CONTROL OF LEAF DEVELOPMENT IN *THEOBROMA CACAO* L.

by

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Free "
Bound "

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Total gibberellins
Free "
Bound "

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(a) fully open x 4700

Day 4 of F-2 stage.

(b) partially open x 4700

Day 4 of F-2 stage.

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x 950

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x 1900

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SUMMARY

The present project was undertaken to understand how the cocoa shoot apex activity and leaf development in well-watered cocoa seedlings might be endogenously controlled. The results showed that the water status of the leaf during development was a balance between the potential water loss of a large surface area lacking a significant cuticle and the restriction of water loss effected by the orientation of the leaf, degree of pigmentation, the presence of leaf hairs and a restricted stomatal development. It was suggested that the structural characteristics of the young leaf at F-1 and early F-2 will tend to limit the transpiration loss. Maximum water loss only occurred after late F-2 when the leaves were fully expanded, horizontally positioned and the stomata fully developed. The water uptake in response to the transpiration loss showed a large increase at the end of the F-2 and beginning of the I-1 stages. It is possible that the large transpiration loss after the late F-2 of the flush cycle causes a water deficit in the young seedling plant. It was found that there was a cyclic change in water deficit in the plant as shown by the changes in leaf RWC and Ψ_{leaf}. The late stage of F-2, I-1 and the beginning of I-2 showed a water deficit whereas the remaining part of I-2, F-1 stage and the beginning of F-2 stage did not show this deficit. It was thought that the water deficit may lead to build up of ABA in the shoot.

The effects of drought on the plant was to reduce the RWC and Ψ_{leaf}. The initiation of bud break still occurred during the period of water stress but leaf expansion only took place after the water deficit was relieved. Defoliation of either the NF leaves or PF leaves of cocoa at

I-1 and I-2 stages of the cycle reduced the length of the dormant phase of the next flush cycle, demonstrating that the mature leaves were a source of inhibition which exerted an effect on shoot apical activity.

The application of ABA to the NF and PF leaves led to an extension of the dormant phase, whereas application of zeatin or GA_3 decreased it. These results suggested that the control of the timing of leaf production is hormonal rather than due to carbohydrate availability. These results were confirmed by studying the movement of radioactive labelled ABA, GA_3, IAA and BAP from a donor leaf to the different parts of the plant. On the basis of RSA values, the bud was shown to accumulate ^{14}C-ABA during the dormant phase (I-1 and I-2), while the growth promoters ^{14}C-IAA, ^{14}C-GA_3 and ^{14}C-BAP accumulated in the bud during the periods of active growth (F-1 and F-2). The gibberellin levels in the NF or PF leaves were at a maximum during leaf expansion and declined during the dormant phase. There was a correlation between water status of the cocoa seedlings (as measured from NF leaves) and activity of the apex. Thus periods of active growth (bud burst, leaf production and leaf expansion) were associated with high RWC and Ψ_{leaf} and an increased export of growth promoters from the leaves to the shoot apex and a decline in the export of growth inhibitors. When the shoot apex was dormant the water deficit in the leaves led to an increase in export of inhibitors from the leaves to the apex causing apical dormancy; when there is sufficient water, then the plant is relieved from the water deficit and the apex resumes its activity.

CHAPTER 1

General Introduction

Cocoa, like the great majority of tropical trees, does not grow continuously throughout the year but behaves as an intermittently growing evergreen, exhibiting periods of intensive leaf growth alternating with periods of vegetative rest (Greenwood and Posnette, 1950; Alvim, 1964; Greathouse et al. 1971).

This type of growth is referred to as "flush growth", and the period from when the apex becomes active, producing the first leaf of one flush, up to the corresponding stage for the next flush is called a flush cycle of growth. One of the features of this cycle is a rapid production of leaves, which are referred to as flush leaves. These leaves are red to pale green during expansion, and only develop the dark green colour typical of the mature leaves during the dormant period. The number of flush leaves produced in one flush varies, but usually increases with the age of the trees. The frequency of flushing declines with plant age. In mature trees in the field the frequency is about four times per year, the actual number depending on environmental conditions, while in seedlings the flush cycle lasts between 30 - 49 days.

The periodicity in vegetative growth of temperate species can usually be correlated with the well defined seasonal variations in the field, for example, day length, rainfall and temperature. But where less well defined seasons occur, as in the tropics, the factors controlling shoot growth rhythms are not so well understood. However, a number of studies have been carried out with cocoa in an attempt to establish possible causal relationships between the flush growth cycle and environmental factors.

Sale (1968) demonstrated under growth room conditions that flushing in cocoa was not induced by an increase in temperature to about 83°F, as previously advanced by Humphries (1944) and Greenwood and Posnette (1950), or by an increase in temperature range as suggested in earlier work by Alvim (1957). Sale (1968) found that no critical temperatures were required for the induction of flush growth, but the different components of leaf growth responded in different ways to temperature. Whereas the rate of flush production increased with an increase in night and day temperatures, both the number of leaves produced per flush and the individual leaf areas decreased with either an increase in day or night temperature. Further work suggested that the total shoot growth extension per plant and not the growth extension per flush or per leaf was directly controlled by temperature (Sale, 1969).

While temperature has been suggested to affect flush growth, a study by Piringer and Downs (1960) demonstrated that growth of cocoa was also very much influenced by photoperiod, the plant growing faster and producing more leaves under 12- and 16-hour days than under 8 hours of light per day. Thus there is the possibility that flush growth is also controlled by the amount of solar radiation received by the plant, the correlation with temperature being only indirect (Alvim, 1964).

The effects of other environmental factors on growth rhythms of cocoa led McDonald (1933) to suggest that soil moisture and/or air relative humidity are important in the flushing of cocoa. Later Alvim et al. (1964) on the basis of field studies in Brazil, proposed that (a) decreased rainfall (or increased moisture stress)

increased the rate of leaf abscission; (b) abscission leads to bud burst; (c) flushing occurs soon after abscission or, under conditions of severe moisture stress, after the onset of rains.

The stimulating effect of watering after a period of water deficit was confirmed by Sale (1970) who demonstrated that vigorous flushing occurred about 10 days after watering. This led to the conclusion that rain or irrigation appeared to be necessary in order to initiate flush growth but was not necessarily related to bud break.

The importance of moisture stress in breaking dormancy has been clearly demonstrated in the case of coffee flower buds (Alvim, 1960). A similar type of response to a relatively dry period followed by a wet period has been observed with the flowering and flushing cycles in citrus (Cassin et al. 1969) and is probably of common occurrence among tropical trees. Alvim (1964) suggested the term "hydroperiodicity" for this physiological response to a succession of dryness and wetness.

Alvim et al. (1969) suggested that when cocoa was water stressed apical bud development was arrested before leaf expansion took place, i.e. leaf primordia were formed but did not expand. Leaf primordia remained "ready to grow" until water stress was relieved when there was then rapid expansion of these primordia. He further suggested that periodic water stress was an absolute requirement for the continuation of leaf initiation and expansion in cocoa. As evidence of this link between water stress and leaf production he cited the fact that cocoa growing without overhead shade flushes more intensively than shaded cocoa, suggesting this was due more to higher internal

moisture stress developing in these trees than to light intensity, (Alvim et al. 1972), but photosynthetic performance of non-shaded trees would usually be higher and so some interrelationship between carbohydrate level and intensity of flushing could also be possible here.

Studies on temperate plants have shown that moisture stress increases the amount of abscisic acid in the leaves, this being the main factor controlling leaf shedding (Wareing & Saunders, 1971). To take into account the probable role of ABA in controlling flushing under moisture depletion Alvim et al. (1972) suggested that moisture stress increases the concentration of ABA, thus accelerating senescence and leaf shedding. Translocation of ABA (or another inhibitor(s) with similar physiological activity) from the leaves to the bud decreases as a result of abscission layer formation in the leaf petioles or due to the leaf fall. In time, the bud starts growing and finally the shoots and leaves expand, provided water is not limited for growth.

The presence of ABA in stressed plants showed that during water stress in the cocoa plants, the inhibitor ABA increased in the buds and the cytokinins declined, while after abscission or on watering the ABA declined and the promoter increased thus promoting bud break (Alvim et al. 1974).

While all the above reports have related the control of flush growth in cocoa to environmental factors, observations that in field-stands of cocoa adjacent trees are often at different stages of flush cycle development indicates that factors other than climate must be influencing the timing of the flush growth (McKee, 1944). Although asynchronous growth has been noticed, differences in soil and micro-climate, e.g. shade, could account for some of the variation.

Nevertheless, when cocoa plants were grown in growth chambers under "constant" conditions asynchronous growth was observed; some plants with expanding flush leaves being adjacent to plants in the dormant state (Greathouse et al. 1971). The observation that growth remains intermittent under these conditions, together with the observations of asynchronous growth in the field, strongly suggest that flushing in cocoa is based on an endogenous mechanism. This basic endogenous control system may however be overridden by environmental stress when periodicity of flushing will then become irregular.

Growth studies on flush growth on oak (Borchert, 1975) revealed growth patterns that agreed with a computer-predicted model of rhythmic growth based on an assumed dependence of shoot growth on a functional equilibrium between the shoot and root system. If, over prolonged periods, the shoot grows faster than the root system, such that the roots cannot supply the shoot with sufficient water and nutrients, inhibition or a slowing down of shoot growth will occur until the root can grow to a size that can support shoot growth again. It was further suggested that the production of an internal water deficit, resulting from the upset of the shoot/root ratio, was a likely physiological basis for the restriction or inhibition of shoot growth found in the dormant periods of the flush cycle.

An internal water deficit inhibits growth by a direct action on stomatal aperture, transpiration, photosynthesis, enzymatic activity, and/or other physiological processes involved in growth, or indirectly by altering nutrient, mineral and hormonal relations

(Kozlowski, 1968; Da Silva, 1973; Pasternak and Wilson, 1974; Plaut, 1971; Arad et al. 1973). One of the major effects of water stress on hormonal levels in plants is a dramatic increase in ABA either in wilted leaves (Wright, 1969) or in stressed plants (Mizrahi et al. 1970; Wright, 1972; Most, 1971; Zeevart, 1971). Removal of the stress then caused a decline in ABA levels (Mizrahi et al. 1972; Wright, 1969; Wright, 1972). At the same time as ABA production occurred under water stress, the cytokinin level also declined (Hsiao, 1973). It seems likely that effects of water stress on growth may be due to an effect on a number of growth regulatory substances.

The studies of Alvim et al. (1974) with field grown cocoa plants showed an accumulation of ABA in the flush leaves and in buds of cocoa plants under soil moisture stress. However, even under controlled growth conditions, well-watered cocoa plants showed a large increase in levels of ABA in the leaves when the apex was dormant (Orchard et al. 1980). Alvim et al. (1974) considered the flush leaves to play an important role in controlling the apex activity by supplying inhibitory amounts of ABA to the bud during periods of internal water stress. After defoliation, the shoot apex activity was also resumed which Alvim et al. (1974) explained was as a result of a decline in ABA levels and an increase in the cytokinin content in the buds. In the same way Vogel (1975) showed that foliar growth rhythms of cocoa may be controlled by the effect of the developing flush leaves on the bud. For instance, removal of these leaves before the leaves finished expansion caused continued shoot apex activity.

The role of hormones in causing bud break in cocoa was investigated more closely by Orchard et al. (1979). They found that with excised

shoot apices of cocoa bud break occurred only in the presence of kinetin or gibberellic acid, while ABA inhibited bud break. Measurement of levels of cytokinins and auxins were also made by Orchard et al. (1981) for the whole plant. Auxin compounds were present in the flush leaves during leaf expansion (F-2) but, immediately after reaching full size and during the period of leaf maturity, no auxins could be detected. The cytokinin levels were also high during leaf expansion, declined in the recently matured leaves, then increased again just prior to the renewal of apical bud growth. The changes in auxin and cytokinin in relation to growth in these plants were thus very similar to the changes in the same growth substances in the field grown plants.

These earlier investigations indicated how the shoot apex activity and growth of flush leaves in cocoa may be endogenously controlled. The suggestion is that in the beginning of the cycle, activation of the terminal bud by a growth promoting substance, e.g. cytokinin, leads to the production of flush leaves. This is followed by expansion and shoot elongation which proceeds at a faster rate than root growth and results in an "unbalanced" root/shoot ratio. This means that not enough water can be supplied to the aerial part of the plant by the now "inadequate" root system, a situation which causes a water deficit in the newly expanded flush leaves. There is a cessation of shoot and leaf growth even though environmental conditions are favourable for continued growth. The water deficit may inhibit growth either directly by affecting turgor pressure or indirectly by affecting the levels of growth substances

such as cytokinins and ABA which subsequently affect metabolic processes. Flush growth is only renewed when the root/shoot ratio is restored in favour of the root system, e.g. by removal of the flush leaves (decreasing the demand for water) or by root growth. The return of a more favourable root/shoot ratio restores the water balance of the plant and leads to a decline in the production of growth inhibitory substances which releases the shoot from the growth inhibition.

Clearly this hypothesis is a complex one but nevertheless several aspects are amenable to investigation and thus the hypothesis can be evaluated. It seems that information on three particular aspects of the hypothesis was lacking:

i) water balance of the plant during flush growth cycles;

ii) the role of the new flush leaves in control of shoot apex activity, and iii) the movement of growth substances from sites of synthesis or accumulation to the shoot apex. These areas were thus chosen for extensive investigation. Experimental work in each section included:

i) Examination of those surface characteristics of the leaves of cocoa which might affect water loss, e.g. stomatal development, cuticle development, leaf angle, stomatal and cuticle diffusive resistance. Plant water status as measured by relative water content, water potential and ABA levels of the new flush leaves at different times of the flush growth cycle.

ii) The effect of the flush leaves on the apex. This can be shown by altering the supply of growth substances from the leaves, either by removing leaves or applying growth substances to the leaves.

iii) Movement of the growth substances to the bud. The export of ABA, cytokinins and GA_3 to the bud can be determined by the pattern of transport of ^{14}C labelled growth substances from the donor leaf to the bud and the rest of the plant.

An immediate problem was the choice of plant material. Theobroma cacao var. Amazon was chosen because of the reasonable uniformity of growth between the plants and the ease with which the different stages of the flush cycle were observed. The age of the plant had to be standardised and because the old plants were too large to keep in numbers sufficient for adequate replication of experiments, it was decided to use young seedlings approximately 6 to 9 months old. The use of such plants also has the advantage that the interval between flushes is shorter than in older specimens and therefore more economic use of time was possible. To ensure that the control of flush growth of cocoa is considered in terms of the proposed endogenous control mechanism, non-environmentally stressed plants were used throughout this work.

In undertaking this research it was considered of importance to understand how development in cocoa is controlled for a number of reasons, e.g. for its own intrinsic interest as a control system in cocoa; because the exploitation of the cocoa species commercially - as a very important world crop - could only benefit from a detailed

knowledge of the control of shoot development when expensive services such as irrigation and nutrient provision could perhaps be more effectively and economically executed; and for its possible implications in assessing control of growth in other species.

CHAPTER 2

Materials and Methods

2.1 Plant material

Theobroma cacao L. (var. Amazon) seedlings were grown in a glass house with a RH of 70-100% and supplementary lighting (warm white fluorescent tubes 80 Wm^{-2} of bench) on a 12hr day length. The day temperatures were 25°C to 30°C, and the minimum night temperature 15°C. The plants of 6 - 8 months age, were selected for treatment at specific stages of the flush cycle according to the description of the stages by Greathouse, Laetsch and Phinney (1971) and Orchard *et al.* (1981) as outlined below:-

F-1 : Bud swelling, leaf initiation and unfolding.

F-2 : Leaf expansion. Leaves thin with anthocyanin pigment prominent. Apical bud dormant.

I-1 : Leaf expansion complete. Rapid greening. Apical bud dormant.

I-2 : Newly produced leaves dark green with a thick cuticle. Apical bud dormant.

2.2 Leaf growth

Leaf areas of the new flush leaves were calculated from the equation devised for cocoa by Reynolds (1971):-

$$\log Y = -0.632 + 1.987 \log X$$

where Y is the leaf area and X the length of the leaf along the mid rib. Actual determinations of the leaf area showed that this relationship held for plants grown under the present conditions. The orientation or position of each leaf with respect to its petiole

and the petiole to the main stem was determined by the leaf/petiole and petiole/stem angle.

2.3 Stomatal and cuticle development

Discs were taken from flush leaves of cocoa seedlings at different times of the growth cycle and immediately immersed in 20% (v/v) acetone for 20 minutes. The discs were further dehydrated by immersion in the following series of acetone solutions:

% (v/v) acetone	Duration (minutes)
25	20
50	10
75	10
85	10
95	10
100	10

After dehydration the acetone was removed from the discs in a critical point dryer (Poleron E300); the discs were stuck on "stubs" and then coated with 60% gold/palladium. Coated specimens were viewed in a Cambridge Stereoscan Mark 2A scanning electron microscope.

Stomatal counts were made on the upper and lower surfaces of the leaves with a epidermal impression method similar to that described by Brown and Rosenberg (1970). Nail varnish was applied to the leaf surface, allowed to dry for 1hr, stripped off with clear cellophane tape, and mounted on a microscope slide. Stomata were counted directly from the slide with a light microscope at x100 magnification using an ocular grid.

The average figure was obtained from a count of 30 fields from each leaf. Measurements were taken at the same time for guard cell size, distance between stomata and aperture length and width.

The thickness of the cuticle was measured by taking very thin sections of leaf tissue, dehydrating in alcohol, followed by staining in Sudan III. After the material was washed free of excess stains, the stained cuticle was measured with a light microscope and grid.

2.4 Stomatal Diffusive Resistance

Measurements of diffusion resistance to water vapour transfer were made on both the upper and lower surfaces of the same flush leaf, either with a Lambda Diffusion Resistance Meter (Kanemasu et al. 1969) or a porometer constructed of stainless steel and with automatic timing (Crump Scientific Cat. No. 502). The whole leaf diffusive resistance was calculated from an equation:

$$\frac{1}{R} = \frac{1}{R_L} + \frac{1}{R_U}$$

where R_L is the resistance of the lower surface and R_U the resistance of the upper surface. For experiments, calibration graphs for both instruments were made before each set of measurements were taken (Figs. 1 and 2).

2.5 Relative Water Content

The technique originally developed by Stocker (1928) and modified by Weatherley (1950) involves floating weighed leaf discs (2 cm.) on water for 24hr in diffuse light to obtain full turgor when a

Fig. 1. Typical calibration curve for L.D.R.M. Time lapse measured between 20 to 40 μA (HUM-1).

