Stewart, W.D.P., Gallon, J.R. Academic Press.

Akkermans, A.D.L., Roelofsen, W., Blom, J., Huss-Danell, K., Harkink, R., 1983. Utilization of carbon and nitrogen compounds by Frankia in synthetic media and in root nodules of Alnus glutinosa, Hippophaë rhamnoides and Datisca cannabina. Can.J. Bot. 61: 2793-2800.

Alvim, R., Hewett, E.W., Saunders, P.F., 1976. Seasonal variations in the hormone content of willow. I. changes in abscisic acid content and cytokinin activity in the xylem sap. Plant Physiol. 57: 474-476.

* Angulo Carmona, A.F., 1974. La formation des nodules fixateurs d'azote chez Alnus glutinosa (L.) Vill. Acta Bot. Neerl. 23: 257-303.

Astle, M.C., Rubery, P.H., 1980. A study of abscisic acid uptake by apical and proximal root segments of Phaseolus coccineus (L.). Planta 150: 312-320.

Back, A., Richmond, A.E., 1971. Interrelations between gibberellic acid, cytokinins and abscisic acid in retarding leaf senescence. Physiol. Plant. 24: 76-79.

Baker, D., Torrey, J.G., Kidd, G.H., 1979. Isolation by sucrose-density fractionation and cultivation in vitro of actinomycetes from nitrogen fixing root nodules. Nature 281: 76-78

Bangearth, F., 1982. Changes in the ratio of cis-trans to trans-trans abscisic acid during ripening of apple fruits. Planta 155: 199-203.

Behl, R., Jeschke, W.D., 1979. Effects of ABA on transport, accumulation and uptake of K^+ and Na^+ in excised barley roots. Plant Physiol. (Suppl.) 63: 14.

Behl, R., Jeschke, W.D. Hartung, W., 1981. A compartmental analysis of abscisic acid in roots of Hordeum distichon. J. Exp. Bot. 32: 889-897.

Belke, C.J., Volkert, S., Brenner, M.L., 1980. Metabolism and phloem transport of [2- 14C] ABA in tomato leaves. Plant Physiol. 65: S94.

Bellandi, D.M., Dörffling, K., 1974. Transport of abscisic acid 2-C-14 in intact pea seedlings. Physiol. Plant. 32: 365-368.

Bengtson, C., Falk, S.O., Larsen, S., 1977. The after-effect of water stress on transpiration rate and changes in the abscisic acid content of young wheat plants. Physiol. Plant. 41: 149-154.

Berrie, A.M.M., 1984. In: Advanced plant physiology. Ed. Wilkins, M.B. Pitman.

Berrie, A.M.M., Robertson, J., 1976. Abscisic acid as an endogenous component in lettuce fruits, Lactuca Sativa (L.) cv. Grand Rapids. Does it control thermodormancy? Planta 131: 211-215.

Berry, A., Torrey, J.G., 1979. Isolation and characterization in vivo and in vitro of an actinomycetous endophyte from Alnus rubra Bong. In: Symbiotic nitrogen fixation in the management of temperate forests. Eds: Gordon, J.C., Wheeler, C.T., Perry, D.A. Forestry publication, School of Forestry, Oregon State University, Corvallis.

Biddington, N.L., Dearman, A.S. 1982. The effect of abscisic acid on root and shoot growth of cauliflower plants. Plant Growth Regulation 1: 15-24.

Binkley, D., 1982. Nitrogen fixation and net primary production in a young sitka alder stand. Can. J. Bot. 60: 281-283.

Blom, J., 1981. Utilization of fatty acids and NH_4^+ by Frankia AVC I1. FEMS Microbiology Lett. 10: 143-145.

Blom, J., Harkink, R., 1981. Metabolic pathways for gluconeogenesis and energy generation in Frankia AVC I1. FEMS Microbiology Lett. 11: 221-224.

* Böttger, M., 1970. Hormonal regulation of petiolar abscission in Coleus rehneltianus explants. Planta 93: 205-213.

Bond, G., 1983. See p200, reference 1.

Boussiba, S., Richmond, A.E., 1976. Abscisic acid and the after effect of stress in tobacco plants (Nicotiana rustica (L.)). Planta 129: 217-219.

Brabham, D.E., Biggs, R.H., 1981. cis-trans photoisomerization of abscisic acid. Phytochemistry and Photobiology 34: 33-37.

Browning, G., Saunders, P.F., 1977. Membrane localized gibberellins A_9 and A_4 in wheat chloroplasts. Nature 265: 375-377.

Burschka, C., Tenhunen, J.D., Hartung, W., 1983. Diurnal variations in abscisic acid and stomatal response to applied abscisic acid in leaves of irrigated and non-irrigated Arbutus unedo plants under naturally fluctuating environmental conditons. Oecologia 58: 128-131.

Callaham, D., Del Tredici, P., Torrey, J.G., 1978. Isolation and cultivation in vitro of the actinomycete causing root nodulation in Comptonia. Science 199: 899-902.

Callaham, D., Torrey, J.G., 1977. Prenodule formation and primary nodule development in roots of Comptonia (Myricaceae). Can.J.Bot. 55: 2306-2318.

Catalano, M., Hill, T.A., 1969. Interaction between gibberellic acid and kinetin in overcoming apical dominance, natural and induced by IAA in tomato (Lycopersicon esculentum Mill. cv. Potentate). Nature 222: 985-986.

Chanson, A., Pilet, P.E., 1982. Transport and metabolism of [2-^{14}C] abscisic acid in maize root. Planta 154: 556-561.

Choveaux, N.A., Van Staden, J., 1981. The effect of 1-naphalene acetic acid on the endogenous cytokinin content of aseptically cultured bark segments of Salix babylonica. Plant and Cell Physiol. 22: 1207-1214.

Ciha,A.J., Brenner, M.L., Brun, W.A., 1977. Rapid separation and quantification of ABA from plant tissues using HPLC. Plant Physiol. 59: 821-826.