S.E = $\overline{\varphi}$

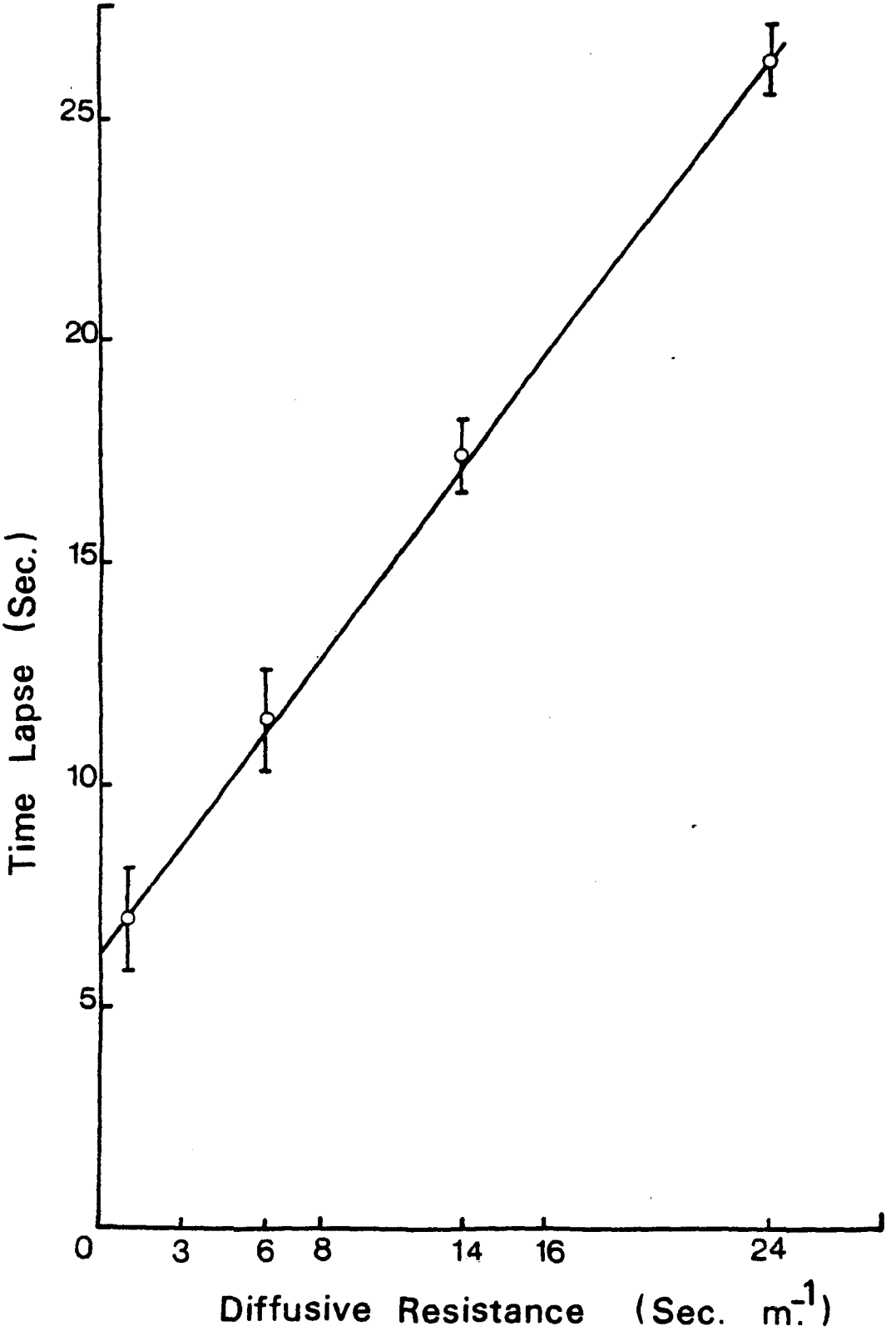
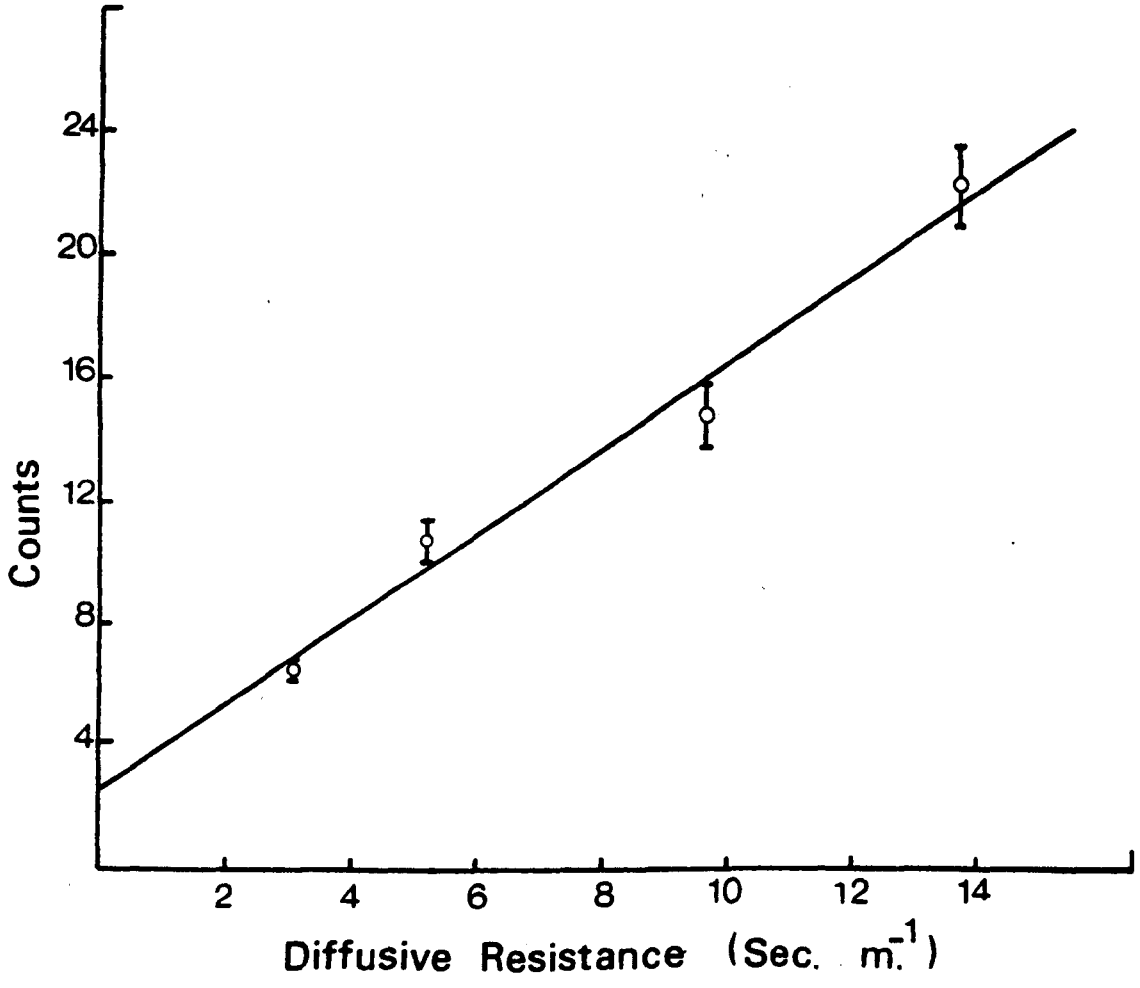

25
20
15
10
5
0
3
6
8
14
16
24
Time Lapse (Sec.)
Diffusive Resistance (Sec. m.-1)

Fig. 2. Typical calibration curve for the Crump Scientific Diffusion Resistance Meter. S.E. = $\bar{\pm}$


Counts
24
20
16
12
8
4
2
4
6
8
10
12
14
Diffusive Resistance (Sec. m.-1)

second weighing is taken. The leaf discs were oven dried at 80°C for 24hr to a constant dry weight. The RWC was then calculated from the equation:

$$RWC = \frac{(\text{Initial fresh weight} - \text{Dry weight})}{(\text{Fully turgid weight} - \text{Dry weight})} \times 100$$

The determination of RWC from the young expanding leaves at the F-2 stage was not possible because of the continued increase in fresh weight due to tissue expansion during the period of incubation. It was therefore decided that in all the estimations of RWC, measurements would start after the F-2 stage when the leaf was fully expanded.

2.6 Leaf water potential (Ψ)

Leaf samples were isolated in a sealed chamber and measurement of relative vapour pressure after a period of equilibrium gave the Ψ_{leaf} of the sample directly. A thermocouple psychrometer "Wescor H-33 dew point" calibrated using standard solutions of sodium chloride was used throughout the whole course of experimentation. An increase in negative values denotes lower water potential (see appendix).

Samples for determination of Ψ_{leaf} were taken as small discs (0.5cm) from the developing flush leaves between 11.00 a.m. and 1.30 p.m. to avoid any change in Ψ_{leaf} and RWC due to a diurnal rhythm (Fordham, 1971; Alvim et al. 1972; Fordham and George, 1972). The small discs were left to equilibrate for 1 hr. in the Wescor H-33 psychrometer chamber (one hour was found to be sufficient for the discs to reach equilibrium in the chamber (Fig. 3). Leaf water potential of the disc was calculated from the standard curve (Fig. 4). Results and discussion for work on water potential are

Fig. 3. Galvanometer readings for discs from flush leaves at (I-2) with different equilibration times.

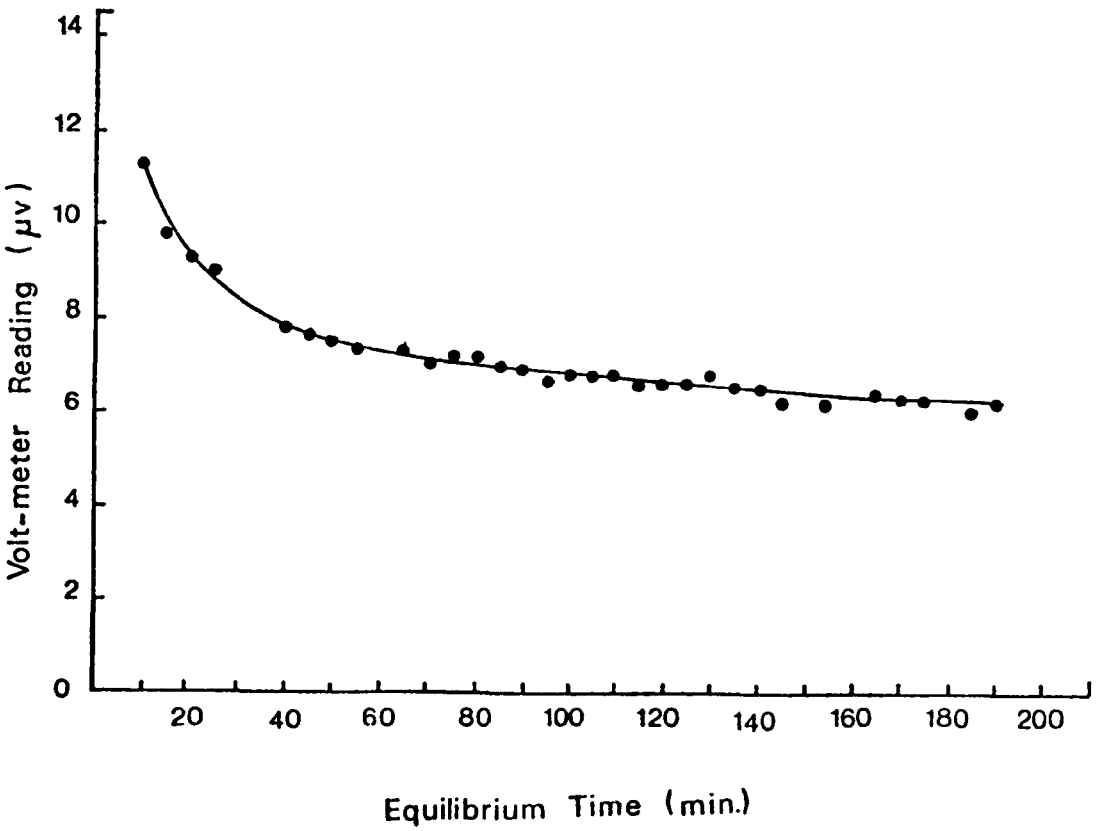
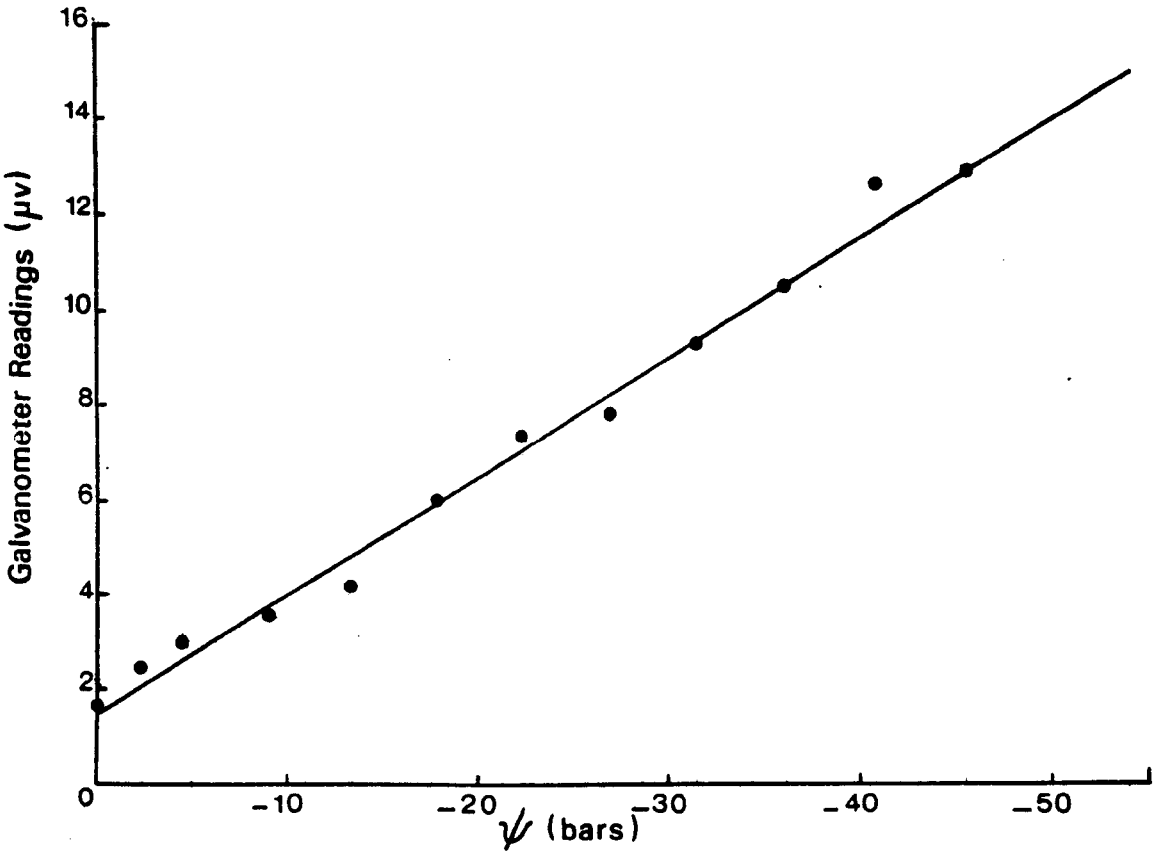

14
12
10
8
6
4
2
0
Volt-meter Reading (μv)
20
40
60
80
100
120
140
160
180
200
Equilibrium Time (min.)

Fig. 4. Calibration curve for the Wescor H-33 psychrometer. Constructed with standard sodium chloride solutions of known water potential.


Galvanometer Readings (μv)
16
14
12
10
8
6
4
2
0
-10
-20
-30
-40
-50
ψ (bars)

presented with "bars" as the unit. These can be converted to "Pascals" (the S.I. unit) by multiplying by 10^5.

2.7 Water uptake

Plants with at least four flushes were selected for uniformity at the F-2 stage of the new flush cycle. Each plant was placed in $^1/10$ conc. Arnon culture solution (Arnon, 1949) in a black painted sealed plastic container and aerated using an air pump. The decrease in the volume of the solution during 24hr. or 48hr. was taken to indicate the rate of water uptake by each plant. This measurement of water uptake was continued for two flush cycles. No measurable amount of water was lost from a control container lacking a plant.

2.8 Extraction and purification of growth hormones

2.8.1 Extraction of abscisic acid

Abscisic acid was extracted according to the method of Goldschmidt *et al.* (1973). Fresh leaf material was homogenized in 95% ethanol then the homogenate was left overnight at 5°C. After filtering through Whatman No. 1 filter paper to remove the tissue, the ethanolic extract was washed three times with equal volumes of petroleum ether (b.p. 40 - 60°C) and the petroleum ether fractions discarded. The ethanolic extract was evaporated *in vacuo* at 45°C to leave an aqueous residue which was then adjusted to pH 7.2 with 1.0M sodium hydroxide and partitioned three times with equal volumes of diisopropyl ether to give the neutral fraction.

The aqueous phase was then acidified with 1.0M hydrochloric acid to pH 3.0, extracted three times with equal volumes of diethyl ether to give a free acidic fraction.

To obtain the ABA glucosides which is the "bound" form of ABA, the remaining aqueous phase was subjected to alkaline hydrolysis to release the free ABA. The aqueous phase was adjusted to pH 11.0; kept at 60°C for 1hr; cooled and readjusted to pH 3.0 before being partitioned three times with equal volumes of diethyl ether.

All of the three fractions produced were evaporated in vacuo at 40°C then resuspended in 5cm^3 of ethanol and stored at -20°C until required.

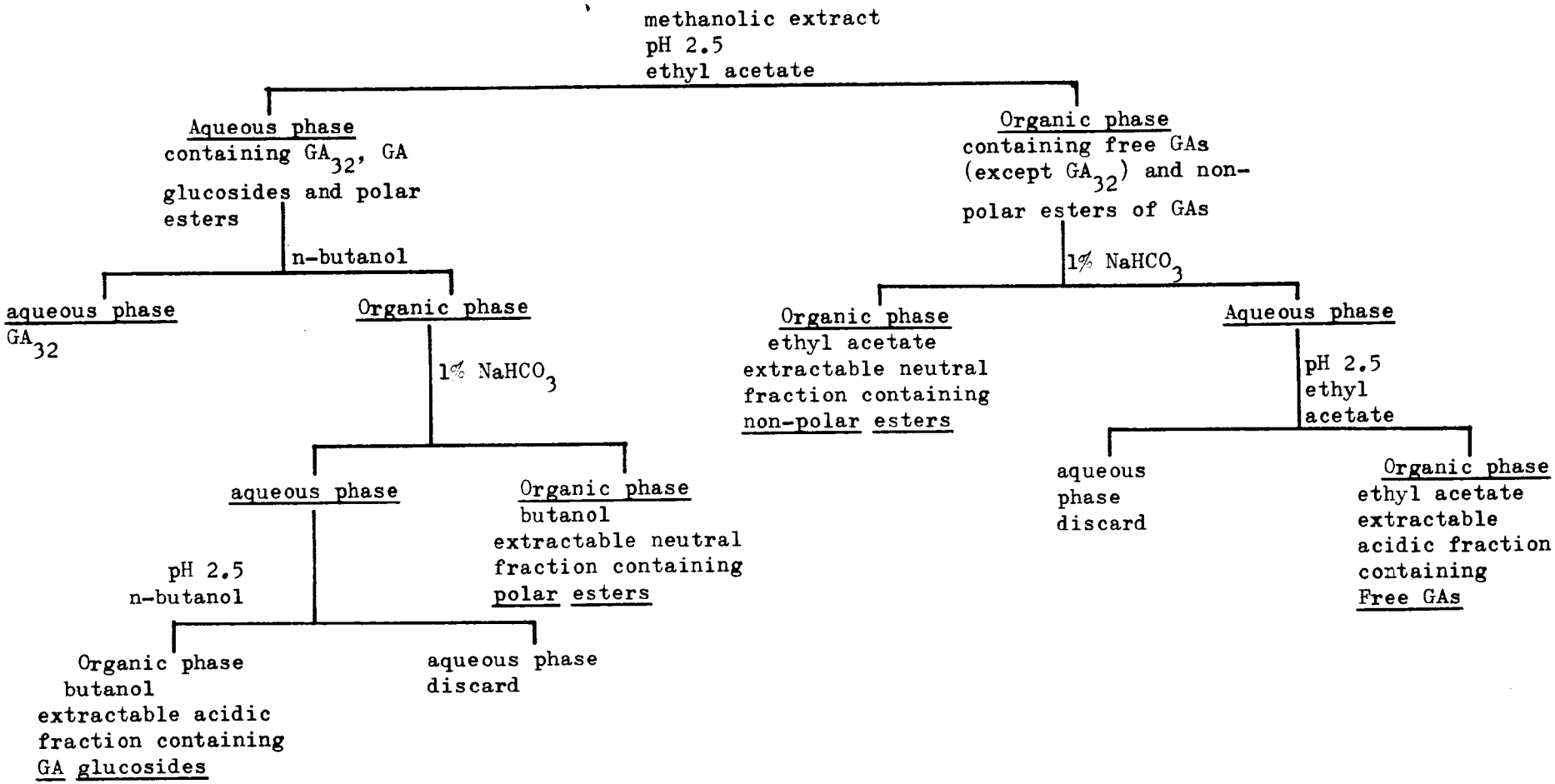
2.8.2 Purification of abscisic acid

The ABA in each fraction was purified further by paper chromatography. Aliquots of each fraction were loaded onto Whatman 3MM paper, the chromatograms were developed in isopropanol:ammonia:water (90:1:9) in an ascending system, at room temperature, until the solvent front had travelled 20cms. The area corresponding to synthetic ABA (R_f 0.6 - 0.8) was cut out and eluted in methanol for 20hr. The use of standard ABA gave a recovery of 75%.

2.8.3 Extraction of gibberellins (GA_3)

The plant material (a minimum of 40g fresh weight of leaf tissue was found to be necessary for satisfactory estimations) was first extracted in methanol using a blender (Kende, 1967). The organic solvent was removed under reduced pressure and the aqueous concentrate

A scheme for the general extraction procedure for free gibberellins, their esters and glucosides (Russell ,1975)



Russell, S. (1975). Extraction, purification and chemistry of gibberellins In Gibberellins and Plant Growth. (ed.) h. N. Krishnamoorthy, Haryana Agricultural Univ., Hissar. Wiley Eastern Ltd. New Delhi.

acidified to pH 2.5 then extracted with ethyl acetate (Cross et al. 1963). The ethyl acetate phase was back-extracted with 1% (w/v) sodium bicarbonate, and the aqueous solution was adjusted to pH 2.5 and re-extracted with ethyl acetate. The extract was evaporated under reduced pressure to give a mixture containing gibberellins. The resulting organic phase after extraction with sodium bicarbonate contains the non-polar esters. The ethyl acetate fraction in Phaseolus seeds-extract was found to contain the glucosyl esters of GA_{34}/GA_{37} (Hiraga et al. 1972). Only one free gibberellin, GA_{32}, cannot be extracted by this general procedure (Yamaguchi et al. 1970). This is because the partition coefficient of GA_{32} between water and ethyl acetate favours the aqueous phase.

Bound gibberellins which are more polar than free gibberellins are not extracted from aqueous solution by partitioning with ethyl acetate. However, they can be extracted with n-butanol (Sembdner et al. 1964; Yokota et al, 1971). Usually the aqueous phase resulting after first extraction with ethyl acetate is partitioned with n-butanol, and the butanol extract is then back-extracted with 1% (w/v) sodium bicarbonate yielding a butanol-soluble acidic fraction which contains the glucosides of gibberellins (Yokota et al. 1971b; Schreiber et al. 1970) and a butanol-soluble neutral fraction which contains very polar esters such as the glucosyl ester of gibberellin A_{38} (Hiraga et al. 1972).

2.8.4 Purification of gibberellins

The acidic and neutral extracts obtained by the isolation procedures previously described contain a mixture of gibberellins

and other substances such as phenolics. Glenn *et al.* (1972) have shown that the passage of these extracts through insoluble polyvinyl pyrrolidone (PVP) column or slurry removes the phenols, with no loss of gibberellin-like activity.

The PVP slurry for use here was prepared by adding PVP at concentrations of 75 mg cm^{-3} to buffer solutions (0.1 M phosphate pH 8.0) containing the extracts or standard solution then slurried thoroughly with constant shaking for 30 min. The PVP was filtered off by vacuum filtration and the residue washed with successive aliquots of buffer. Fresh PVP was then added to the filtrate and the shaking and filtration procedure repeated a further two times.

Recovery of gibberellins from the combined filtrates was achieved by acidifying the solutions to pH 2.8 with hydrochloric acid and partitioning five times against ethyl acetate (buffer-ethyl acetate, 5:3 v/v). The combined extracts were dried, filtered and evaporated to dryness *in vacuo*. Residues were taken up with successive aliquots of methanol-ethyl acetate (1:1 v/v) and prepared for analysis by TLC or paper chromatography.

After purification of the ethyl-acetate-soluble gibberellins and the n-butanol extractable gibberellins, samples were taken to hydrolyse the non-polar esters, polar esters and GA-glucosides. 1% cellulose (Sigma) in 0.2M acetate buffer (pH 4) was added to an aliquot of the sample and allowed to stand at 37°C for 16 hr. The solution was concentrated to dryness and the resulting residue was extracted with methanol. The extract was examined by TLC or paper chromatography with a solvent system consisting of diisopropyl ether : acetic acid (95 : 5 v/v) or isopropanol : ammonia : water

(10:1:1 v/v). Following chromatography, the plate or the paper was divided into RF strips and the areas with gibberellin activity were assayed. A recovery of 70% was obtained when standard GA_3 was used.

2.8.5 Extraction of auxins

The method used for extraction of auxins was that originally described by Morris et al. (1969). Plant tissues were extracted in 85% ethanol for 24hr. at 5°C in darkness. This was repeated three times and the washings were collected and made up to volume. This method will extract only ethanol-soluble compounds. To extract the more firmly bound auxins (ethanol insoluble complexes), the residue after extraction in 85% ethanol was washed three times with aliquots of 95 % ethanol . The washed residues were dried in vacuo, resuspended in three times their packed volume of 2M sodium hydroxide (1.0 - 5.0cm^3), and extracted for 15hr. in darkness at 21°C. After extraction the residues were washed in a further volume of 2M sodium hydroxide and twice in water, and the extracts and washings were combined and made up to volume with distilled water. Aliquots of this extract were assayed for radioactivity.

2.8.6 Purification of auxins

The indolic compounds in the plant extracts were purified and identified as follows. An aliquot of the ethanolic extract was reduced to dryness in vacuo at 25 - 27°C, the residue was taken up in 0.5cm^3 methanol, and then an aliquot was spotted onto Whatman No. 1 paper. Ascending chromatograms were developed over a distance

of 20cm. at 20°C in darkness using solvent systems of isopropanol: 30% ammonia : water (10:1:1 or 8:1:1 v/v) and n-butanol : glacial acetic acid : water (8:1:22 v/v). The chromatogram was cut into 20 equal sections each of which was eluted (in polythene counting vials) in 2.0cm^3 methanol for 30 - 60 min. Eight cm^3 of scintillation fluid was added to each vial and the radioactivity determined. Comparisons were made with authentic, unlabelled indole compounds including IAA and indole-3-aldehyde (IAld), indole-3-L-aspartic acid (IAAsp), indole aceto nitrile (IAN) and indole-3-acetaldehyde (IAAld), all of which were chromatographed in parallel with the samples under investigation. Colour development after spraying with Ehrlich reagent, ninhydrin or KNO_2 in HNO_3/ethanol; and fluorescence under UV radiation was used to confirm the identity of developed spots.

The Ehrlich reagent gives a red-violet colour which is useful for identification on paper chromatograms when used as a solution of 1 volume of 10% (w/v) p-dimethyl aminobenzaldehyde in concentrated HCl to 4 volumes of acetone (Jepson, 1958). There is another test, which is similar to but much more sensitive than the original Ehrlich test. This employs p-dimethyl aminocinnamaldehyde made up as 1% solution in a 50:50 mixture of ethanol and 6M HCl (Harley-Mason and Archer, 1958). This, applied as a spray, gives a blue reaction in the presence of indoles and is claimed to detect as little as 0.1µg IAA. A recovery of 85% was achieved when radioactive IAA was used during the whole extraction procedure.

2.8.7 Extraction of cytokinins

The plant material was extracted in cold 80% methanol with a Polytron homogenizer according to the method of Reda (1976). The homogenate was left overnight at 5°C then filtered through Whatman No. 1 filter paper. The filter paper and the crude debris were shaken with 80% methanol, filtered, and the debris washed again with methanol until "bleached" then the mixture finally filtered.

2.8.8 Purification of cytokinins

Each filtrate was evaporated *in vacuo* at 40°C to give an aqueous residue, which was then centrifuged at 15,000 r.p.m. for 30 min. to remove chloroplasts and other debris. PVP was added to the supernatant, to form a suspension, so that substances such as pigments and phenols were removed. After this "slurry" had been shaken overnight the PVP was removed by filtration with Whatman No. 1 filter paper.

The aqueous filtrate was adjusted to pH 3.0 with 1M hydrochloric acid and partitioned three times with equal volumes of ethyl acetate. After discarding the ethyl acetate the aqueous phase was adjusted to pH 7.0 with 1M sodium hydroxide, then partitioned three times with equal volumes of water-saturated butan-1-ol. The butanol phase (containing free cytokinins) was stored at -20°C. The aqueous fraction was evaporated *in vacuo* at 40°C then treated with alkaline phosphatase (1 mg in 10cm^3 extract) in 0.1M Tris buffer and 0.01M magnesium chloride for 24hr. at 30°C. The enzyme catalyses

the conversion of the inactive bound cytokinins (cytokinin nucleotides) to free cytokinins. After 24hr. the cytokinin enzyme solution was adjusted to pH 7.0 with 1M hydrochloric acid and partitioned three times with equal volumes of water-saturated butan-1-ol.

All of the combined butan-1-ol fractions were evaporated to dryness under vacuum at 40°C, the residues were redissolved in methanol then each solution was applied to Whatman 3MM chromatography paper, then developed in propan-1-ol : ammonia : water (10:1:1). The solvent front was allowed to run to 20cm. before the chromatograms were air-dried in darkness over-night. The chromatograms were then divided into 10 equal strips and each strip was placed into a vial with 10 cm^3 scintillation fluid to measure its radioactivity. The R_f values of standard non-radioactive and radioactive cytokinins were used to identify the position of cytokinin components in the extracts. A recovery of 87% of a radioactive BAP was obtained by this method of extraction and purification.

2.9 Measurement of growth hormones

2.9.1 Abscisic acid

Wheat grains were germinated in darkness for 66hr. at 25°C according to the procedure used by Wright (1956). Seedlings of at least 2cm coleoptile length were selected then cuts made 2, 4 and 6 mms below the tip, the tip section was discarded and the other two 2mm sections used in the bioassay. The segments were incubated in a Petri-dish containing 2 cm^3 of distilled water and a section of the chromatogram

including a control section. After 24hr. incubation in the dark at 25°C the coleoptiles were measured with the aid of a photographic enlarger.

A dose-response curve was constructed for standard ABA in which the concentration of ABA was plotted against growth of coleoptile segments expressed as a percentage of the control (Fig. 5). From this curve statistically significant growth-inhibitory responses could be converted to ABA equivalents.

2.9.2 Gibberellic acid

Lettuce seeds were sown in the dark at 25°C according to the procedure used by Frankland and Wareing (1950). After 2 days seedlings were selected with radicles of a similar length (6 - 8mm) and "planted" in 9 cm Petri dishes lined with filter paper moistened with 6 cm^3 of test solution. Ten seedlings were used per treatment. They were placed 15 cm. below a light source consisting of two daylight fluorescent tubes (5 ft., 80 watt), in which position the temperature was stable at 28°C. Each hypocotyl length was measured after 5 days, to the nearest mm. The concentration of gibberellic acid in any extract was calculated from a standard curve which showed a linear relationship between hypocotyl elongation and the of gibberellic acid concentration (Fig. 6).

2.10 Application of growth hormones to plants

The stage of the flush cycle at which the donor leaf was selected for hormone application varied according to the type of hormone used.

Fig. 5. Dose-response curve for abscisic acid in the wheat coleoptile bioassay.

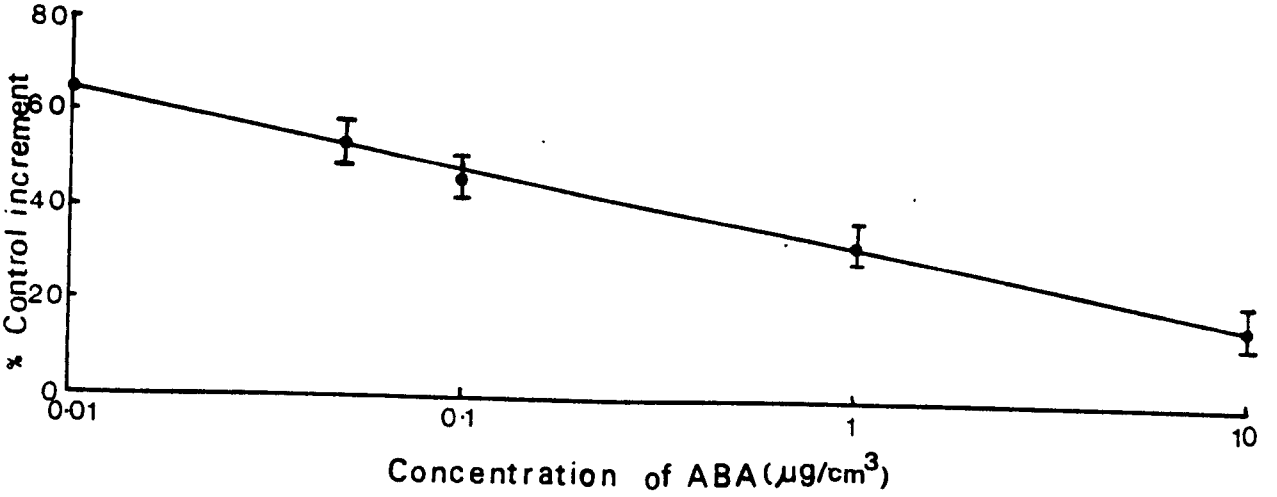
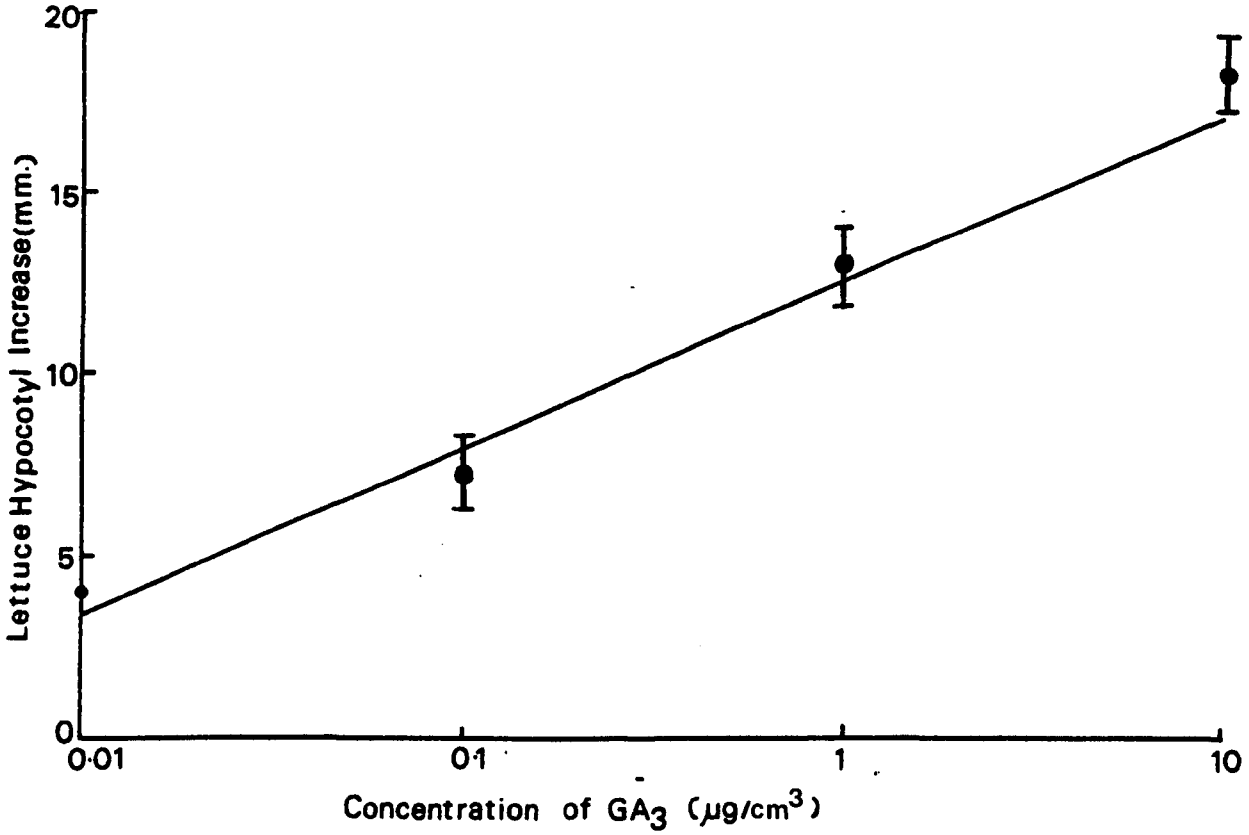

80
60
40
20
0
% Control increment
0·01
0·1
1
10
Concentration of ABA (μg/cm3)

Fig. 6. Dose-response curve for gibberellic acid (GA_3) in the lettuce hypocotyl bioassay.


20
15
10
5
0
Lettuce Hypocotyl Increase(mm.)
0·01
0·1
1
10
Concentration of GA3 (µg/cm3)

Thus ABA was applied to the leaf at the end of the dormant phase of the cycle, i.e. I-2, so that any delay in the onset of growth would be more clearly demonstrated, whereas the growth promoters were applied at the beginning of the dormant stage, i.e. I-1, so that any reduction in the length of the dormant period would also be clearly shown.

Abscisic acid, gibberellic acid (GA_3) and zeatin were dissolved separately in 0.8% agar at concentrations of 1×10^{-4} M for ABA and zeatin and 3×10^{-3} M for GA_3. The agar block (1.0 x 1.0 x 0.5cm) containing one of the growth hormones was placed on the upper surface of the donor leaf after lightly abrading the leaf surface with carborundum powder. The donor leaf was the first developed leaf of the new or previous flush (PF_1). The leaf was loosely enclosed in a clear polythene bag to prevent dehydration of the agar block, then the plants were returned to the glasshouse. The radioactive ^{14}[C] ABA (0.165µg per block, a racemic mixture with a specific activity of 45µCimg^{-1}), ^{14}[C] GA_3 (0.5µCi per block with specific activity 3.65 mCi/mmol.), ^{14}[C] BAP (0.4µCi per block with specific activity 54 mCi/mmol.), ^{14}[C] IAA (0.5µCi per block with specific activity 57.5 mCi/mmol.) were dissolved separately in agar and the agar block was applied to the plant as previously described.

2.11 Determination of radioactivity

The radioactivity in extracted plant material was determined by liquid scintillation spectrometry.

Two cm^3 of the extract was transferred to a scintillation vial and then reduced to dryness *in vacuo*. Ten cm^3 of a scintillation mixture comprising 2,5-diphenyl oxazole (PPO)

dissolved in toluene ($4g.dm^{-3}$) were added to each vial. The samples were assayed in a Packard-Tri-Carb scintillation spectrophotometer. It was appreciated that there would be quenching of the radioactivity by the presence of chlorophyll, solid material and other chemicals in the extracts. No attempt was made to calculate the total quenching and therefore to correct the figure for radioactivity of each sample. It was felt that a correction for quenching would not result in a major alteration to the pattern of radioactivity, and so in all cases the original figures for radioactivity have been used with correction for background only. The radioactivity in each plant part was expressed as a percentage of the total exported from the donor leaf to provide an overall picture of the distribution of radioactivity. The radioactivity data were also expressed in terms of relative specific activity (RSA). This calculation is claimed to give a value which allows a more meaningful description of the ability of the different parts of a plant to accumulate activity of a supplied radioactive compound (Mor and Halevy, 1979; Robinson et al., 1980). The RSA was calculated by dividing counts min^{-1} g^{-1} fresh weight of tissue in a specified part of the plant by counts min^{-1} g^{-1} of the whole plant, excluding the source leaf.

CHAPTER 3

Structural changes during leaf development

3.1 Introduction

It seems clear from previous studies by Alvim *et al.* (1974) and Orchard *et al.* (1980) that changes in ABA levels occur in both field grown plants and greenhouse seedlings of cocoa. Although changes in ABA in field grown plants can be correlated with changes in soil water deficit, changes in ABA in seedlings (Orchard *et al.* 1980) occur despite them being maintained in soil at field-capacity. The reasons why a water deficit may occur in seedlings under these conditions has been discussed briefly in Chapter 1 and is further discussed in Chapter 4. A main component of the present hypothesis regarding the control of flush growth (either in the field or glasshouse conditions) is this variation in water status of cocoa plants, and in particular in this case, seedlings. It was considered necessary to understand more of how new flush leaves develop and in particular development of those features of the leaves which could affect water loss and thus lead to significant changes in seedling water status.

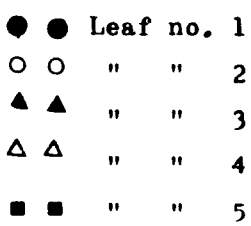
An internal water deficit in the seedling could arise by an imbalance developing between water uptake by the roots and the sum of that lost in transpiration by the leaves plus that used to support leaf expansion, i.e. increase in leaf fresh weight.
It was decided therefore to examine those physical parameters of the leaves of seedling plants most likely to affect the transpiration loss from the new flush leaves at different stages of the flush cycle. The term transpiration is used here to mean the process

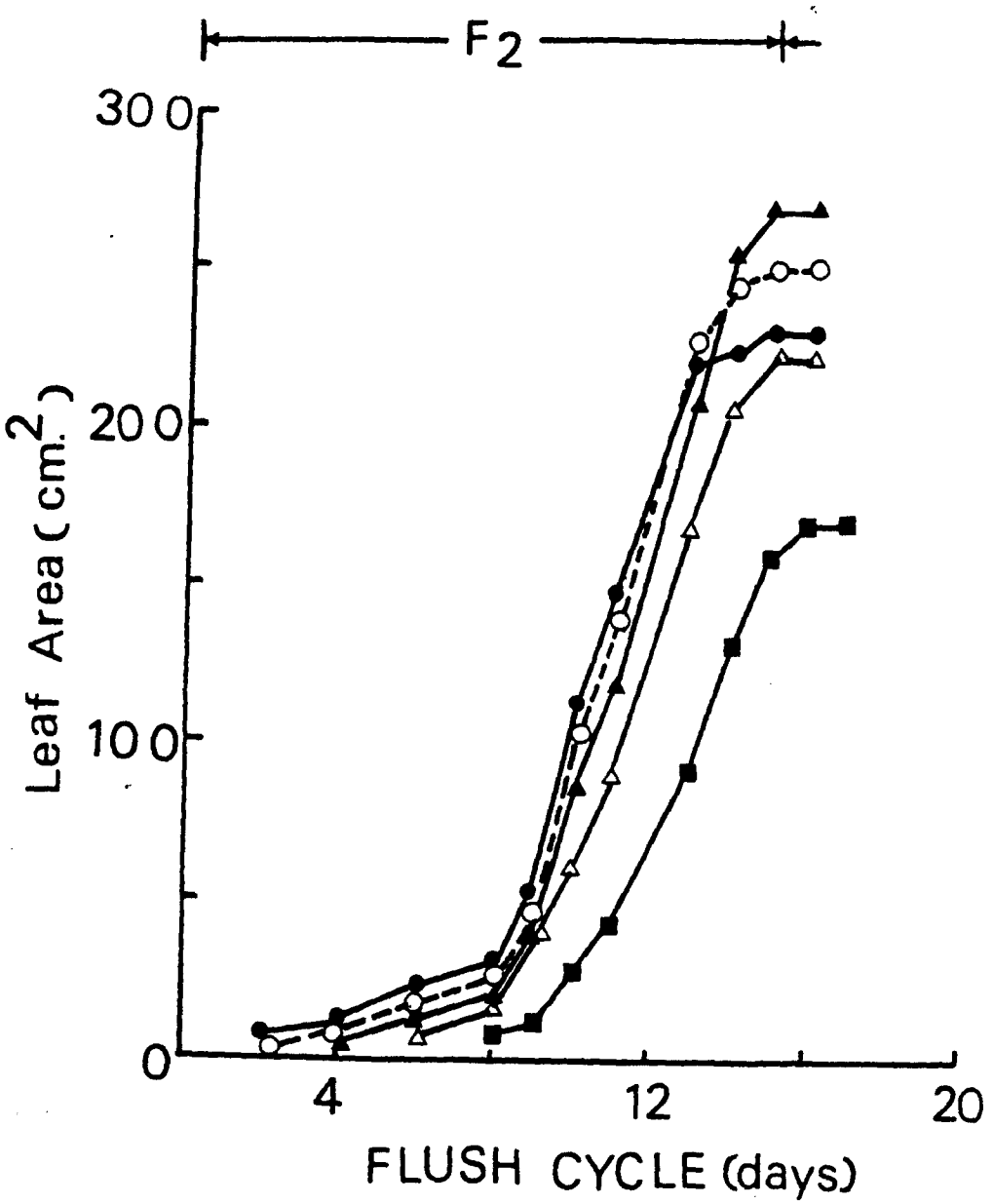
leading to loss of water (as water vapour) from the stomata and cuticle combined, i.e. total water loss from a particular leaf surface. These parameters were leaf position, cuticle thickness and stomatal number and development. The changes in these parameters were correlated then with measurements of the cuticle and stomatal diffusive resistance to water vapour which was a measure of the potential total water loss from the leaves. Plants used in this analysis were the same age as those described in Chapter 2 and methods are also described in details in Chapter 2.