Cohen, D.B., Dumbroff, E.G. Webb., D.P., 1978. Seasonal patterns of ABA in roots of Acer saccharum. Plant Sci. Lett. 11: 35-39.

Colquhoun, A.J., Hillman, J.R., 1972. The effects of abscisic acid on senescence in leaf discs of radish Raphanus sativus (L.). Planta 105: 213-224.

Colquhoun, A.J., Hillman, J.R., 1975. Endogenous abscisic acid and the senescence of Phaseolus vulgaris (L.). Z. Pflanzenphysiol. 76: 326-332.

Cornforth, J.W., Milborrow, B.V., Ryback, G., 1965b. Synthesis of (+) abscisin II. Nature 206: 715.

Cornforth, J.W., Milborrow, B.V., Ryback, G., Wareing, P.F., 1965a Chemistry and physiology of 'dormins' in sycamore. Nature 205: 1269-1270.

Cornforth, J.W., Milborrow, B.V., Ryback, G., 1966. Identification and estimation of (+)- Abscisin II ('Dormin') in plant extracts by spectropolarimetry. Nature 210: 627-628.

Cram, W.J., 1976. In: Encyclopedia of plant physiology. Eds. Pirson, A., Zimmerman, M.H. 2A: 284-307.

Crozier, A., 1980. The analysis of plant hormones in trees. In: Control of shoot growth in trees. Ed. Little, C.H.A. Canadian Forest Service, Fredericton, New Brunswick.

Crozier, A., 1981. See p200, reference 2.

Davenport, T.L., Jordan, W.R., Morgan, P.W., 1977. Movement and endogenous levels of abscisic acid during water stress-induced abscission in cotton seedlings. Plant Physiol. 59: 1165-1168.

Davidson, R.M., Young, H., 1974. Abscisic acid content of xylem sap. Planta 109: 95-98.

Davis, L.A., Addicott, F.T., 1972. Abscisic acid correlations with abscission and with developments in the cotton fruit. Plant Physiol. 49: 644-648.

De Bell, D.S., 1979. Future potential for use of symbiotic nitrogen fixation in forest management. In: Symbiotic nitrogen fixation in the management of temperate forests. Eds. Gordon, J.C., Wheeler, C.T., Perry, D.A. Oregon State University, Corvallis, Oregon.

Dela-Fuente, R.K., Leopold, A.C., 1972. Two components of auxin transport. Plant Physiol. 50: 491-495.

Dewdney, S.J., McWha, J.A., 1978. The metabolism and transport of abscisic acid during grainfill in wheat. J. Exp. Bot. 29: 1299-1308.

Dixon, R.O.D., Wheeler, C.T. 1983. Biochemical, physiological and environmental aspects of symbiotic nitrogen fixation. In: Biological nitrogen fixation in forest ecosystems: foundations and applications. Eds. Gordon, J.C., Wheeler, C.T. (Martinus Nijhoff, Dr. W. Junk publishers).

Dörffling, K., 1976. Correlative bud inhibition and abscisic acid in Acer pseudoplantanus and Syringa vulgaris. Physiol. Plant. 38: 319-322.

Dörffling, K., Böttger, M., Martin, D., Schmidt, V., Borowski, D., 1978. Physiology and chemistry of substances accelerating abscission in senescent petioles and fruit stalks. Physiol. Plant. 43: 292-296.

Dumbroff, E.G., Cohen, D.B., Webb, D.P., 1979. Seasonal levels of abscisic acid in buds and stems of Acer saccharum. Physiol. Plant. 45: 211-214.

Dunlap, J.R., Morgan, P.W., 1981. Preflowering levels of phytohormones in sorghum. II. Quantification of preflowering internal levels. Crop Sci. 21: 818-822.

Eagles, C.F., Wareing, P.F., 1964. The role of growth substances in the regulation of bud dormancy. Physiol. Plant. 17: 697-709.

El-Antably, H.M.M., Larsen, P., 1974. Distribution of gibberellins and abscisic acid in geotropically stimulated Vicia faba roots. Physiol. Plant. 32: 322-329.

El-Antably, H.M.M., Wareing, P.F., Hillman, J.R., 1967. Some physiological responses to D, L abscisin (Dormin). Planta 73: 74-90.

Even-chen, Z., Itai, C., 1975. The role of abscisic acid in senescence of detached tobacco leaves. Physiol. Plant. 34: 97-100.

Everat-Bourbouloux, A., 1982. Transport and metabolism of labelled abscisic acid in broad bean plants (Vicia faba (L.)). Physiol. Plant. 54: 431-439.

Everat-Bourbouloux, A., Charnay, D., 1982. Endogenous abscisic acid levels in stems and axillary buds of intact or decapitated broad bean plants (Vicia faba (L.)). Physiol. Plant. 54: 440-445.

Feldman, L.J., 1981a. Root cap inhibitor formation in isolated root caps of Zea Mays L. J. Exp. Bot. 32: 779-788.

Feldman, L.J., 1981b. Light-induced inhibitors from intact and cultured caps of Zea roots. Planta 153: 471-475.

Fiscus, E.L., Stermitz, F.R., Amoros, L.G., 1982. Abscisic acid transport coefficients of Phaseolus root systems. Plant Physiol. 69: 1353-1355.

Fuchs, Y., Mayak., S., Fuchs, S., 1972. Detection and quantitative determination of abscisic acid by immunological assay. Planta 103: 117-125.

Fujioka, S., Yamaguchi, I., Murofushi, N., Takahashi, N., Kaihara, S., Takimoto, A. 1983. The role of plant hormones and benzoic acid in flowering of Lemna paucicostata 151 and 381. Plant and Cell Physiol. 24: 241-246.

Gillard, D.F., Walton, D.C., 1976. Abscisic acid metabolism by a cell-free preparation from Echinocystis lobata liquid endosperm. Plant Physiol. 58: 790-795.