3.2 Leaf growth

The various stages of the flush cycle have been described by Greathouse et al. (1971) for cocoa plants grown under conditions of controlled temperature and light intensity and by Vogel (1975) for cocoa seedlings in a shaded tropical environment. Although the length of the stages differed in the present greenhouse-grown material compared with the other two growth situations, the same basic pattern of development was shown. The beginning of the cycle was marked by a swelling of the apical bud followed by unfolding of all the leaves of one flush in rapid succession. As shown by the leaf area changes of a typical flush (Fig. 7), all the leaves began to expand at approximately the same time and all reached a maximum size within a short period, although the final size of the later formed leaves was always smaller. The cycle was completed in six weeks and was repeated with little change in both the summer and winter.

Fig. 7. Leaf area changes of a typical flush




F2
300
200
100
0
Leaf Area (cm.2)
4
12
20
FLUSH CYCLE (days)

3.3 Leaf orientation

Measurements of the petiole/stem and lamina/ petiole angle were made on three replicate plants during a flush cycle. The measurements were made by a protractor.

The orientation of the leaves alters considerably during the flush cycle (Figs. 8 and 9). Immediately following leaf emergence the leaves at the F-1 stage are red and are held almost vertically upwards. However, with expansion (F-2 stage) the petiole moves into a horizontal position and the lamina hangs vertically downwards. During this stage the laminae are thin and of a flaccid appearance. This position is maintained throughout expansion until the leaves begin to green (start of I-1 stage), then both the petiole and the lamina begin to adopt a more vertical position. By the time the leaf has become fully green, and rigid with a shiny cuticle, the lamina/petiole angle increased (from horizontal) and this, together with a near vertical position of the petiole, means that the lamina is held horizontally. The mechanism by which these changes in angle are achieved is unknown but it is likely to be related to the turgor changes in the pulvinus at the base of both the leaf lamina and the petiole.

3.4 Stomatal development

All measurements were made on three plants each replicated three times of stomatal number, shape and resistance.

Preliminary observations of both surfaces of the flush leaves confirmed the report of Metcalfe and Chalke (1950) that

Fig. 8. The change in petiole-stem angle of the new flush leaf during a flush cycle.

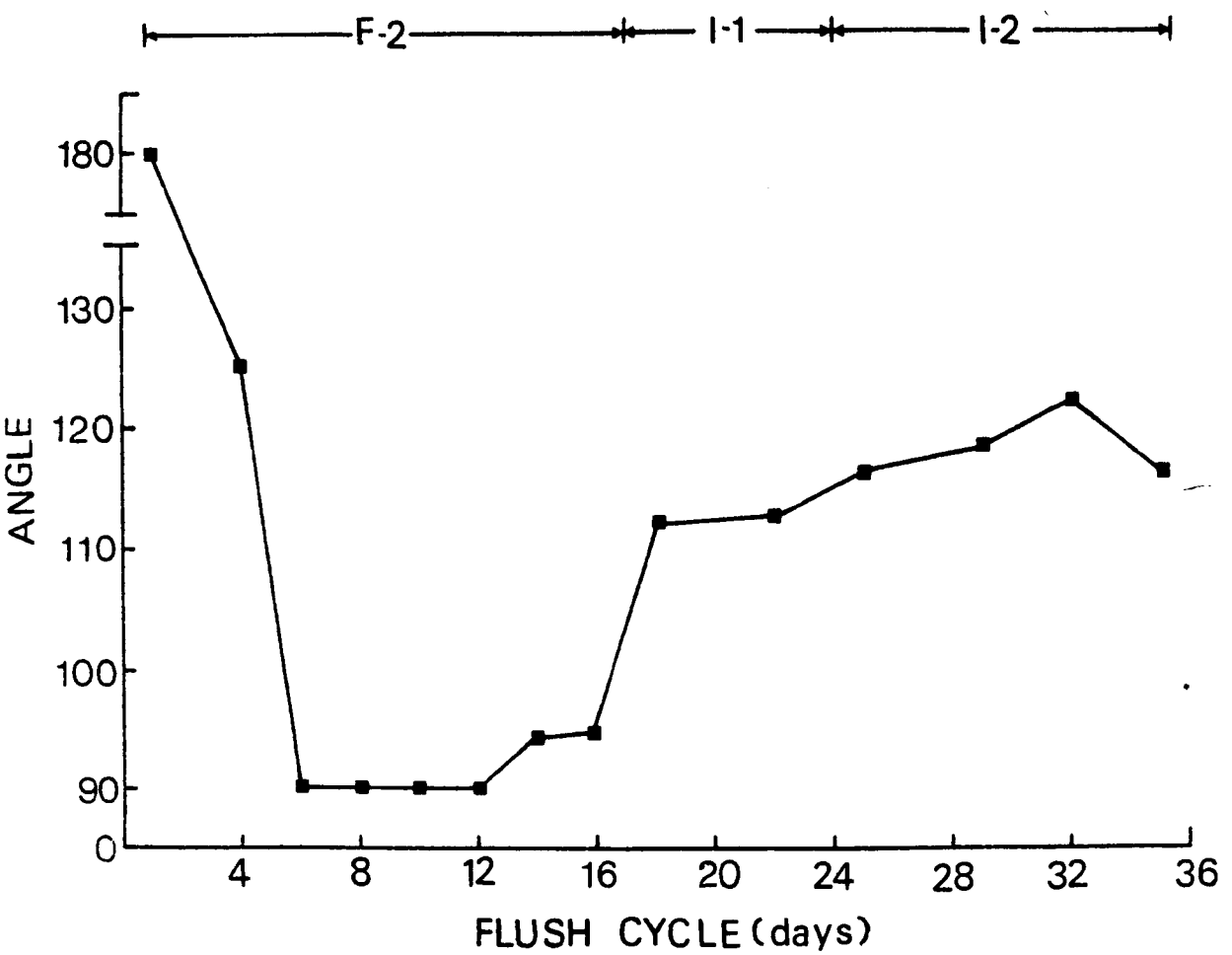
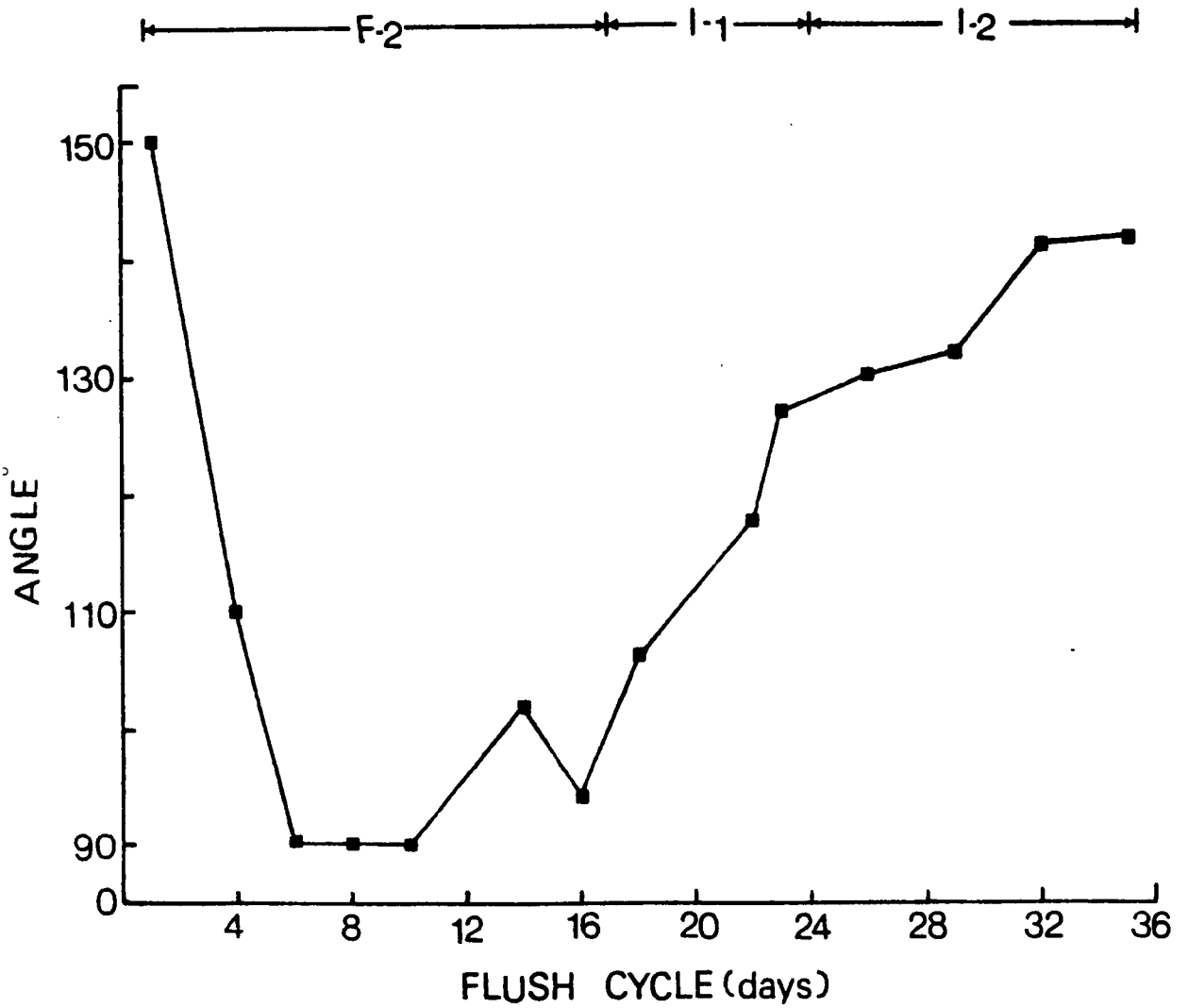

F-2
I-1
I-2
180
130
120
110
100
90
0
ANGLE
4
8
12
16
20
24
28
32
36
FLUSH CYCLE (days)

Fig. 9. The change in Lamina-petiole angle of the new flush leaf during a flush cycle.


F-2
I-1
I-2
150
130
110
90
0
ANGLE°
4
8
12
16
20
24
28
32
36
FLUSH CYCLE (days)

stomata are only present on the lower surface of the cocoa leaf. On the very young four days old flush leaf stomata were present only on the mid-rib, lateral and smaller veins (Plate 1) and then only those on the mid-rib were fully developed and open. The stomata on the lateral and smaller veins opened within a further two days and at the same time stomata began to develop on the lamina (Plate 2) and later to open. This change in development was also indicated by the increase in stomatal aperture width and length (Fig. 10c & d) and an increase in the guard cell width and length (Fig. 10e & f). A large number of the stomata became open during leaf expansion and at maximum leaf size all the stomata were open (Plate 3). Although the stomatal density actually declined during F-2 (Fig. 10a), stomatal development did appear to follow an ordered pattern closely correlated with the growth of the leaf. It was interesting to note, the correlation between stomatal development/functioning and chlorophyll. In young leaves, up to mid F-2, the veins are green and these are the only part of the plant to possess functional stomata. As the inter-veinal regions green so stomata develop here too.

The cuticle development showed that there was a slow increase in cuticle thickness of the new flush leaf during F-2 and then a more rapid increase in the cuticle thickness was observed at late F-2, but most of the cuticle development occurred during the I-1 stage (Fig 11).

3.5 Stomatal and cuticular diffusion resistance

The cuticular diffusion resistance was measured from the upper surfaces of the leaves and stomatal diffusion resistance was measured from the lower surfaces of the leaves. The measurements of

Plate 1. Open stomata on main vein:

(a) fully open x4700

Day 4 of F-2 stage

(b) partially open x4700

Day 4 of F-2 stage

Plate 1(a)

Plate 1(b)

Plate 2 : Guard cells fully formed, but not functional.
x 950
Day 8-10 of F-2 stage.

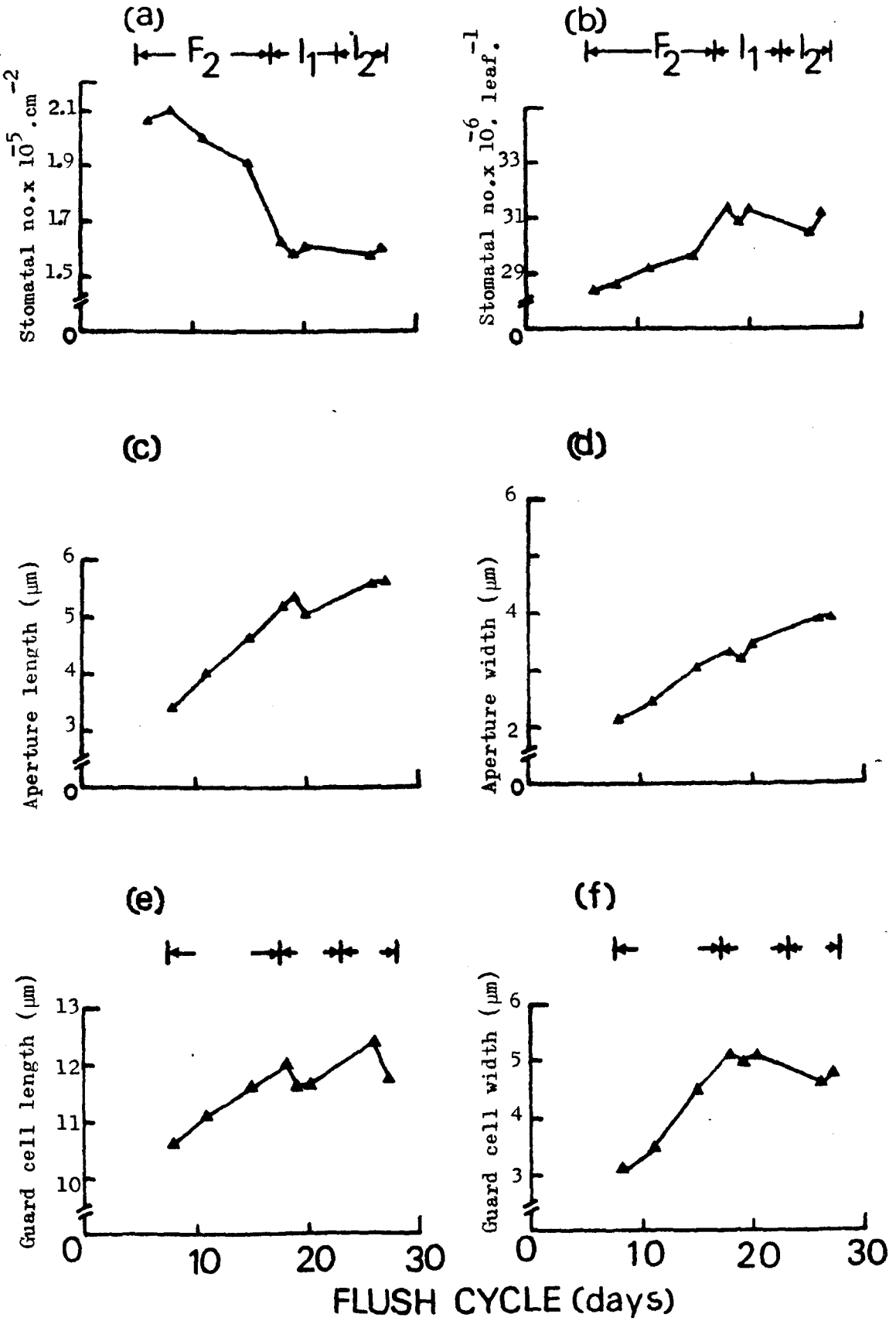
Plate 3 : Open stomata on lamina.
x 1900
Day 17 of F-2 stage.

Plate 2

Plate 3

Fig. 10. The stomatal development of the new flush leaves during a flush cycle:

(a) stomatal number per cm^2.
(b) " " per leaf.
(c) aperture length.
(d) aperture width.
(e) guard cell length.
(f) guard cell width.


(a)
F2
I1
I2
Stomatal no.x 10^-5 .cm^-2
2.1
1.9
1.7
1.5
0
(b)
F2
I1
I2
Stomatal no.x 10^-6. leaf^-1
33
31
29
0
(c)
Aperture length (µm)
6
5
4
3
0
(d)
Aperture width (µm)
6
4
2
0
(e)
Guard cell length (µm)
13
12
11
10
0
10
20
30
(f)
Guard cell width (µm)
6
5
4
3
0
10
20
30
FLUSH CYCLE (days)

stomatal resistance of the lower surface correspond to the sum of stomatal and cuticle water loss of the lower surface.

The cuticular resistance of the upper surface showed very low values at early F-2, but rapidly increased during late F-2 and the maximum values of cuticular diffusion resistance were obtained during the I-1 stage (Fig. 11). The stomatal resistance (Fig. 12) in the young leaf was very similar to the cuticular resistance of the upper surface but then in contrast to the cuticle resistance progressively reduced during leaf expansion until by late F-2 it was about 5 sec. cm^{-1}. The rapid decline in resistance of the lower surface during F-2 implies that most water loss from the mature leaves is from the lower rather than the upper surface and via the stomata rather than the cuticle. During the I-1 stage there was some fluctuation in resistance from the upper and lower surfaces.

The environmental variables in the greenhouse of light, temperature and relative humidity were recorded during all measurements of diffusion resistance. The statistical analysis showed that only one factor which might have influenced the stomatal diffusion resistance was the light intensity (Fig. 13). The increase in light intensity was accompanied by lower diffusion resistance while at the lower light intensities stomatal closure occurred as measured by the high values of leaf diffusive resistance.

3.6 Discussion

In the cocoa it is clear that the stomatal density decreased with leaf age (Fig. 10a). This correlation was also shown in other

Fig. 11. The change in cuticle thickness of the new flush leaves during the flush cycle.

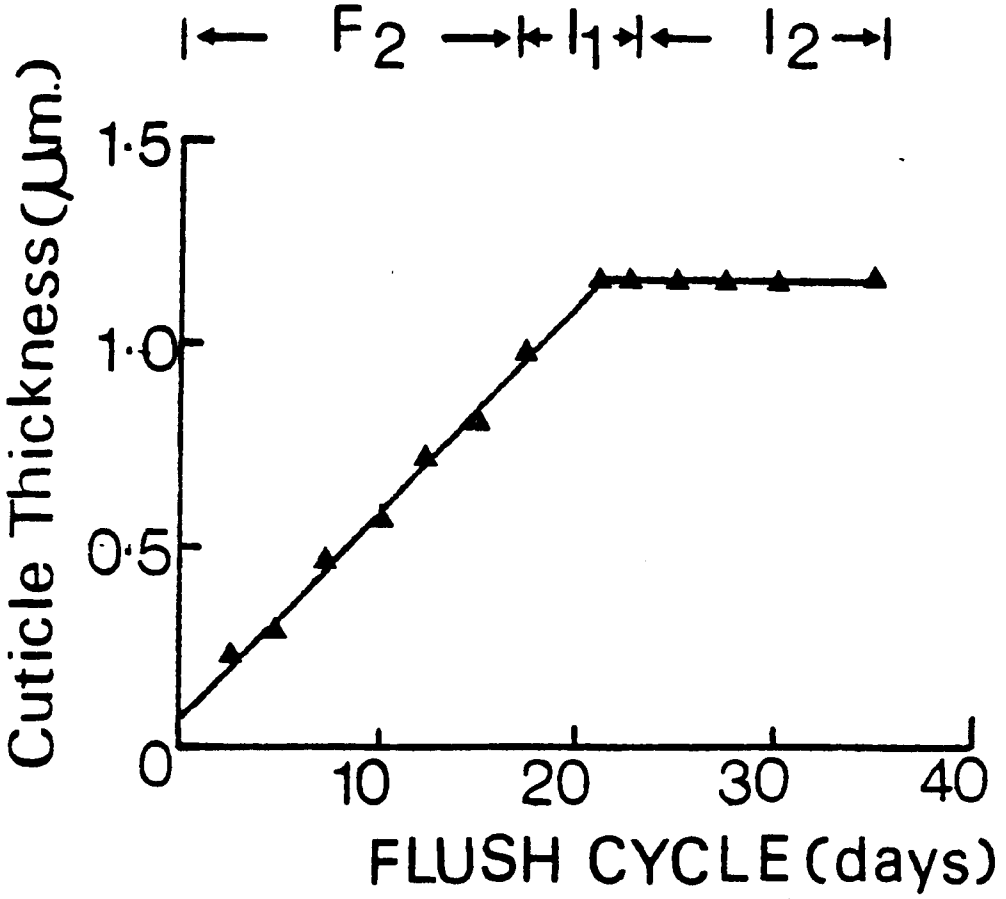

F2
I1
I2
Cuticle Thickness (μm.)
1·5
1·0
0·5
0
10
20
30
40
FLUSH CYCLE (days)

Fig. 12. The change in diffusive resistance of the new flush leaves during a flush cycle.