Glenn, J.L., Kuo, C.C., Durley, R.C., Pharis, R.P., 1972. Use of insoluble polyvinyl pyrolidone for purification of plant extracts and chromatography of plant hormones. Phytochem. 11: 345-351.

Glinka, Z., 1980. Abscisic acid promotes both volume flow and ion release to the xylem in sunflower roots. Plant Physiol. 65:537-540.

Goldbach, H., Goldbach, E., 1977. Abscisic acid translocation and influence of water stress on grain abscisic acid content. J. Exp. Bot. 28: 1342-1350.

Gray, R.T., Mallaby, R., Ryback, G., Williams, V.P., 1974. Mass spectra of methyl abscisate and isotopically related analogues J. Chem. Soc. Perkin Trans II: 919-924.

Guinn, G., 1982. Fruit age and changes in abscisic acid content, ethylene production and abscission rate of cotton fruits. Plant Physiol. 69: 349-352.

* Hansen, E.A., Dawson, J.O., 1982. Effect of Alnus glutinosa on hybrid Populus height growth in a short-rotation intensively cultured plantation. Forest Sci. 28: 49-59.

Hardy, R.W.F., Holsten, R.D., Jackson, E.K., Burns, R.C., 1968. The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. Plant Physiol. 43: 1185-1207.

Harrison, M.A., Saunders, P.F., 1975. The abscisic acid content of dormant birch buds. Planta 123: 291-298.

* Hartung, W., 1976. The basipetal transport of $[2\text{-}^{14}C]$ ABA in roots of intact seedlings of runner beans and its significance for root geotropism. Planta 128: 59-62.

* Hartung, W., 1977. Transport of $[2\text{-}^{14}C]$ abscisic acid from the root system of runner bean seedlings to the aerial parts of the plant. Z. Pflanzenphysiol. 83: 81-84.

Hartung, W., Abou-Mandour, A.A., 1980. Abscisic acid in root cultures of Phaseolus coccineus (L.). Z. Pflanzenphysiol. 97: 265-269.

* Hartung, W., Behl, R., 1974. See p 200, reference 3.

* Hartung, W., Behl, R., 1975. Localisation of acropetal transport of [2-^{14}C] abscisic acid in roots of Phaseolus coccineus (L.) and evidence for a radial ABA-translocation between stele and cortex. Planta 122: 53-59.

Heilmann, B., Hartung, W., Gimmler, H., 1980. The distribution of abscisic acid between the chloroplasts and the cytoplasm of leaf-cells and the permeability of the chloroplast envelope for abscisic acid. Z. Pflanzenphysiol. 97: 67-78.

Henson, I.E., Alagarswamy, G., Mahalakshmi, V., Bidinger, F.R., 1982. Diurnal changes in endogenous abscisic acid in leaves of pearl millet (Pennisetum americanum (L.) Leeke) under field conditions. J. Exp. Bot. 33: 416-425.

Hillman, J.R., Young, I., Knights, B.A., 1974. Abscisic acid in leaves of Hedera helix (L.). Planta 119: 263-266.

Hirai, N., Fukui, H., Koshimizu, K., 1978. A novel abscisic acid metabolite from seeds of Robinia pseudoacacia. Phytochem. 17: 1625-1627.

Hirai, N., Koshimizu, K., 1981. Chirality of the acyl group of β-hydroxy-β-methylglutarylhydroxy-abscisic acid. Phytochem. 20: 1867-1869.

Hoad, G.V., 1973. Effect of moisture stress on abscisic acid levels in Ricinus communis (L.) with particular reference to phloem exudate. Planta 113: 367-372.

Hoad, G.V., 1967. (+) Abscisin II, ((+)-dormin) in phloem exudate of willow. Life Sci. 6 1113-1118.

Hoad, G.V., 1975. Effect of osmotic stress on abscisic acid levels in xylem sap of sunflower (Helianthus annuus (L.)). Planta 124: 25-29.

Hoad, G.V., Gaskin, P., 1981. Abscisic acid and related compounds in phloem exudate of Yucca flaccida Haw. and coconut (Cocos nucifera). Planta 150: 347-348.

Hocking, T.J., 1973. Movement and metabolism of ABA-2-^{14}C in plants. Ph. D. thesis, University of Glasgow.

Hocking, T.J., Clapham, J., Cattell, K.J., 1978. Abscisic acid binding to subcellular fractions from leaves of Vicia faba. Planta 138: 303-304.

Hocking, T.J., Hillman, J.R., Wilkins, M.B., 1972. Movement of abscisic acid in Phaseolus vulgaris plants. Nature New Biol. 235: 125-125.

Hocking, T.J., Hillman, J.R., 1975. Studies on the role of abscisic acid in the initiation of bud dormancy in Alnus glutinosa and Betula pubescens. Planta 125: 235-242.

Horgan, R. 1980. Modern methods for plant hormone analysis. In: Progress in Phytochemistry 7. Eds. Reinhold, L., Harborne, J.B., Swain, T. Pergamon Press.

Houwers, A., Akkermans, A.D.L., 1981. Influence of inoculation on yield of Alnus glutinosa in the Netherlands. Plant and Soil 61: 189-202.

Hubick, K.T., Reid, D.M., 1980. A rapid method for the extraction and analysis of ABA from plant tissue. Plant Physiol. 65: 523-525.

Huss-Danell, K., Roelofsen, W., Akkermans, A.D.L., Meijer, P., 1982. Carbon metabolism of Frankia spp. in root nodules of Alnus glutinosa and Hippophaë rhamnoides. Physiol. Plant. 54: 461-466.

Huss-Danell, K., Sellstedt, A., Flower-Ellis, A., Sjöström, M., 1982. Ammonium effects on function and structure of nitrogen-fixing root nodules of Alnus incana (L.) Moench. Planta 156: 332-340.

Ingersoll, R.B., Smith, O.E., 1971. Transport of abscisic acid. Plant and Cell Physiol. 12: 301-309.

Ingram, T.J., Reid, J.B., Potts, W.C., Murfet, I.C., 1983. Internode length in Pisum. The effect of the Le gene on gibberellin metabolism. Physiol. Plant. 59: 607-616.

Jackson, M.B., Osborne, D.J., 1972. Abscisic acid, auxin and ethylene in explant abscission. J. Exp. Bot. 23: 849-862.

Jacobs, W.P., 1959. What substance normally controls a given biological process? 1. Formulation of some rules. Dev. Biol. 1: 527-533.

Jewer, P.C., Incoll, L.D., Howarth, G.L., 1981. Stomatal response in isolated epidermis of the crassulacean acid metabolism plant Kalanchoe daigremontiana Hamet et Perr. Planta 153: 238-245.

Jones, R.J., Mansfield, T.A., 1970. Suppression of stomatal opening in leaves treated with abscisic acid. J. Exp. Bot. 21: 714-719.

Jones, W.W., Coggins, C.W., Embleton, T.W., 1976. Endogenous abscisic acid in relation to bud growth in alternate bearing 'Valencia' orange. Plant Physiol. 58: 681-682.

Juntilla, O., 1977. Shoot tip abortion in Salix as affected by growth hormones and defoliation. Z. Pflanzen physiol. 87: 455-462.

Kannangara, T., Durley, R.C., Simpson, G.M., 1982. Diurnal changes of leaf water potential, abscisic acid, phaseic acid and indole-3-acetic acid in field grown Sorghum bicolor L. Moench. Z. Pflanzen physiol. 106: 55-61.

Kelly, M., 1969. Comparisons and cross-reactions of nitrogenase from Klebsiella pneumoniae, Azotobacter chroococcum and Bacillus polymyxa. Biochim et Biophys. Acta. 191: 527-540.

Kende, H., Gardner, G., 1976. Hormone binding in plants. Ann. Rev. Plant Physiol. 27: 267-290.

Kobriger, I.M., Tibbitts, T.W., Brenner, M.L.,1982. Diurnal abscisic acid levels of peas grown in a field and in a controlled environment. Plant Physiol. (Suppl.) 69: 38.

Koshimizu, K., Inui, M., Fukui, H., Mitsui, T., 1968. Isolation of (+)- abscisyl-β -D-glucopyranoside from immature fruits of Lupinus luteus. Agric. Biol. Chem. 32: 789-791,

Krekule, J., Kohli, R.K., 1981. See p 200, reference 4.

Kubik, M., Antoszewski, R., 1983. Altering the sensitivity of strawberry plants to exogenous abscisic acid. Physiol. Plant. 57: 505-508.

Lalonde, M., 1977. See p 200, reference 5.

Larsen, P. 1955. Nomenclature of plant growth substances. News and notes, Plant Physiol.(Suppl.) 30 : 190-191.

Laver, M.L., Zaerr, J.B., Lavender, D.P., 1978. Seasonal variation of abscisic acid in the dormant shoots of Douglas fir. Can. J. Bot. 57: 534-538.

Lehmann, H., Preiss, A., Schmidt, J., 1983. A novel abscisic acid metabolite from cell suspension cultures of Nigella Damascena. Phytochem. 22: 1277-1278.

Lehmann, H., Schutte, H.R., 1980. Biochemistry of phyto effectors. 16. Purification and characterisation of an abscisic acid glucosylating enzyme from cell-suspension-cultures of Macleaya microcarpa. Z. Pflanzen-physiol. 96: 277-280.

Lenton, J.R., Bowen, M.R., Saunders,P.F., 1968. Detection of abscisic acid in the xylem sap of willow (Salix viminalis L.) by gas liquid chromatography. Nature 220: 86-87.

Lenton, J.R., Perry, V.M., Saunders, P.F., 1972. Endogenous abscisic acid in relation to photoperiodically-induced bud dormancy. Planta 106: 13-22.

Lesham, Y., Philosoph, S., Wurzburger, J., 1974. Glycosylation of free trans ABA as a contributing factor in bud dormancy break. Biochem. Biophys. Res. Comm. 57: 526-531.

Lindoo, S.J., Noodén, L.D., 1978. Correlation of cytokinins and abscisic acid with monocarpic senescence in soybeans. Plant and Cell Physiol. 19: 887-1006.

Liu, W., Carns, H.R., 1961. Isolation of abscisin, an abscission-accelerating substance. Science 134: 384-385.

Lovelock, J.E., 1963. Electron absorption detectors and technique for use in quantitative and qualitative analysis by gas chromatography. Analytic. Chem. 35: 474-481.

Loveys, B.R., 1979. The influence of light quality on levels of ABA in tomato plants, and evidence for a novel abscisic acid metabolite. Physiol. Plant. 46: 79-84.

Loveys, B.R., Kriedemann, P.E., 1974 . Internal control of stomatal physiology and photosynthesis. I. Stomatal regulation and associated changes in endogenous levels of abscisic and phaseic acids. Aust. J. Plant Physiol. 1: 407-415.

Loveys, B.R., Leopold, A.C., Kriedemann, P.E., 1974 . Abscisic acid metabolism and stomatal physiology in Betula lutea following alteration in photoperiod. Ann. Bot. 38: 85-92.

Loveys, B.R., Milborrow, B.V., 1981. Isolation and characterisation of 1'-O-abscisic acid-β -D-glucopyranoside from vegatative tomato tissue. Aust. J. Plant Physiol. 8: 571-589.

Loveys, B.R., Milborrow, B.V., 1984. Metabolism of abscisic acid. In: Society for Experimental Biology seminar series 23: The biosynthesis and metabolism of plant hormones. Eds. Crozier, A., Hillman, J.R. CUP.

Lowe, D.J., Smith, B.E., Eady, R.R. 1980. The structure and mechanism of nitrogenase. In: Recent advances in biological nitrogen fixation. Ed. Subba Rao, N.S. Edward Arnold.