Key: A - Upper surface 'cuticular'

B - Lower surface 'stomatal'

C - Whole leaf resistance

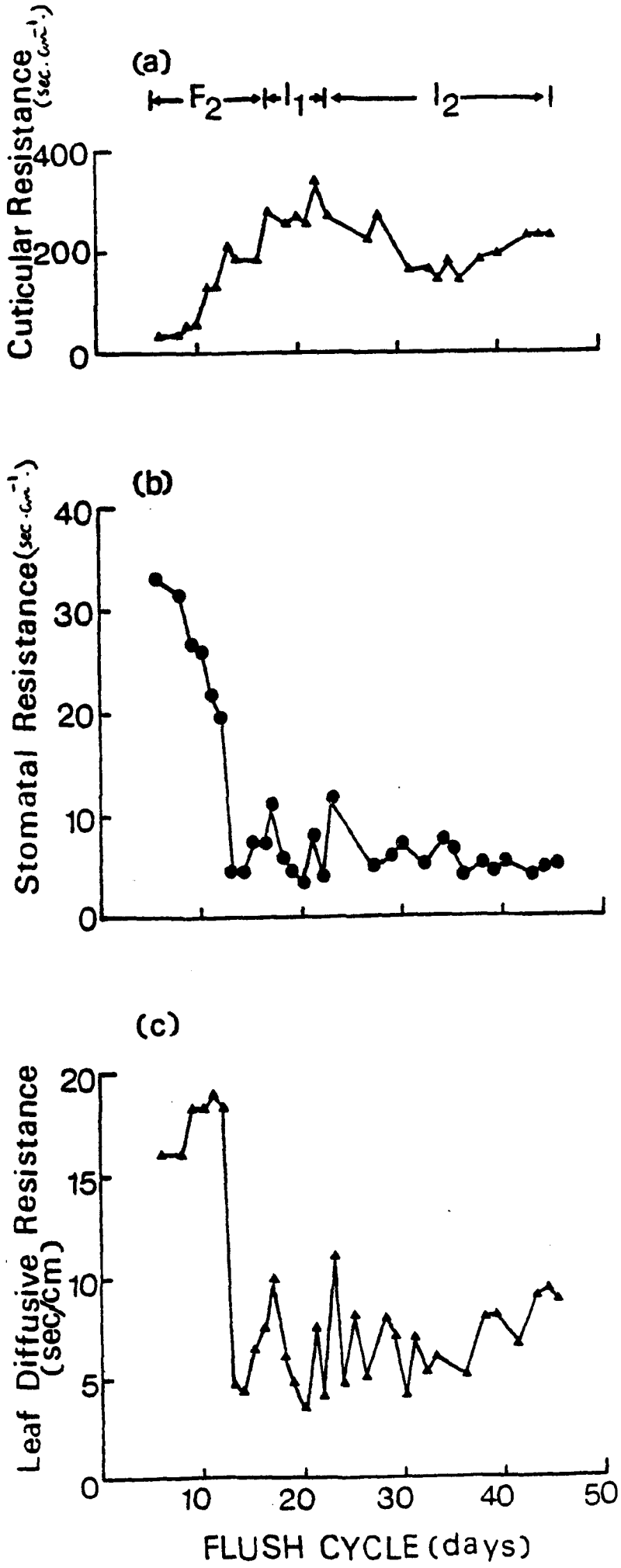
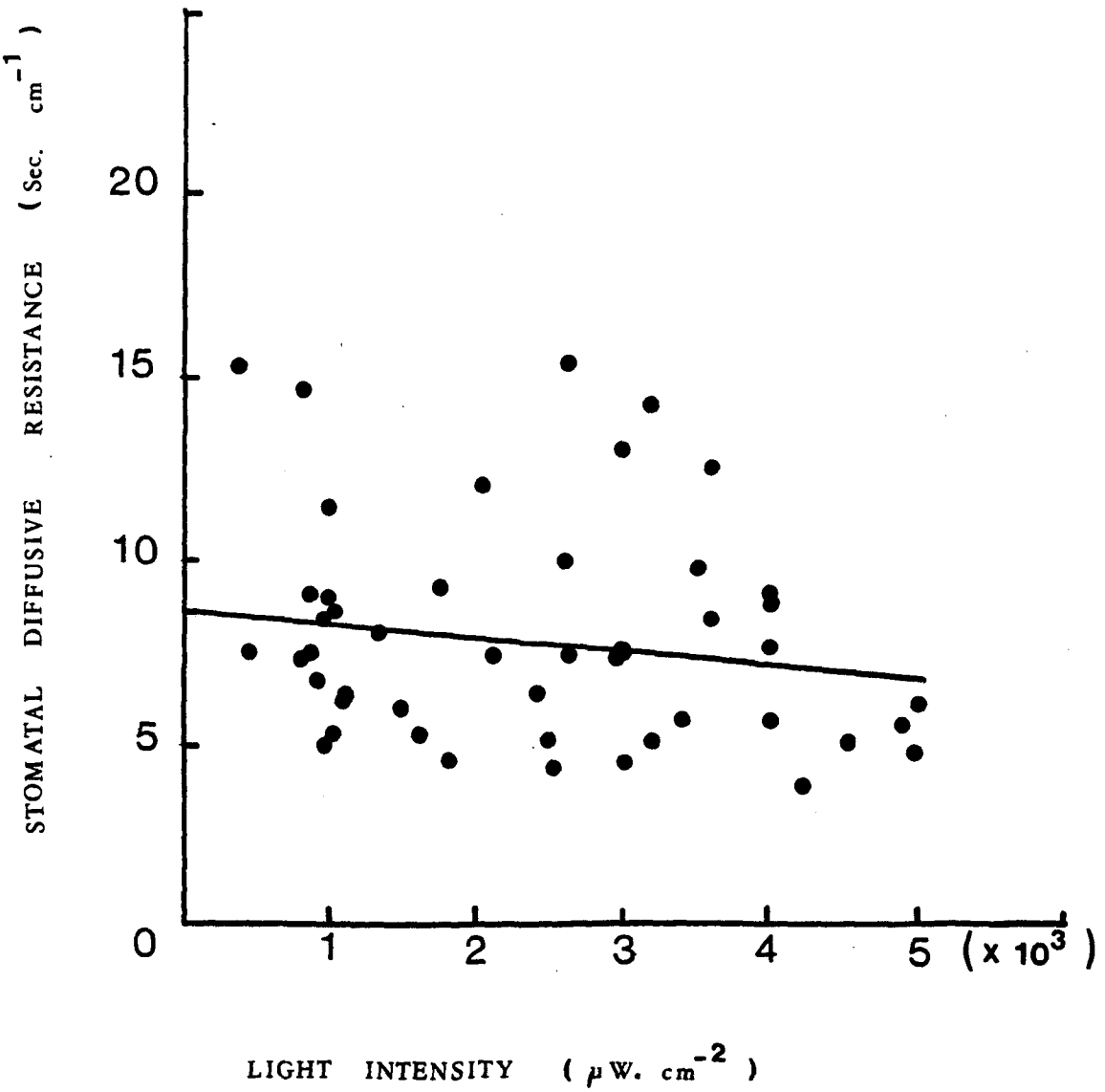

(a)
F2
I1
I2
Cuticular Resistance (sec. cm-1)
400
200
0
(b)
Stomatal Resistance (sec. cm-1)
40
30
20
10
0
(c)
Leaf Diffusive Resistance (sec/cm)
20
15
10
5
0
10
20
30
40
50
FLUSH CYCLE (days)

Fig. 13. The influence of light intensity on the stomatal diffusion resistance of mature cocoa leaves . The relationship was plotted according to the bivariate regression analysis. The correlation coefficient (=0.666) is highly significant at 0.05 level.


STOMATAL DIFFUSIVE RESISTANCE (Sec. cm^{-1})
20
15
10
5
0
1
2
3
4
5 (x 10^3)
LIGHT INTENSITY (μW. cm^{-2})

species. For instance, Brown and Rosenberg (1970) found the average stomatal density in sugar beet leaves of different ages to be 9,710 stomata cm^{-2}. This mean density of stomata decreased with age from 11,350 stomata cm^{-2}. for the young immature leaf to 5,950 stomata cm^{-2}. for the oldest leaf. The decrease in stomatal density could be attributed to the continuous expansion of the leaf at a nearly constant rate. In cocoa there was also a change in length of the stomata with the age, again in agreement with the sugar beet leaves (Brown and Rosenberg, 1970) where the mean stomatal length of different physiological ages increased gradually from 10.6μm on the youngest leaf to 24.8μm on the oldest leaf. As the leaf expands the size of the stomata increases.

In an examination of the same parameters, density and size of stomata in leaves of French bean in relation to diffusion resistance, it was found that the decrease in density was balanced to some extent by the growth of the stomatal pore (Solarova, 1973). The stomatal density declined with leaf maturation from 950 to $400mm^{-2}$ on the lower surface whereas the size of stomata on the upper surface increased from 15μm to 35μm, but from 10μm to ca. 30μm on the lower surface.

Schönherr and Bukovac (1978) also found that stomatal frequency in the French bean was three times greater on the lower than the upper surface and was inversely proportional to leaf size in young expanding leaves. Thus fully expanded leaves from 10 day old plants had approximately 100 stomata mm^{-2} on the upper surface.

The flush type of shoot growth in cocoa, in which all the leaves of a new flush are produced rapidly and expand at approximately the same time, must represent a sudden and massive increase in area of potential transpiration loss. However, there are a number of characteristics of developing cocoa leaves which may contribute to reduce the potential for high transpiration during leaf expansion when water is required for rapid leaf expansion and hence likely to increase the possibility of a water deficit occurring in the plant. For instance, although the absence of a cuticle on the very young leaves facilitates rapid water loss, the cuticle is synthesized during expansion so that the resistance rapidly increases to a very high value. Thus even during expansion when the young leaf is thin and flaccid in appearance and appears not to have a cuticle, the cuticular resistance is nevertheless high and in comparison to the potential water loss via the stomata represents a very small component of the total water loss. Another characteristic of the young leaf which might well help to limit water loss is the vertical position of the leaves during the expansion stages. This means that the leaves are going to be less exposed to direct light in comparison with the horizontally positioned mature leaves and therefore less likely to develop a high leaf temperature with its stimulatory effect on transpiration. The red anthocyanin pigment in the leaf hairs and epidermal cells may also serve the same purpose since it will tend to reflect those wavelengths which cause the greatest temperature increase, and the hairs will increase the depth of the boundary layer.

The transpiration rate of the leaf during development will therefore be a balance between the potential for water loss of a large surface area lacking a significant cuticle and one protected by various physical parameters such as leaf position, pigmentation, leaf hairs and a restricted stomatal development. It is suggested that the structural characteristics of the young leaf at F-1 and early F-2 will tend to limit the water loss from the rapidly expanding leaves. Maximum water loss will then only occur after late F-2 when the leaves are horizontally positioned and the stomata fully developed. The particular characteristics of leaf development revealed here may thus indicate an evolutionary response of cocoa to conserve water during the F-2 phase when water is required for rapid leaf expansion. If root growth continues during the period of leaf expansion of one flush then again the low rate of increase in demand for water over a long period will mean a chance for root growth to maintain at least a reasonably favourable shoot:root ratio.

It is significant that the large increase in potential water loss during I-1 and I-2 coincides with the period of highest ABA levels in both the NF and PF leaves as shown by Orchard *et al.* (1980). It is thus possible that the large transpiration loss after the late F-2 stage of the flush cycle might still cause an internal water deficit in the young seedling plant and therefore a build up of ABA in the shoot. Investigation of any correlation between the leaf/plant water status and ABA levels of the shoot thus forms the subject for the next chapter.

CHAPTER 4

Plant-Water Status and Abscisic Acid Levels in Cocoa Seedlings

4.1 Introduction

Field-grown cocoa plants usually experience an extended period of water deficit during the dry season which is now considered to be one of the major factors initiating flush growth in this species (Alvim et al.,1964). The sequence of events suggested is that as a result of the water deficit, there is a build up of ABA in the cocoa leaves and finally in the bud, which is maintained in a state of dormancy by the accumulated ABA. High ABA levels in the leaves also accelerate leaf senescence and abscission. The reduction in transpiration surface caused by the leaf fall eventually relieves the water deficit and effects a decline in the ABA level in the plant. The decline in ABA and corresponding increase in growth promoting cytokinin eventually releases the bud from dormancy (Alvim et al., 1974). This sequence of bud break, leaf production and bud dormancy is repeated a number of times during the wet season in field grown plants. It is also shown by seedling plants grown under well-irrigated conditions (Orchard et al., 1979, 1980, 1981). An examination of the seedlings showed leaf ABA to increase during the stages of the cycle when the bud was dormant then decline around bud break (Orchard et al., 1980). There is also a rise in cytokinin level prior to bud break so that further growth of the bud may be due in part to the changed balance between inhibitor and promoter rather than just a decrease in inhibitor level (Orchard et al..1981). Since there is a close correlation between high ABA levels and water deficit in the field-grown cocoa (Alvim et al., 1974) and in other species (Clemens and Jones, 1978, Wright and Hiron, 1969, Wright, 1972,

Loveys and Krideman, 1973; Beardsell and Cohen, 1974 & 1975; Zabadal, 1974; Wright, 1977), it may be that the fluctuations in the levels of ABA in the cocoa leaves of seedlings are an indication of cyclic changes in seedling water status during the flush cycle. It must however be emphasised that in seedlings, such as those used by Orchard and in this investigation where plants were maintained at (water) field-capacity any such changes in internal water deficits, and ABA level, occur despite optimal irrigation. To evaluate the role of water status in the control of the flush cycle in seedling cocoa, the following experiments were performed.

1. Assessment of the water status of the new flush leaves of well-watered seedlings through two flush growth cycles by measuring leaf water potential, relative water content and water uptake (in the latter case from plants grown in nutrient solution culture). Sampling began with leaves of a new flush (NF) and continued with this flush throughout the following two flush cycles. At F-1 (36 day) these leaves were then PF_1 leaves and at the second F-1 (day 74) became PF_2 leaves. This sampling procedure was used because these particular leaves were near to the shoot apex and will be more likely to strongly interact with it.

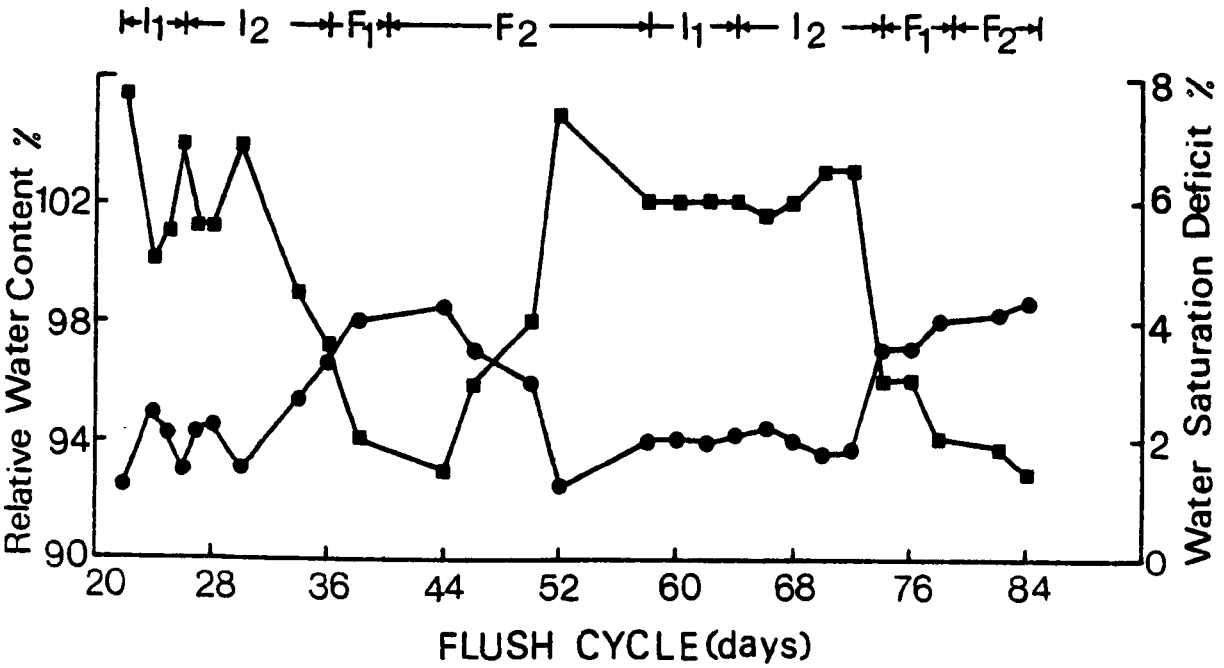
2. Measurements were made of the influence of water stress on the growth flush pattern in cocoa seedlings. Seedlings were subjected to water stress by droughting; watering was stopped for a period, then the different physiological changes due to water stress were compared with control well-watered plants. The droughted plants were

rewatered after 16 days. To demonstrate the effect of moisture stress on the water status of the plant, the RWC and ψ_{leaf} which will reflect any change in the water content of the seedling was followed from NF leaves. Since the ABA may build up in the leaves in the same way as in other species in the presence of soil moisture deficits the ABA levels were measured for both NF and PF_1 leaves of the droughted plants. The sensitivity of the stomata in cocoa leaves under drought conditions as shown by the stomatal diffusion resistance was also measured in the NF leaves. Finally, the effect of drought on the growth of the whole seedling was followed by noting the change in the bud activity and the leaf growth throughout the course of drought. Plants were of the same age as those used previously and the methods are described in chapter 2.

4.2 Relative Water Content (RWC) of the NF leaves throughout two flush cycles

Measurements were made in triplicate on each of three plants over two flush cycles. In experiments on the effect of droughting five control and five droughted plants were used. The values for RWC are shown in Fig. 14. The well-watered plants showed the lowest values of RWC during I-1 and the beginning of I-2, then there was an increase in RWC during late I-2 stage, which continued during F-1 to a value of 98%. This high level was maintained during most of the F-2 stage, then the RWC declined to a low value, 94% by the end of F-2 and remained low through the second I-1 and I-2 stages, and then again increased during F-1 and the early period of F-2 to 98%.

Fig. 14. The change in relative water content (●) or water saturation deficit (■) of the flush leaves during a flush cycle.


I1 I2 F1 F2 I1 I2 F1 F2
Relative Water Content %
102
98
94
90
Water Saturation Deficit %
8
6
4
2
0
20
28
36
44
52
60
68
76
84
FLUSH CYCLE (days)

The RWC shows a minimum during the I-1 and early I-2 stages and a maximum in the final part of I-2, F-1 and the first part of F-2. Although only one particular leaf-flush was sampled, the pattern of change in RWC with stage of cycle was very similar whether this flush represented NF, PF_1 or PF_2 leaves. This means that the pattern seen is probably occurring throughout the plant.

In an attempt to see whether this pattern could be upset by an imposed water stress, the RWC was measured from NF leaves of plants subjected to drought conditions. The RWC of the NF leaves of control plants showed the same changes as those described above. However, in the plants deprived of water the RWC of the NF leaves declined rapidly to 80% when wilting occurred. After watering, there was a very rapid return to a high water content (Fig. 15).

4.3 Water Potential (ψ_{leaf}) of the NF leaves throughout two flush cycles

The values for water potential are shown in Fig. 16. The well-watered plants showed the highest values (least negative) at the time of rapid expansion of the NF leaves, i.e. during the F-2 stage, when the measured value was -7.5 bars. This declined at the end of F-2 to ca.-10 bars, then there was a further decline in the value of ψ_{leaf} throughout I-1 until it was -16 bars. This value of ψ_{leaf} remained constant during the early part of the I-2 stage, but showed a rapid increase during mid I-2 and continued to increase to reach a high value (-8.5 bars) at the start of the next flush. The pattern of change in the ψ_{leaf} was very similar throughout the rest of the sampling period as the leaves became PF_1 then PF_2, suggesting that the pattern of change in ψ_{leaf} in leaves throughout a cycle is likely to be similar for all of the leaves of the seedlings. Thus the ψ_{leaf} shows a maximum during F-1, F-2 and late I-2 stages and a minimum at late F-2, I-1 and the beginning of I-2 stages.