McMichael, B.L., Hanny, B.W., 1977. Endogenous levels of abscisic acid in water stressed cotton leaves. Agron. J. 69: 979-982.

McNiel, R.E., Carpenter, P.L., 1979. The effect of temperature, solar radiation and moisture on the reduction of acetylene by excised root nodules of Alnus glutinosa. New Phytol. 82: 459-465.

McVean, D.N., 1953. Biological flora of the British Isles. Alnus glutinosa (L.) Gaertn. J. Ecol. 41: 447-466.

Markhart, A.H., 1982. Penetration of soybean root systems by abscisic acid isomers. Plant Physiol. 69: 1350-1352.

Martin, G.C., Scott, I.M., Neill, S.J., Horgan, R., 1982. Identification of abscisic acid glucose ester, indole 3-acetic acid, zeatin and zeatin abscisate in receptacles of pear. Phytochem. 21: 1079-1082.

Mertens, R., Deusneuman, B., Weiler, E.W., 1983. Monoclonal antibodies for the detection and quantitation of the endogenous plant growth regulator, abscisic acid. FEBS Letters 160: 269-272.

Mertens, R., Stünning, M., Weiler, E.W., 1982. Metabolism of tritiated enantiomers of abscisic acid prepared by immuno-affinity chromatography. Nature-Wissenschaften 69: 595-597.

Mikola, 1983. In: Biological nitrogen fixation in forest ecosystems: foundations and applications. Eds. Gordon, J.C., Wheeler, C.T., Martinus Nijhoff/Dr. Junk Publishers.

Milborrow, B.V., 1966. The effects of synthetic dl -dormin (abscisin II) on the growth of the oat mesocotyl. Planta 70: 155-171.

Milborrow, B.V., 1967. The identification of (+) abscisin II [(+)- Dormin] in plants and measurements of its concentrations. Planta 76: 93-113.

Milborrow, B.V., 1969. Identification of 'metabolite C' from abscisic acid and a new structure for phaseic acid. J. Chem. Soc. 966-967.

Milborrow, B.V., 1970. The metabolism of abscisic acid. J. Exp. Bot. 21: 17-29.

Milborrow, B.V., 1971. Abscisic acid. In: Aspects of terpenoid chemistry and biochemistry. Ed. Goodwin, T.W. Academic Press.

Milborrow, B.V., 1972. Stereochemical aspects of formation of double bonds in abscisic acid. Biochem. J. 128: 1135.

Milborrow, B.V., 1974. The chemistry and physiology of ABA. Ann. Rev. Plant Physiol. 25: 259-307.

Milborrow, B.V., 1978. The stability of conjugated abscisic acid during wilting. J. Exp. Bot. 29: 1059-1066.

Milborrow, B.V., 1980. A distinction between the fast and slow responses to abscisic acid. Aust. J. Plant Physiol. 7: 749-754.

Milborrow, B.V., Mallaby, R., 1975. Occurrence of methyl (+) abscisate as an artefact of extraction. J. Exp. Bot. 26: 741-748.

Milborrow, B.V., Robinson, D.R., 1973. Factors affecting the biosynthesis of abscisic acid. J. Expt. Bot. 24: 537-548.

Milborrow, B.V., Vaughan, G.T., 1979. The long term metabolism of (±) - [2-^{14}C] abscisic acid by apple seeds. J. Exp. Bot. 30: 983-995.

Milborrow, B.V., Vaughan, G.T., 1982. The 4'-O-β-D-glucoside of dihydrophaseic acid. Abstracts of the eleventh international conference on plant growth substances, Aberystwyth.

Minchin, F.R., Witty, J.R., Sheehy, J.E., Müller, M., 1983. A major error in the acetylene reduction assay:decreases in nodular nitrogenase activity under assay conditions. J. Exp. Bot. 34: 641-649.

Mulkey, T.J., Evans, M.L., Kuzmanoff, K.M., 1983. The kinetics of abscisic acid action on root growth and gravitropism. Planta 157: 150-157.

Neill, S.J., Horgan, R., Heald, J.K., 1983. Determination of the levels of abscisic acid glucose ester in plants. Planta 157: 371-375.

Neill, S.J., Horgan, R., Walton, D.C., Lee, T.S., 1982. The biosynthesis of abscisic acid in Cercospora rosicola. Phytochem. 21: 61-65.

Nitsch, J.P., 1967. Progress in the knowledge of natural plant growth regulators. Ann. N.Y. Acad. Sci. 144: 279-294.

Normand, P., Lalonde, M., 1982. Evaluation of Frankia strains isolated from provences of two Alnus species. Can. J. Microbiol. 28: 1133-1142.

Ohkuma, K., Lyon, J.L., Addicott, F.T., Smith, O.E., 1963. Abscisin II, an abscission-accelerating substance from young cotton fruit. Science 142: 1592-1593.

Ohkuma, K. et al., 1965. See p 200, reference 6.

Ohlrogge, J.B., Garcia-Martinez, J., Adams, D., Rappaport, L., 1980. Uptake and subcellular compartmentation of gibberellin A_1 applied to the leaves of barley and cowpea. Plant Physiol. 66: 422-427.

Pauls, K.P., Chambers, J.A., Dumbroff, E.G. and Thompson, J.E., 1982. Perturbation of phospholipid membranes by gibberellins. New Phytol. 91: 1-17.

Peng, C.T., 1977. See p 200, reference 7.

Perry, T.O., Hellmers, H., 1973. Effects of abscisic acid on growth and dormancy of two races of red maple. Bot. Gaz. 134: 283-289.

Philipson, J.J., Coutts, M.P., 1979. The induction of root dormancy in Picea sitchensis (Bong.) Carr. by abscisic acid. J. Exp. Bot. 30: 371-386.

Phillips, D.A., 1971. Abscisic acid inhibition of root nodule initiation in Pisum sativum. Planta 100: 181-190.

Phillips, I.D.J., 1975. Apical dominance Ann. Rev. Plant Physiol. 26: 341-367.

Phillips, I.D.J., Miners, J., Roddick, J.G., 1980. Effects of light and photoperiodic conditions on abscisic acid in leaves and roots of Acer pseudoplatanus (L.). Planta 149: 118-122.

Pierce, M., Raschke, K., 1981. Synthesis and metabolism of ABA in detached leaves of Phaseolus vulgaris (L.) after loss and recovery of turgor. Planta 153: 156-165.

Pilet, P.E., 1978. The role of the cap in the geotropism of roots exposed to light. Z. Pflanzenphysiol. 89: 411-426.

Pizelle, G., 1984. Seasonal variations of the sexual reproductive growth and nitrogenase activity (C_2H_2) of mature Alnus glutinosa. Plant and Soil 78: 181-188.

Proebsting, W.M., 1983. Transmissible factors regulating shoot growth of Cornus sericea (L.). Z. Pflanzenphysiol. 112: 191-198.

Potts, W.C., Reid, J.B., 1983. Internode length in Pisum. III The effect and interaction of the Na/na and Le/le gene differences on endogenous gibberellin-like substances. Physiol. Plant. 57: 448-454.

Quarrie, S.A., 1984. Abscisic acid and drought resistance in crop plants. News Bulletin, British Plant Growth Regulator Group, 7: 1-15.

Reeve, D.R., Crozier, A., 1980. In: Hormonal regulation of development I. Molecular aspects of plant hormones. Ed. MacMillan, J. Encyclopedia of plant physiology, new series 9: 203-280 Springer-Verlag, Heidelberg.

Rivier, L., Milton, H., Pilet, P.E., 1977. GCMS determinations of ABA in the caps and apex of maize roots. Planta 134: 23-27.

Robertson, J., Berrie, A.M.M., 1977. Abscisic acid and germination of thermodormant lettuce fruits (Lactuca sativa) cv. Grand Rapids; fate of isotopically-labelled abscisic acid. Physiol. Plant. 39: 51-59.

Rudnicki, R., Czapski, J., 1974. Uptake and degradation of 1-C-14 abscisic acid by apple seeds during stratification. Ann. Bot. 38: 189-192.

Sagee, O., Goren, R., Riov, J., 1980. Abscission of citrus leaf explants. Plant Physiol. 66: 750-753.

Salama, A.M.S., El-D.A., Wareing, P.F., 1979. Effects of mineral nutrition on endogenous cytokinins in plants of sunflower (Helianthus annuus (L.)). J. Exp. Bot. 30: 971-981.

Saleh, A.N., Hemberg, T., 1980. The influence of kinetin on the endogenous content of indoleacetic acid in swelling seeds of Phaseolus, Zea and Pinus and young plants of Phaseolus. Physiol. Plant. 50: 99-102.

Samet, J.S., Sinclair, T.R., 1980. Leaf senescence and abscisic acid in leaves of field grown soybean. Plant Physiol. 66: 1164-1168.

Sandberg, G., Oden, P.C., Dunberg, A., 1982. Population variation and diurnal changes in the content of indole-3-acetic acid of pine seedlings (Pinus sylvestris (L.)) grown in a controlled environment. Physiol. Plant. 54: 375-380.

Saunders, P.F., 1978. Phytohormones and bud dormancy. In:Phytohormones and related compounds - a comprehensive treatise Vol.II. Eds. Letham, D.S., Goodwin, P.B., Higgins, T.J.V.

Schlenk, H., Gellerman, J.L., 1960. Esterification of fatty acids by diazomethane on a small scale. Analyt. Chem. 32: 1412-1414.
Scholander, P. F., et al.,1965. See p 200, reference 8.
Schwintzer, C.R., Berry, A.M., Disney, L.D., 1982. Seasonal patterns of root nodule growth, endophyte morphology, nitrogenase activity and shoot development in Myrica gale. Can.J. Bot. 60: 746-757.

Scott, A., Gardner, I.C., McNally, S.F., 1981. Localization of citrulline synthesis in the alder root nodule and its implication in nitrogen fixation. Plant Cell Reports 1: 21-22.

Seeley, S.D., Powell, L.E., 1970. Electron capture GC for sensitive assay of ABA. Analyt. Biochem. 35: 530-533.
Sembdner, G., 1980. See p 200, reference 9.
Setter, T.L., Brun, W.A., 1981. Abscisic acid translocation and metabolism in soybeans following depodding and petiole girdling treatments. Plant Physiol. 67: 774-779.

Sharkey, T.D., Raschke, K., 1980. Effects of phaseic acid and dihydrophaseic acid on stomata and the photosynthetic apparatus. Plant Physiol. 65: 291-297.

Shipton, W.A., Burggraaf, A.J.P., 1982. A comparison of the requirements for various carbon and nitrogen sources and vitamins in some Frankia isolates. Plant and Soil 69: 149-161.

Silvester, 1983. In: Biological nitrogen fixation in forest ecosystems: foundations and applications. Eds. Gordon, J.C., Wheeler, C.T. Martinus Nijhoff/Dr. Junk Publishers.

Skoog, F., Miller, C.O., 1957. Chemical regulation of growth and organ formation in plant tissues cultured in vitro. Symposia of the Society for Experimental biology, XI: 118-131.

Snaith, P.J., Mansfield, T.A., 1982. Control of the CO_2 responses of stomata by IAA and ABA. J.Exp. Bot. 33: 360-365.

Sondheimer, E., Galson, E.C., Chang, Y.P., Walton, D.C., 1971. Asymmetry, its importance to the action and metabolism of abscisic acid. Science 174: 829-831.

Sprent, J.I., 1979. The biology of nitrogen-fixing organisms. McGraw-Hill.