Fig. 15. The change in relative water content of the new flush leaves of control well-watered plants (■) or droughted plants (●) at different times of the flush cycle. The arrow points to the time of watering.

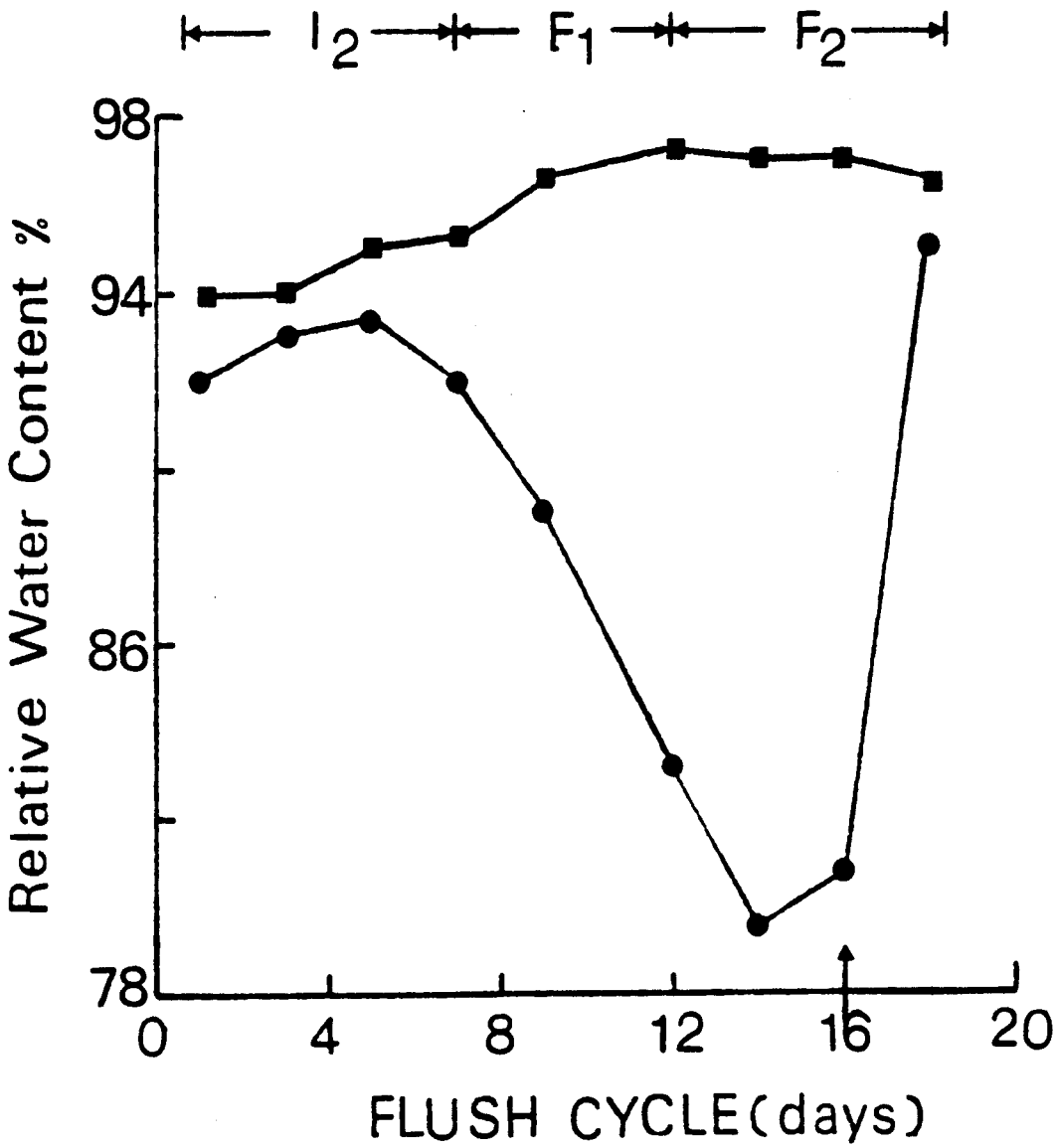
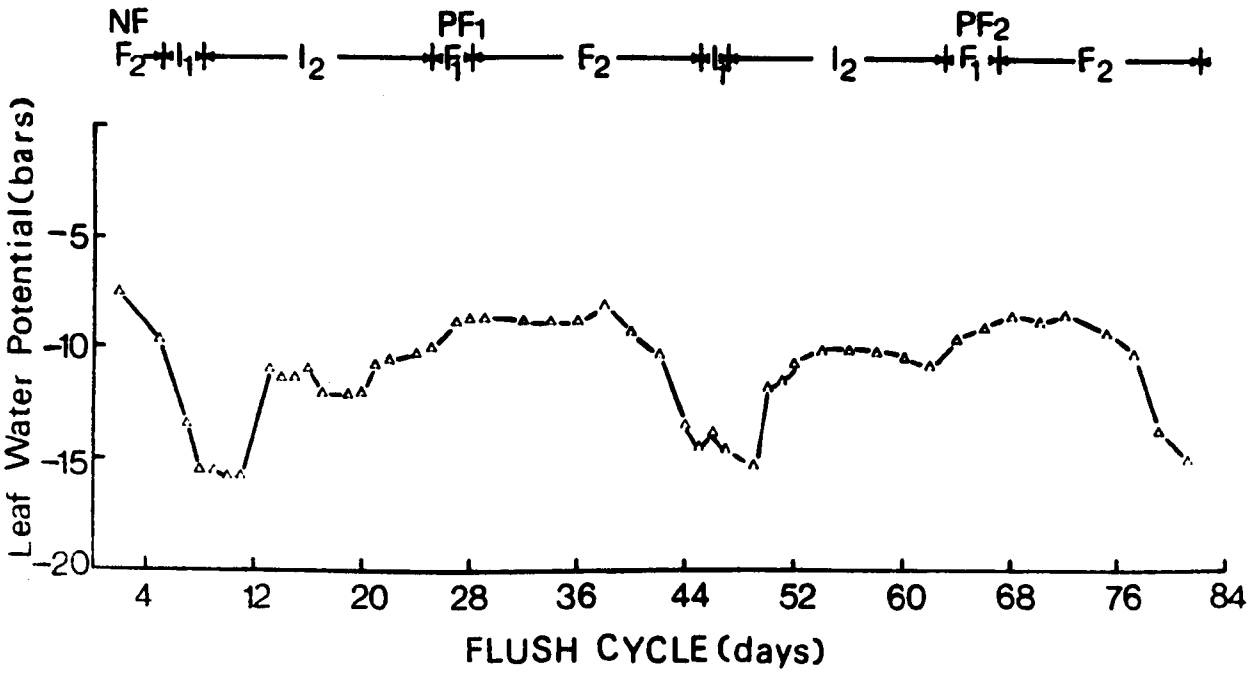

I2
F1
F2
98
94
86
78
Relative Water Content %
0
4
8
12
16
20
FLUSH CYCLE(days)

Fig. 16. The change in water potential of the flush leaves during a flush cycle.


NF
PF1
PF2
F2 I1 I2 F1 F2 I1 I2 F1 F2
Leaf Water Potential(bars)
-5
-10
-15
-20
4
12
20
28
36
44
52
60
68
76
84
FLUSH CYCLE (days)

Water potential was also measured for NF leaves of plants subjected to drought conditions. Measurements of ψ_{leaf} of the NF leaves of control plants showed the same changes as those described previously for well-watered plants. However the pattern of change in ψ_{leaf} with water stressed plants was different. Initially the ψ_{leaf} showed a gradual decline but then the decline was more rapid during the drought period showing a change from -14 bars to -20.88 bars. Water stress thus leads to a change in the pattern of ψ_{leaf} in the cocoa leaf compared to the well-watered plants. However, after watering the droughted plants the ψ_{leaf} increased from -20.88 to -18.32 after two days, and recovered fully 4 days after rewatering to give the same ψ_{leaf} as the well-watered plants (-10.85 bars) see Fig. 17.

4.4 Water uptake of the seedlings throughout a flush cycle

The water uptake from three replicate plants is shown in Fig. 18 The graph showed a fluctuation pattern in which a number of trends can be seen. In the first flush cycle the end of the F-2 stage was characterised by an increase in water uptake from 12 cm^3 day^{-1} until a new level of uptake was established by I-1 of approximately 30cm^3 day^{-1}. The uptake fluctuated during the I-2 stage of the flush cycle . These fluctuations may have been due to environmental variations affecting transpiration rate. As the NF leaves became PF leaves and a new flush cycle began the water uptake gradually increased to 40 cm^3 day^{-1} then at the end of F-2 and in I-1 showed a sharp rise to establish a new level of approximately 70cm^3 day^{-1}.

4.5 Abaxial (lower surface) diffusive resistance of the NF leaves

The responsiveness of the stomata of cocoa seedlings to changes in leaf RWC and ψ_{leaf} was measured in well-watered and those plants which

Fig. 17. The change in water potential of the new flush leaves of control well-watered plants (■) and droughted plants (▲) at different times of the flush cycle. The arrow points to the time of watering.

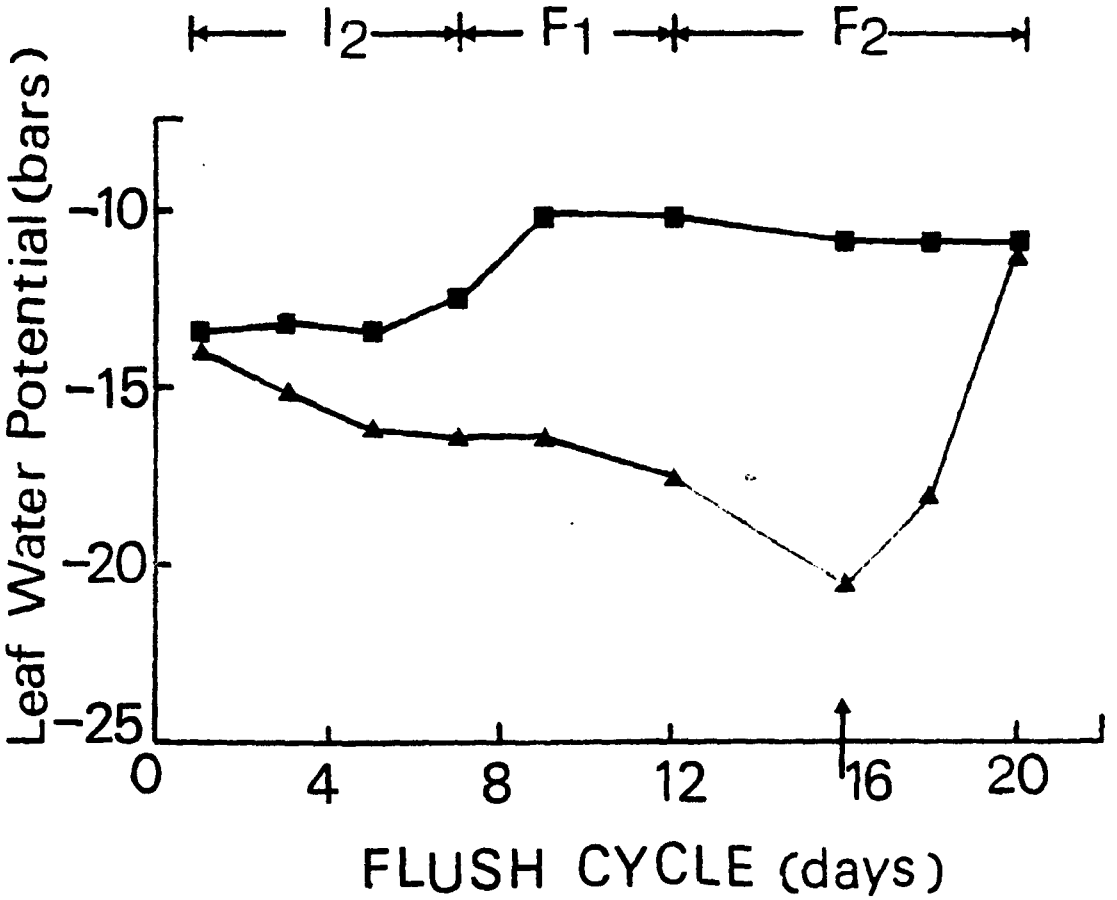
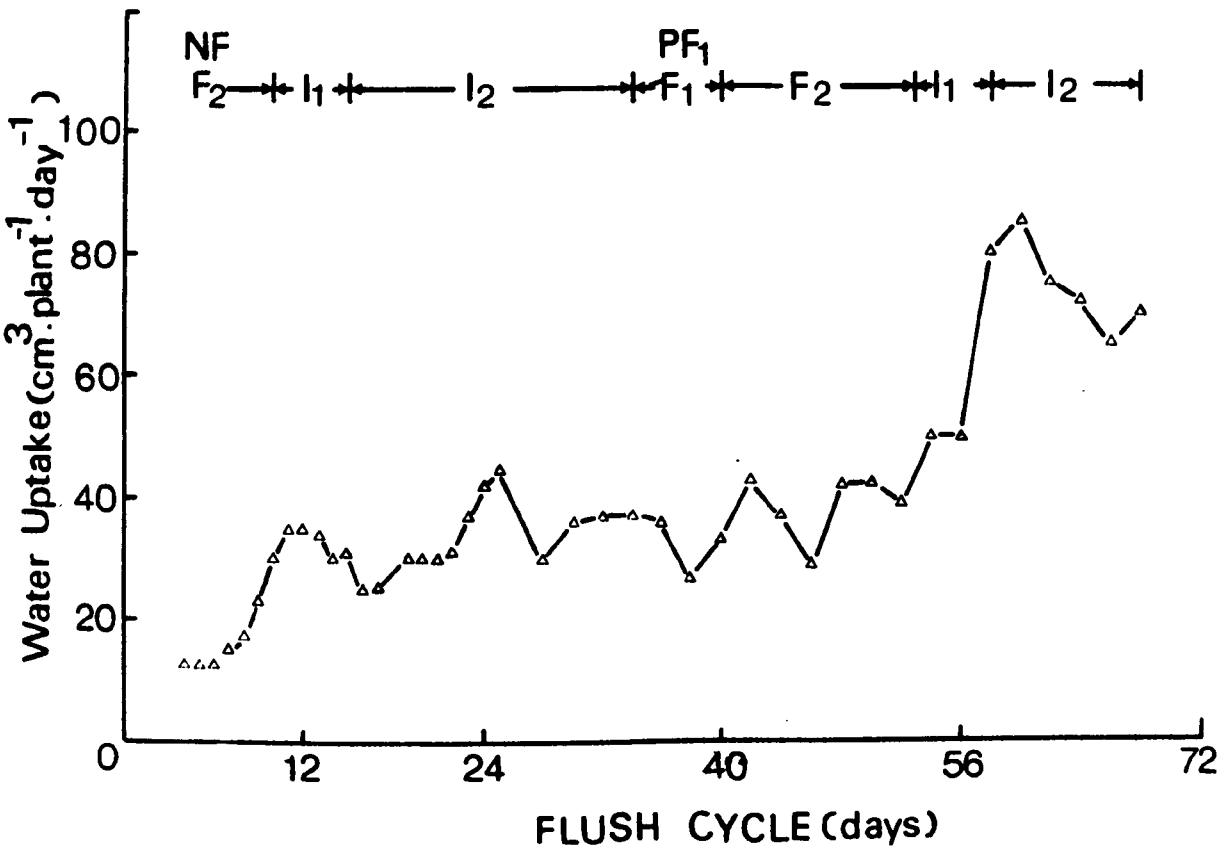

I2
F1
F2
Leaf Water Potential (bars)
-10
-15
-20
-25
0
4
8
12
16
20
FLUSH CYCLE (days)

Fig. 18. The rate of water uptake by cocoa seedlings over the course of two flush cycles.


NF
PF1
F2
I1
I2
F1
F2
I1
I2
Water Uptake(cm3.plant-1.day-1)
0
20
40
60
80
100
12
24
40
56
72
FLUSH CYCLE (days)

were not watered for 16 days. Measurements of stomatal diffusive resistance were made on NF leaves of five replicate plants during the drought period and also for a period after rewatering of the plants (Fig. 19). In well-watered control plants the diffusive resistance was 8 sec. cm^{-1}. during the I-2 stage but declined gradually to 6 sec. cm^{-1} by the end of the I-2, and declined further at F-1 and F-2 (2.15 - 4.54 $sec.cm^{-1}$).

In the stressed plants, however, a gradual increase in diffusive resistance ($\overset{*}{R}$) was shown during the first five days, i.e. 8 to 26.97 $sec.cm^{-1}$, then $\overset{*}{R}$ showed a constant value for the next four days followed by a further increase. Thus at the twelfth day $\overset{*}{R}$ was 54.52 sec. cm^{-1} (ca. 18 times greater than the control). A further increase in $\overset{*}{R}$ was shown by the fourteenth day (87.86 $sec.cm^{-1}$) then the value of $\overset{*}{R}$ remained constant to day sixteen at which time the plants were wilting.

After watering the stressed plants, there was a rapid reduction in $\overset{*}{R}$. The value declined within 1 hr from 87.63 $sec.cm^{-1}$ to 61.83 $sec.cm^{-1}$ and after 2hr $\overset{*}{R}$ had further decreased to 45.08 $sec.cm^{-1}$. After 1 day $\overset{*}{R}$ was 12.56 $sec.cm^{-1}$ and after two days 8.25 $sec.cm^{-1}$. Diffusive resistance continued to decline until the end of the experiment when $\overset{*}{R}$ reached a value similar to well-watered control plants.

The relationship between RWC and ψ_{leaf} was linear so that any decline in RWC of the leaf was associated with a decline in ψ_{leaf} (Fig.20). The changes in the stomatal diffusive resistance were also linearly correlated with the changes in the leaf RWC and ψ_{leaf} (Figs.21 & 22).

Fig. 19. The change in stomatal diffusive resistance of the new flush leaves of control well-watered plants (▲) or droughted plants (■) at different times of the flush cycle. The arrow points to the time of watering.

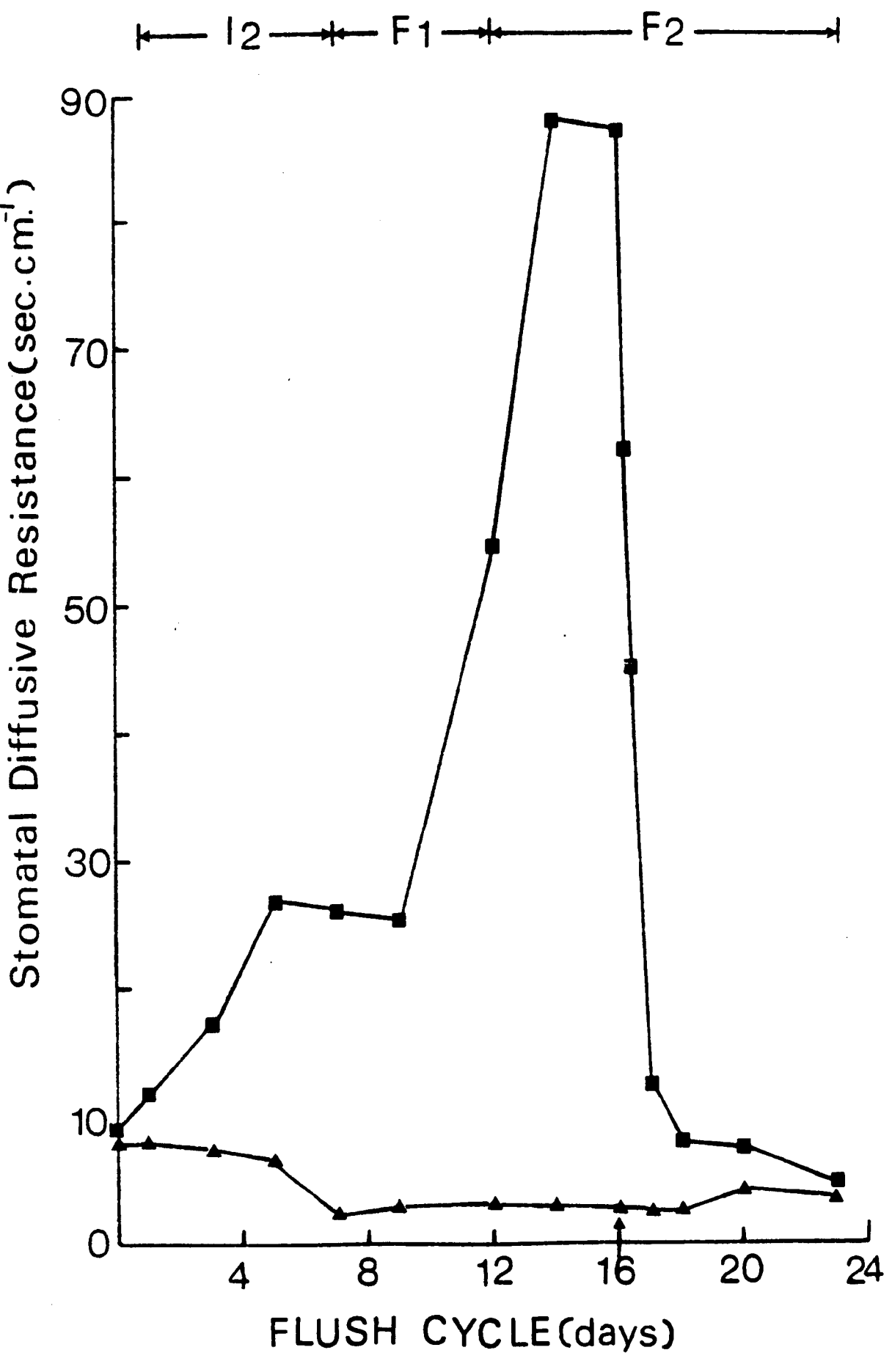

I2
F1
F2
90
70
50
30
10
0
4
8
12
16
20
24
Stomatal Diffusive Resistance(sec.cm.-1)
FLUSH CYCLE(days)

Fig. 20. The relationship between RWC and ψ_{leaf} for the new flush leaves.