Stoddart, J.L., Venis, M.A., 1980. In: Hormonal regulation of development I. Molecular aspects of plant hormones. Ed. MacMillan, J. Encyclopedia of Plant Physiology, new series 9: 203-280. Springer-Verlag, Heidelberg.

Suzuki, T., Kondo, N., Fujii, T., 1979. Distribution of growth regulators in relation to the light-induced geotropic responsiveness in Zea roots. Planta 145: 323-329.

Sweetser, P.B., Vatvars, A., 1976. High performance liquid chromatographic analysis of ABA in plant extracts. Analyt. Biochem. 71: 68-78.

Tal, M., Imber, D., 1971. Abnormal stomatal behaviour and hormonal imbalance in flacca a wiltymutant of tomato. III Hormonal effects on the water status in the plant. Plant Physiol. 47: 849-850.

Tarrant, R.F., 1983. In: Biological nitrogen fixation in forest ecosystems: foundations and applications. Eds. Gordon, J.C., Wheeler, C.T. Martinus Nijhoff/Dr. Junk Publishers.

Tarrant, R.F., Trappe, J.M., 1971. The role of Alnus in improving the forest environment. Plant and Soil (special volume): 335-348.

Taylor, H.F., Smith, T.A., 1967. Production of plant growth inhibitors from xanthophylls: a possible source of dormin. Nature 215: 1513-1514.

Taylor, H.F., Burden, R.S., 1973. Preparation and metabolism of 2-[14 C]-cis, trans xanthoxin. J. Exp. Bot. 24: 873-880.

Taylor, I.B., Rossall, S., 1982. The genetic relationship between the tomato mutants flacca and lateral suppressor, with reference to abscisic acid accumulation. Planta 154: 1-5.

Taylor, J.S., Dumbroff, E.B., 1975. Bud, root and growth-regulator activity in Acer saccharum during the dormant season. Can. J. Bot. 53: 321-331.

Tinelli, E.T., Sondheimer, E., Walton, D.C., 1973. Metabolites of [2- 14C] abscisic acid. Tetrahedron Lett. 2: 139-140.

Tjepkema, J.D., Ormerod, W., Torrey, J.G., 1980. Vesicle formation and acetylene reduction activity in Frankia sp. CPI1 cultured in defined nutrient media. Nature 287: 633-635.

Trewavas, A., 1981. How do plant growth substances work? Plant, Cell and Environment. 4: 203-228.

Trewavas, A.J., 1982. Growth substance sensitivity: the limiting factor in plant development. Physiol. Plant. 55: 60-72.

Tucker, D.J., Mansfield, T.A., 1973. Apical dominance in Xanthium strumarium. J. Exp. Bot. 24: 731-740.

Tukey, H.B., Went, F.W., Muir, R.M., Van Ovenbeek, J., 1954. Nomenclature of chemical plant regulators. Plant Physiol. 29: 307-308.

Uvarov, E.B., Chapman, D.R., 1971. A dictionary of Science. Penguin Books.

Van Dijk, C., 1978. Spore formation and endophyte diversity in root nodules of Alnus glutinosa (L.) Vill. New Phytol. 81: 601-615.

Van Dijk, C., Merkus, E., 1976. A microscopical study of the development of a spore-like stage in the life cycle of the root nodule endophyte of Alnus glutinosa (L.) Gaertn. New Phytol. 77: 73-91.

Van Staden, J., Akkermans, A.D.L., Roelofsen, W., 1977. Nitrogenase activity of endophyte suspensions derived from root nodules of Alnus, Hippophaë, Shepherdia and Myrica spp. Nature 266: 257-258.

Vaughan, G.T., Milborrow, B.V., 1984. The resolution by HPLC of RS [2- 14C] Me 1', 4' - cis diol of abscisic acid and the metabolism of (-)-R- and (+)-S- abscisic acid. J. Exp. Bot. 35: 110-120.

Van Onckelen, H.A., Horemans, S., De Greef, J.A., 1981. Functional aspects of ABA metabolism in cotyledons of Phaseolus vulgaris (L.) seedlings. Plant and Cell Physiol. 22: 507-515.

Venis, M.A., 1977. Receptors for plant hormones. In: Advances in botanical research 5: 53-88. Ed. Woolhouse, H.W. Academic Press.

Walton, D.C., 1980. Biochemistry and physiology of ABA. Ann. Rev. Plant Physiol. 31: 453-489.

Walton, D.C., Harrison, M.A., Cote, P., 1976. The effects of water stress on abscisic acid levels and metabolism in roots of Phaseolus vulgaris and other plants. Planta 131: 141-144.

Wareing, P.F., 1954. Growth studies in woody species. IV. The locus of photoperiodic perception in relation to dormancy. Physiol. Plant. 7: 261-277.

Wareing, P.F., 1977. Growth substances and integration in the whole plant. Symp. Soc. Exp. Biol. 31: 337-365.

Watts, S., Rodriguez, J.L., Evans, S.E., Davies, W.J., 1981. Root and shoot growth of plants treated with abscisic acid. Ann. Bot. 47: 595-602.

Weaver, R.J., Pool, R.M., 1969. Effect of ethrel, abscisic acid and a morphactin on flower and berry abscission and shoot growth in Vitis vinifera. J. Amer. Soc. Hort. Sci. 94: 474-478.

Weiler, E.W., 1980. Radioimmuno-assays for the differential and direct analysis of free and conjugated ABA in plant extracts. Planta 148: 262-272.

* Went, F.W., 1928. Wuchstoff und Wachstum. Rec. Trav. Bot. Neer. 25: 1-116.

Weyers, J.D.B., 1978. The hormonal control of stomatal aperture in Commelina communis (L.) by abscisic acid. Ph.D. thesis, University of Glasgow.

Wheeler, C.T., 1969. The diurnal fluctuation in nitrogen fixation in the nodules of Alnus glutinosa and Myrica gale. New Phytol. 68: 675-682.

Wheeler, C.T., 1971. The causation of the diurnal changes in nitrogen fixation in the nodules of Alnus glutinosa. New Phytol. 70: 487-495.

Wheeler, C.T., Lawrie, A.C., 1976. Nitrogen fixation in root nodules of alder and pea in relation to the supply of photosynthetic assimilates. In: Symbiotic nitrogen fixation in plants. Ed. Nutman, P.S. Cambridge, CUP.

Wheeler, C.T., McLaughlin, M.E., 1979. Environmental modulation of nitrogen fixation in actinomycete nodulated plants. In: Symbiotic nitrogen fixation in the management of temperate forests. Eds. Gordon, J.C., Wheeler, C.T., Perry, D.A. Forestry publication, School of Forestry, Oregon State University, Corvallis.

Wightman, F., Schneider, E.A., Thimann, K.V., 1980. Hormonal factors controlling the initiation and development of lateral roots. II Effects of exogenous growth factors on lateral root formation in pea roots. Physiol. Plant. 49: 304-314.

Wilkins, H., Wain, R., 1974. The root cap and control of root elongation in Zea mays (L.) seedlings exposed to white light. Planta 121: 1-8.

Wilkins, H., Wain, R.L., 1975. Abscisic acid and the response of the roots of Zea mays (L.) seedlings to gravity. Planta 126: 19-23.

Williams, P.M., Sicardi de Mallorca, M., 1982. Abscisic acid and gibberellin-like substances in roots and nodules of Glycine max. Plant and Soil 65: 19-26

Williams, P.M., Sicardi de Mallorca, M., 1984. Effect of osmotically induced leaf moisture stress on nodulation and nitrogenase activity of Glycine max. Plant and Soil 80: 267-283.

Wood, B.W., 1983. Changes in indole acetic acid, abscisic acid, gibberellins and cytokinins during bud-break in Pecan. J. Amer. Soc. Hort. Sci. 108: 333-338.

Wright, S.T.C., 1975. Seasonal changes in the levels of free and bound abscisic acid in blackcurrant (Ribes nigrum) buds and beech (Fagus sylvatica) buds. J. Exp. Bot. 26: 161-174.

Xiloyannis, C., Uriu, K., Martin, G.C., 1980. Seasonal and diurnal variations in abscisic acid, water potential, and diffusive resistance in leaves from irrigated and non-irrigated peach trees. J. Amer. Soc. Hort. Sci. 105: 412-415.

Yamaguchi, T., Street, H.E., 1977. Stimulation of growth of excised cultured roots of soyabean by abscisic acid. Ann. Bot. 41: 1129-1133.

Yokota, T., 1980. In: Hormonal regulation of development I. Molecular aspects of plant hormones. Ed. MacMillan, J. Encyclopedia of Plant Physiology, new series 9: 203-280. Springer-Verlag, Heidelberg.

Zavala, M.E., Brandon, D.L., 1983. Localization of a phytohormone using immunocytochemistry. J. Cell Biol. 97: 1235-1239.

Zeevaart, J.A.D., 1971. Effects of photoperiod on growth rate and endogenous gibberellins in the long day rosette plant spinach. Plant Physiol. 47: 821-827.

Zeevaart, J.A.D., 1977. Sites of abscisic acid synthesis and metabolism in Ricinus communis L. Plant Physiol. 59: 788-791.

Zeevaart, J.A.D., 1980. Changes in the levels of abscisic acid and its metabolites in excised leaf blades of Xanthium strumarium during and after water stress. Plant Physiol. 66: 672-678.

Zeevaart, J.A.D., 1983. Metabolism of abscisic acid and its regulation in Xanthium leaves during and after water stress. Plant Physiol. 71: 477-481.

Zeevaart, J.A.D., Milborrow, B.V., 1976. Metabolism of abscisic acid and the occurrence of epi-dihydrophaseic acid in Phaseolus vulgaris. Phytochem. 15: 493-500.

Zeroni, M., Hall, M.A., 1980. Molecular effects of hormone treatment on tissue. In: Encyclopedia of Plant Physiology NS 9 chapter 6. Eds. MacMillan, J. Springer-Verlag.

Literature omitted from main list

1. Bond, G., 1983. In: Biological nitrogen fixation in forest ecosystems; foundations and applications. Eds. Gordon, J.C., Wheeler, C.T. (Martinus Nijhoff, Dr. W. Junk publishers).

2. Crozier, A., 1981. Aspects of the metabolism and physiology of gibberellins. Adv. Bot. Res. 9:33-149.

* 3. Hartung, W., Behl, R., 1974. Transport and metabolism of [2-^{14}C] abscisic acid in root segments of Phaseolus coccineus L. Planta 120 : 299-305.

4. Krekule, J., Kohli, R.K., 1981. The condition of the apical meristem of seedlings responsive to a promotive effect of abscisic acid on flowering in the short-day plant Chenopodium rubrum. Z. Pflanzenphysiol. 103 : 45-51.

5. Lalonde, M., 1977. Infection process of the Alnus root nodule symbiosis. In : Recent developments in nitrogen fixation pp 569-589. Ed. Newton, W., Postgate, C., Rodriguez-Barrueco, C. Academic Press.

6. Ohkuma, K., Addicott, F.T., Smith, O.E., Thiessen, W.E., 1965. Tetrahedron Lett. 29 : 2529-2535.

7. Peng, C.T., 1977. Sample preparation in liquid scintillation counting. Review 17, The Radiochemical Centre, Amersham.

8. Scholander, P.F., Hammel, H.T., Bradstreet, E.D. and Hemmingsen, E.A., 1965. Sap pressure in vascular plants. Science, 148 : 339-346.

9. Sembdner, G., 1980. In : Hormonal regulation of development I. Molecular aspects of plant hormones. Ed., MacMillan, J. Encyclopedia of Plant Physiology , New Series 9 :203-280. Springer-Verlag, Heidelberg.

ERRATA