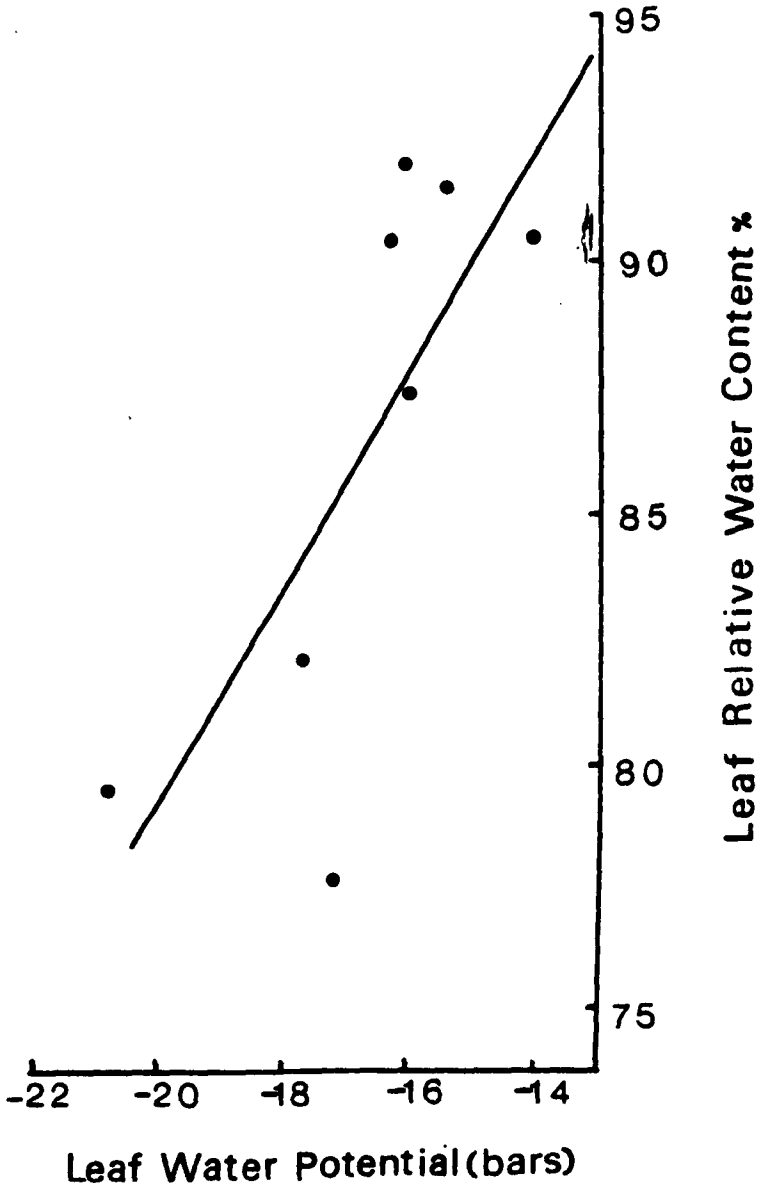

95
90
85
80
75
Leaf Relative Water Content %
-22
-20
-18
-16
-14
Leaf Water Potential (bars)

Fig. 21. The relationship between the stomatal diffusion resistance and RWC.

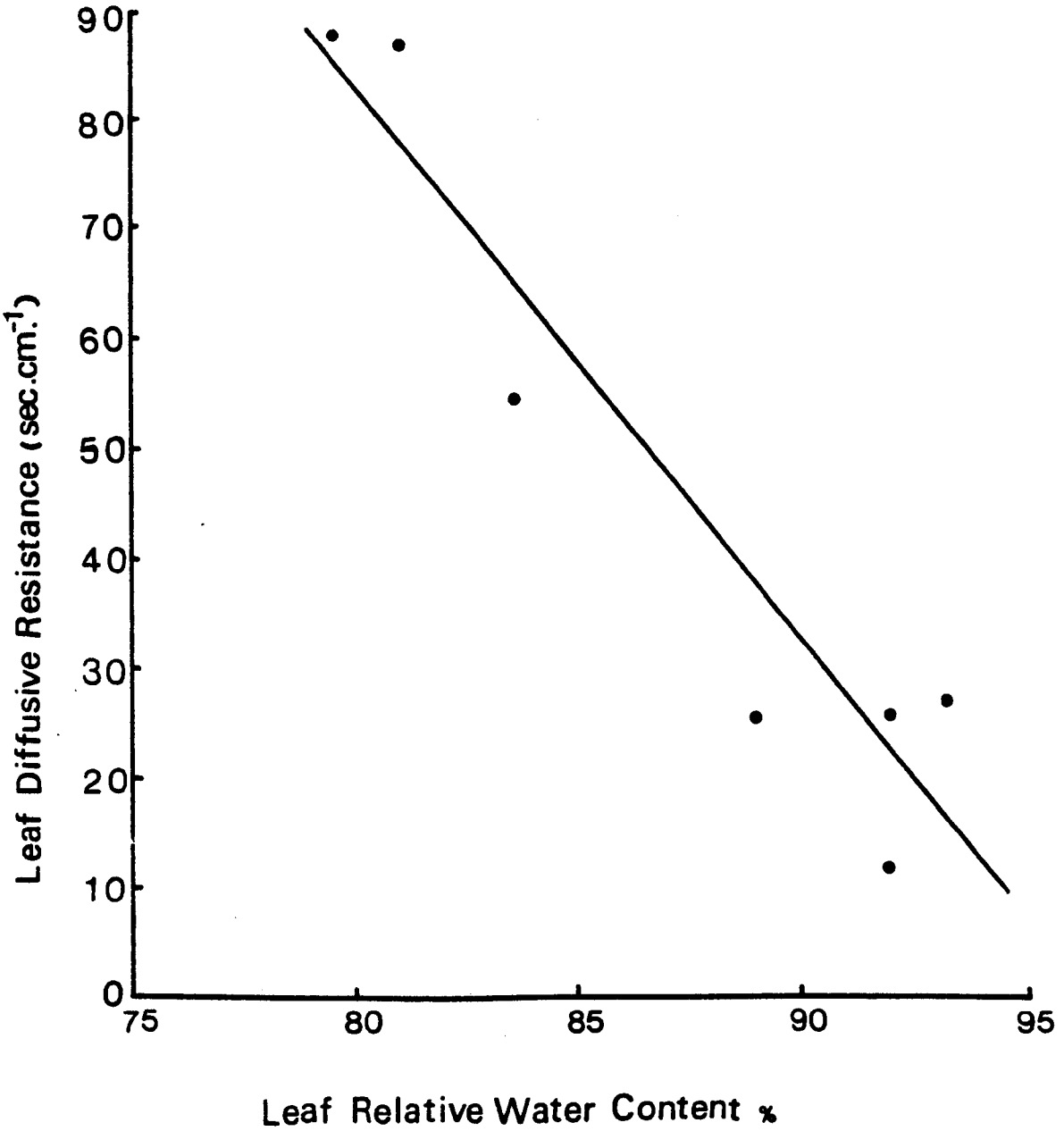
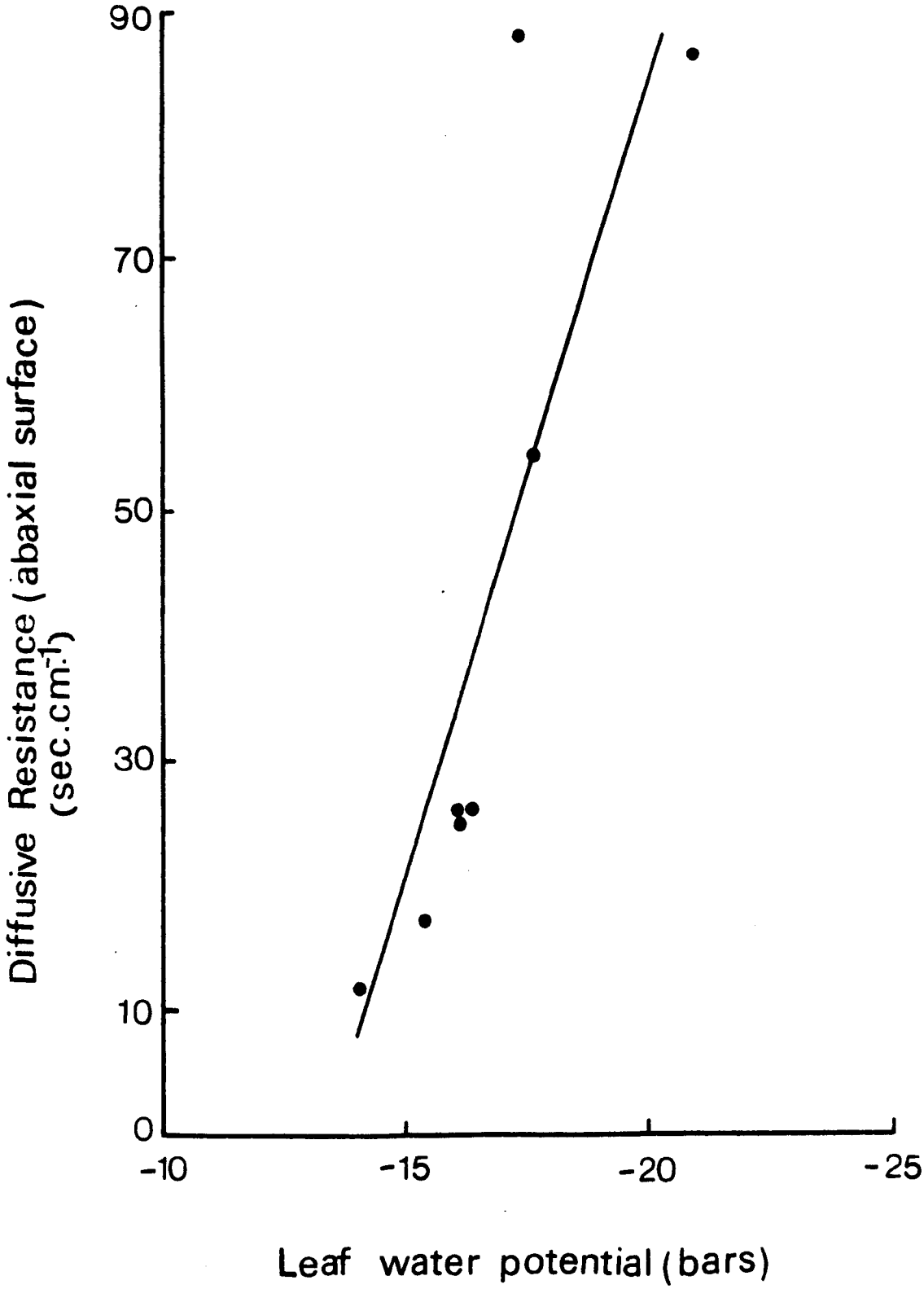

90
80
70
60
50
40
30
20
10
0
75
80
85
90
95
Leaf Diffusive Resistance (sec.cm.-1)
Leaf Relative Water Content %

Fig. 22. The relationship between the stomatal diffusive resistance and the ψ_{leaf}.


90
70
50
30
10
0
-10
-15
-20
-25
Diffusive Resistance (abaxial surface)
(sec.cm-1)
Leaf water potential (bars)

4.6 Bud activity and leaf growth

To assess whether the conditions of water stress achieved in the droughting experiment had any effects on the growth of the cocoa seedlings, the timing of bud burst, the number of new flush leaves and the rate of growth of leaves were measured in comparison with control plants.

In the well-watered plants the bud burst (F-1) occurred seven days after the start of the experiment and lasted for a period of five days before the new leaves commenced expansion (F-2). In contrast bud burst in the water-stress plants did not occur until 10 days after the start of the experiment and bud burst (F-1) lasted 6 days (see Fig. 23).

The expansion phase (F-2) in well-watered plants occupied a period of 15 days until the first leaf was fully expanded. The total leaf area of the flush leaves was 1140cm^2 and the number of the NF leaves an average of five leaves per plant. In droughted plants, the expansion phase (F-2) only commenced after rewatering, but the number of NF leaves produced was reduced to an average of three leaves per plant. The rate of leaf expansion during F-2 was very slow which caused an extension of the F-2 phase to twenty-two days. The mean total leaf area of these NF leaves was only 520cm^2 per plant (see Fig. 23).

4.7 Abscisic acid concentration

The changes in ABA levels were investigated in NF and PF leaves of both control and water stressed plants (Figs. 24 & 25). In well-watered plants the NF and PF leaves both contained less free ABA than bound ABA. The levels of both free ABA and bound ABA were high during the I-2 stage, then declined gradually during the F-1 and F-2 stages, although both NF and PF leaves showed larger amounts of bound than free ABA. In water-atressed plants the pattern of change in both bound and free ABA

Fig. 23. The effects of drought on the time of bud break, leaf area and number of leaves from droughted (a) or control well-watered plants (b). The arrow (↑) points to the time of watering.

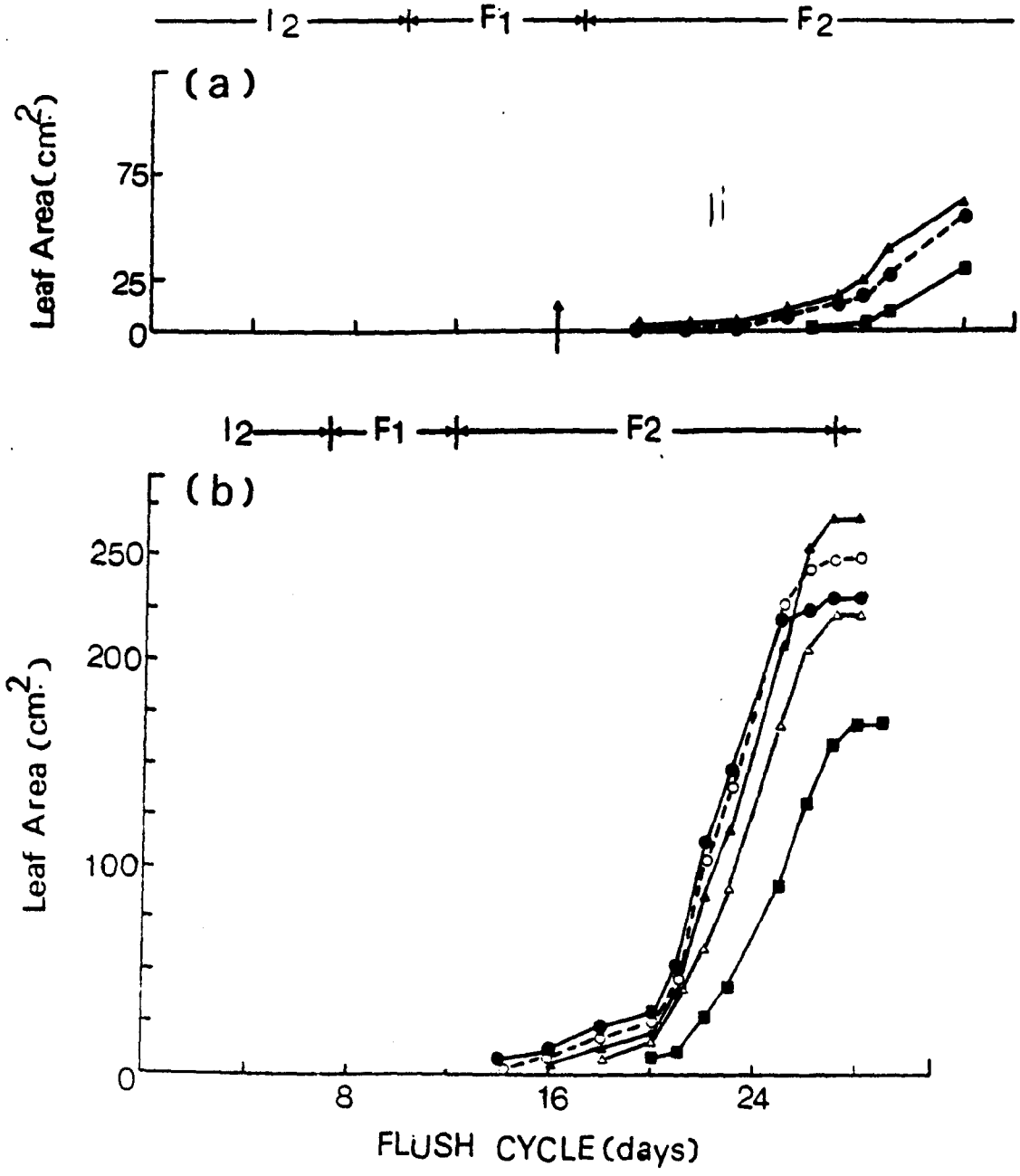

I2
F1
F2
(a)
Leaf Area(cm.2)
75
25
0
I2
F1
F2
(b)
Leaf Area (cm.2)
250
200
100
0
8
16
24
FLUSH CYCLE (days)

Fig. 24. The change in abscisic acid levels in new flush leaves during a flush cycle. The arrow (↑) points to the time of watering.

(a) ▼—▼ Free ABA in control plants
(b) △—△ Bound ABA in control plants
(c) ■—■ Free ABA in droughted plants
(d) ▲—▲ Bound ABA in droughted plants

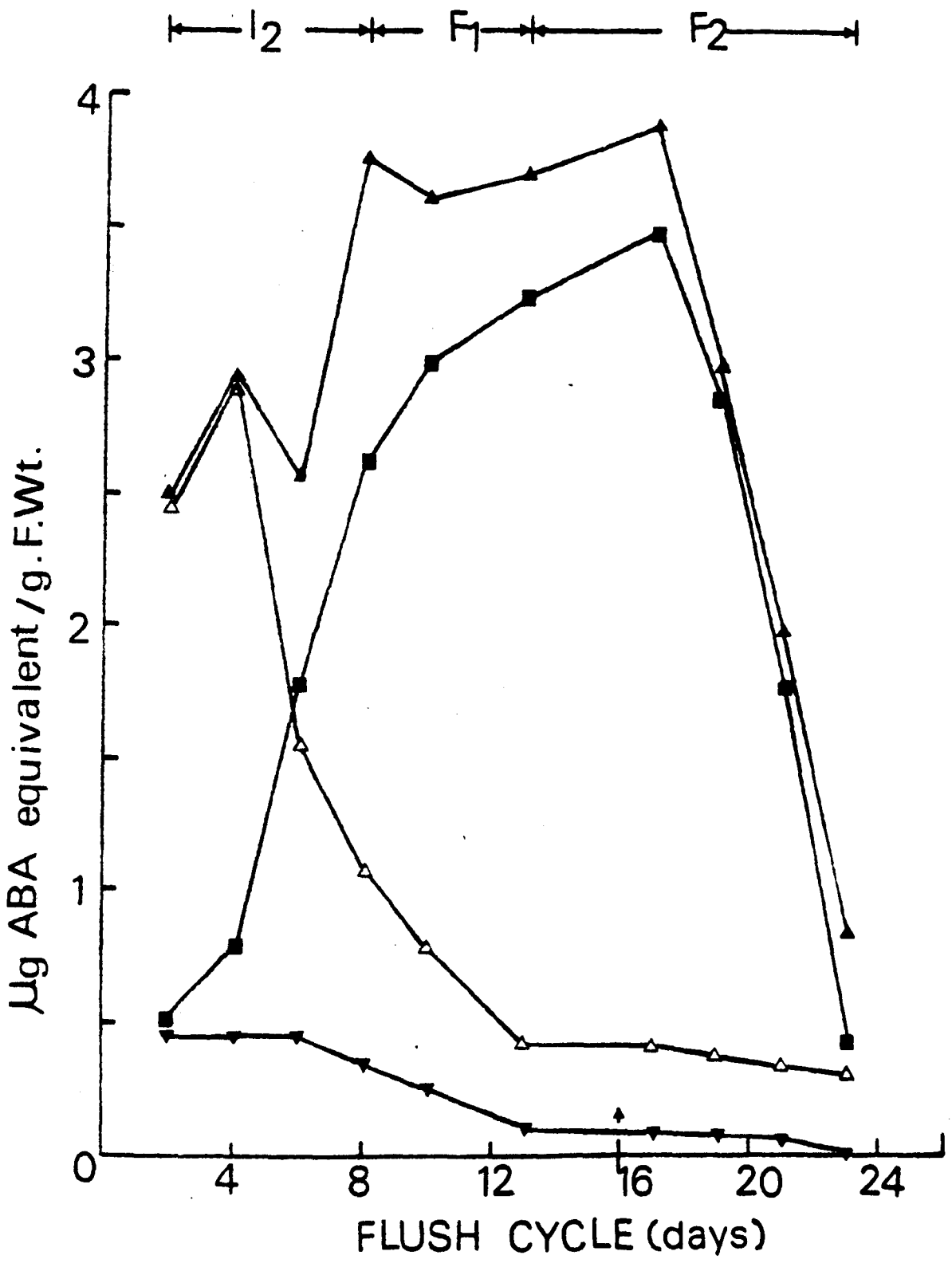
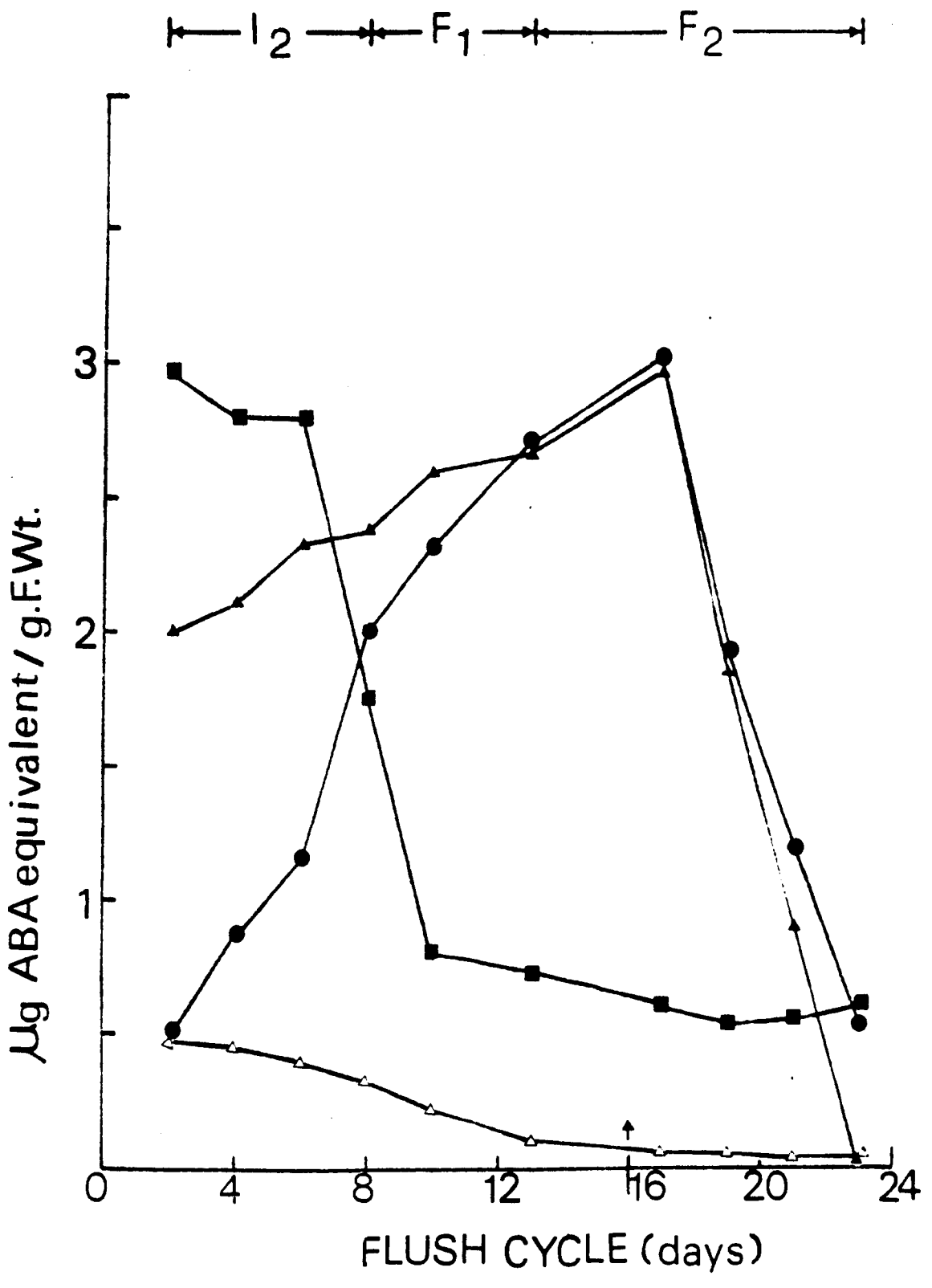

I2
F1
F2
4
3
2
1
0
Jug ABA equivalent/g.F.Wt.
4
8
12
16
20
24
FLUSH CYCLE (days)

Fig. 25. The change in abscisic acid levels of (PF) previous flush leaves during a flush cycle: the arrow (↑) points to the time of watering.

(a) △—△ Free ABA in control plants
(b) ■—■ Bound ABA in control plants
(c) ●—● Free ABA in droughted plants
(d) ▲—▲ Bound ABA in droughted plants


I2
F1
F2
µg ABA equivalent / g.F.Wt.
0
1
2
3
0
4
8
12
16
20
24
FLUSH CYCLE (days)

in both NF and PF leaves was different from that in well-watered plants. The NF leaves showed a large increase in levels of both free and bound ABA during the first seven days but thereafter a more gradual increase occurred. This gave a final increase in free ABA of 32.5 times the control plants. The same pattern of change in the bound ABA was detected. The levels of ABA in the PF leaves followed a similar pattern to the NF leaves except that bound ABA changed very little during the stress period, but still the changes in both forms of ABA in these PF leaves showed that the differences were statistically significant when compared with well-watered plants. A major feature of the amounts in ABA levels, is the larger changes of ABA in NF compared with PF leaves of stressed plants.

After watering the droughted plants there was a rapid decline in the levels of free and bound ABA in both the NF and PF leaves. Within six days of watering the free ABA had reached the original levels of the leaves at the start of the experiment.

4.8 Discussion

As new leaves are produced in the flush cycle the total leaf area of the shoot will increase. Thus the leaf area will show a rapid increase during the F-2 stage then remain constant until the F-2 of the next flush cycle. The transpiration loss will increase with each increase in leaf area which will be reflected in a change of water uptake. However, the changes in water uptake and leaf area do not seem to be directly correlated. The maximum increase in leaf area occurs during the F-2 stage yet during most of this stage water uptake shows only a gradual increase. It is at the end of the F-2 stages and during the I-1 stages that the huge increase in water uptake occurs. This pattern of change in water uptake does in fact correlate with the change in stomatal

development and other leaf surface characteristics. Thus leaves during early and mid F-2 show an incomplete stomatal development and a high diffusion resistance so that the transpiration loss from these leaves will be low. At late F-2 and I-1 stages stomatal development is complete and the diffusion resistance is low. This means that the potential transpiration rate will be high and hence the rapid increase in water uptake occurs.

It was suggested earlier that in seedling plants ABA accumulated in the fully expanded NF leaves at I-1 and early I-2 stages and in the mature PF leaves at the I-1 stage, is exported to the bud and inhibits its further development (Orchard et al. 1980). The problem in attempting to understand the endogenous control of the flush cycle in the seedling plants is to understand why there should be a build up of ABA in plants receiving an adequate water supply. It has already been shown that plants under these optimal conditions showed a pronounced decline in RWC and water potential during late F-2, I-1 and early I-2 stages which coincides with the stages of the flush cycle for which Orchard et al (1980) showed contained the highest levels of free and bound ABA. The effect of increasing the water deficit during I-2 by droughting the plants was to increase the synthesis and accumulation of ABA, which confirmed that the ABA level in the seedling was closely correlated with the internal water deficit. In the droughted plants the delay in bud break was also related to the continued high level of ABA in the leaves. However, despite very high levels of ABA, the bud break was only delayed in the droughted plants which strongly suggests that ABA was involved in maintaining bud dormancy, but also that bud break is influenced by other factors, such as may be an increase in growth promoters.

After bud break the inhibition of leaf expansion in the droughted plants could be due to the presence of either high ABA levels or to a direct effect of the water deficit on cell turgor and cell expansion. The major changes in ABA which are shown here in cocoa plants under drought conditions are similar to most of the changes shown by other workers either in intact plants or excised leaves (Wright and Hiron, 1969; Wright, 1972 & 1977; Loveys and Kriedemann, 1973; Beardsell and Cohen, 1974 & 1975; Zabadal, 1974).

The drought conditions which induced a water deficit in cocoa plants as shown in Figs. 15 and 17 are similar to the conditions imposed on Euphorbia lathyrus (Sivakaumaran & Hall, 1978), lettuce leaves (Aharoni et al. 1977) and on cotton leaves (Cutler and Rains, 1978), in all those examples the drought affected the water status of the leaves leading to a water deficit in the plants.

The water deficit resulting from the water stress caused both delay in the bud growth of the droughted plants and in leaf enlargement. Only after watering did growth occur (Fig. 23). Boyer (1970) found that leaf enlargement in corn, soybean and sunflower was only 25% of the observed maximum at -4 bars, and at -12 bars the leaf enlargement dropped to zero. In cocoa, leaf expansion started at -10.5 bars. The moisture stress in the cocoa affected leaf size, number of leaves and leaf senescence, which is comparable to the effects of water stress on the growth of soybeans (Sivakumar and Shaw, 1978), Panicum maximum v. trichoglume (Ludlow and Ng, 1976) and cotton (Jordan, 1970; Jordan and Ritchie, 1971). The relationship between RWC and ψ_{leaf} in this investigation shows a linear correlation, which is

in agreement with the relationship between RWC and Ψ_{leaf} studied in Agropyron sp. (Maxwell and Redman,1978), and in wheat (Morgan,1977 and Pospisilova,1973). The changes in stomatal diffusive resistance of cocoa seedlings under water stress conditions were also shown to be lineary correlated with the associated changes in RWC& Ψ_{leaf}. This relationship has been found in many different plants (Meidner & Mansfield, 1968; Raschke & Kuhl, 1969; Kanemasu & Tanner, 1969; Mizrahi et al., 1970; Kassam, 1973; Aharoni et al., 1977; Davies, 1977 and 1978; Dörffling et al., 1977; Morgan,1977; Pallardy & Kozlowski, 1979).

This study has shown that even in well-watered plants, the large and suddenly increased transpriation loss following new leaf expansion causes a water deficit which in turn leads to the build up of ABA in the leaves and the enforcement of apical bud dormancy. There are some similarities in behaviour between field grown and seedling plants since in both plants bud dormancy is correlated with internal water deficits which in turn are likely to promote ABA synthesis and accumulation. Although bud break in both types of plants occurs in the presence of high ABA levels, leaf unfolding and expansion will only occur when there is adequate water supply. Since there are these similarities in the effect of water deficit on the response of both seedling and adult plant, the seedling provides a good system for studying the physiology of water relations of the cocoa in more detail.

Since the leaves seem to be very sensitive to any change in the water status of the plant and hence show some differences in the degree of ABA levels. It seems important to study the movement of ABA around the plant and any interaction between the leaves and the apex, this will be the subject of the next chapter.

CHAPTER 5

The work presented in this chapter has already been published in "The New Phytologist". The format of the chapter is essentially that of the paper.

Hormonal Interaction between mature leaves and the shoot apex

5.1 Introduction

The growth of the shoot in cocoa is characterised by alternating periods of growth and dormancy, referred to as the flush cycle (Greenwood and Posnette, 1950). Normally in the field the cycle is triggered off by environmental changes (Sale, 1968), but the cycle will continue under constant conditions (Greathouse, Laetsch and Phinney, 1971; Orchard, Collin and Hardwick, 1980) which indicates that under these circumstances there is some form of endogenous control. Analyses of the new flush (NF) and previous flush (PF) leaves of plants throughout a flush cycle when under both controlled conditions (Orchard, Collin and Hardwick, 1980) and conditions of water stress (Alvim, Alvim, Lorenzi and Saunders, 1974) show the leaves to contain a high level of ABA when the apex is in the dormant phase of the cycle. The correlation between the enhanced level of ABA in the leaves and growth inhibition at the apex suggests that the accumulated ABA in the leaves may be exported to the bud and younger leaves and there either initiates the dormant phase of the cycle, or alternatively, helps maintain the shoot in a dormant state.

Eagles and Wareing (1963), El-Antably, Wareing and Hillman (1967), Hocking and Hillman (1975) have shown that in some temperate perennial species the leaf can act as a source of ABA for the apex.

In their experimental approach the supply of ABA from the leaves to the apex was altered by a combination of leaf defoliation and the application of ABA to the leaf surface, then the effect was measured by a change in growth of the apex. More direct evidence for the export of ABA from the leaf to the apex was obtained by application of ^{14}C-ABA to the leaf then determining the distribution of radioactivity (Hocking, Hillman and Wilkins, 1972). In order to investigate whether cocoa leaves have an inhibitory role as a source of ABA at specific times during the flush cycle a similar approach involving leaf defoliation, hormone application and determination of distribution patterns of ^{14}C-ABA was undertaken with young cocoa plants.

5.2 Materials and Methods

Production of plant material and general methods used in this section of work have already been described in Chapter 2.

When radioactive ABA was applied it was always introduced to either the first produced leaf of a new flush or of the first previous flush.

5.2.1 Leaf defoliation

Leaves belonging to the new (NF) or previous (PF) flush were removed from the plant at a specific stage of the flush cycle. Thus NF leaves were removed from separate batches of plants at F-2; the stage of rapid leaf expansion and low ABA levels in these leaves, and I-2 when the expansion is complete and the leaf

ABA levels high (Orchard et al. 1980). The PF leaves, either those of the first previous flush or all of the previous flush leaves were removed from the plant at I-1 when the endogenous ABA in the PF leaves was known to be high. Leaves were removed at these times so as to compare the effects of removing potential low and high ABA sources on the growth of the apex. For all treatments, the effects of defoliation were determined by noting days to breaking of bud dormancy relative to control plants.

5.3 Metabolism of ^{14}C-ABA

Before deriving any information from data relating to ^{14}C distribution in the various parts of treated plants, it was necessary to determine whether the radioactivity was transported and accumulated as ^{14}C-ABA rather than being metabolised to compounds unrelated to ABA. To establish this the ethanol extract from a recipient NF leaf, when a PF leaf at the I-1 stage of the flush cycle was the donor leaf, was extracted and separated according to the method of Goldschmidt, Goren, Even-Chen and Bittner (1973). It was found that around 70% of the radioactivity in the acid fraction was in ABA and around 80% in the water soluble fraction was conjugated ABA which was probably the ABA-gluco pyranoside noted by Milborrow (1970). Thus the majority of the fed ^{14}C-ABA remained as ABA or was converted to the conjugate. The distribution of radioactivity therefore represents a movement and accumulation of predominantly ABA and not numerous radio-active metabolites.

5.4 Results and Discussion

5.4.1 Leaf defoliation and hormone application

The effects on subsequent apex activity of an alteration in the amount of ABA which would be exported from the leaves to the apex, caused by either defoliation or the application of hormones to the leaves, are shown in Tables 1 and 2 respectively. Removal of the NF leaves at F-2 when the endogenous ABA levels in the NF leaves are low, but the leaves expanding rapidly, reduced the time taken for bud regrowth from 29 to 13 days. Removal of the NF leaves at the later I-2 stage when the NF leaves are mature and contain higher levels of ABA, again caused a reduction in the period of dormancy, this time from 14 to 8 days. Previous flush leaves belonging to the first previous flush contain a high level of ABA at I-1 stage of the flush cycle (Orchard *et al.* 1980). Defoliation of these PF leaves at I-1 reduced the bud dormancy period from 22 to 15 days and when all the PF leaves on the plant were removed the period of bud regrowth changed from 18 to 12 days. The removal of the NF leaves at F-2 caused a relatively greater reduction in the time taken to break dormancy than when defoliation was at the later stage of the cycle, I-2, i.e. a reduction of 55% at F-2 compared with only a 39% at I-2.

It is clear that the leaves do have an inhibitory effect on the growth of the apex. The problem is whether the inhibitory effect is due to the presence of ABA in the leaves and transport of a proportion to the apical bud, to some other growth inhibitors or to other non-hormonal factors. At the F-2 stage in the cycle,

TABLE 1. The effect of defoliation of the NF and PF leaves at specific stages during the flush cycle on bud dormancy

Leaf stage	Leaf age (days)	Defoliated leaves	Days to bud burst control	Days to bud burst treated	% redn. in dormancy period
F-2	3 8	NF	29 ± 1.4	13 ± 1.4	55
I-2	26	NF	14 ± 1.0	8.5 ± 1.5	39
I-1	20	PF	22.3 ± 2.3	15.6 ± 2.6	30
I-1	20	all PF	18 ± 1.0	12.5 ± 1.5	40

TABLE 2. The effects on apical bud growth of applying ABA (1×10^{-4}M), GA_3 (3×10^{-3}M) or zeatin (1×10^{-4}M) to the NF or PF leaves at specific stages during the flush cycle. (Standard errors were calculated where replicates showed variation).

Growth regulator	Stage of application	Site of application	Time to bud burst (days)
ABA	I-2	none	9
		NF	15
		PF	13
GA_3	I-1	none	13.5 ± 0.35
	"	PF	8.5 ± 0.25
	"	NF	7.7 ± 0.24
	F-2	none	25
		PF	25
Zeatin	I-1	none	14.5 ± 0.35
	"	NF	8.5 ± 0.35
	"	PF	7.5 ± 0.35

the NF leaves are expanding rapidly and have a low chlorophyll content. At this stage the leaves are not able to photosynthesize to the capacity of the mature leaves and depend upon the import of photoassimilate to support growth (Baker and Hardwick, 1975). The NF leaves at F-2 represent therefore a powerful sink for nutrient which will be in competition with the shoot meristem. According to Orchard et al. (1980) the NF leaves at this stage contain only a low level of ABA and hence would not be expected to provide a significant supply of ABA to influence the dormancy of the apex. When the NF leaves were removed, however, the apical bud was stimulated to produce new leaves. It is suggested that this stimulus results from the removal of a powerful sink for nutrients rather than a source of ABA. At a later stage of leaf development, I-2, the NF leaves are fully expanded and show maximal photosynthetic ability and are able to export photoassimilate (Baker and Hardwick, 1975). However, removal of NF leaves at I-2 still causes a reduction in the dormant period of the apex. During this period the NF leaves contain higher levels of ABA (Orchard et al. 1980). It is likely, therefore, that the inhibitory effect of the NF leaves at I-2 is due to their enhanced level of ABA and its transport to the apex. Removal of the PF leaves at the I-1 stage also caused a reduction in the dormant period of the apex. These PF leaves were mature and hence exported rather than imported photoassimilate so the leaves would not act as a nutrient sink. They also contained a high level of ABA (Orchard et al. 1980).

Iserentant (1976) compared the effects of defoliating all the leaves on cocoa seedlings at stages equivalent to F-1 and I-2 of the flush cycle. He found release of apical dormancy after defoliation at both these stages and concluded that bud dormancy in the seedlings was due to a correlative inhibition rather than a true dormancy. Essentially, the same results were found here when only NF leaves at F-2 and I-2 were removed. These results suggest that there are no reversible and irreversible stages of dormancy in cocoa seedlings as Vogel (1975) claimed occurred with field grown cocoa trees. However, even in seedlings there appear to be two physiological stages to the period of growth inhibition of the bud.

The application of ABA to either a NF or a PF leaf at the I-2 stage of the cycle increased the dormancy from 9 to 13-15 days (Table 2). The effect of the GA_3 and zeatin, however, was to reduce the period of dormancy from 13-14 days to 7-8 days when applied to either NF or PF leaves at the I-1 stage. However, when GA_3 was applied to a PF_1 leaf at the F-2 stage of the flush cycle there was no reduction in the dormant period.

The results here suggest that the effect of the growth promoters in reducing the dormant period was to overcome the endogenous inhibitors in the leaves. The fact that GA_3 when applied to the PF_1 at the F-2 stage had no effect on reducing the length of the dormant period of the apex reinforces the hypothesis that the apical dormancy at this stage is due to nutrient competition rather than to the presence of hormonal inhibitors in the leaves.

When ABA was applied to the leaves of temperate perennials it was also shown to have an inhibitory effect on the bud (Eagles and Wareing, 1963; El-Antably et al. 1967; McWha and Langer, 1979). It remains a possibility that the relatively large quantities of growth substances applied to the plant in such studies cause plants to respond in an abnormal way. Clearer evidence for the movement of ABA from a source leaf to the apex was provided from the experiments involving the application of ^{14}C-ABA to cocoa leaves.

5.4.2 Movement of ^{14}C-ABA

The distribution of ^{14}C-ABA applied to donor leaves at different times throughout the flush cycle is shown in Figs.26 and 27 . When the first leaf of a new flush at the F-2 stage of the cycle was the donor leaf, 55% of the exported radioactivity was found in the upper young leaves (Fig. 26). A high proportion, 19%, was also found in the shoot above but only 3% in the bud. There was no detectable radioactivity imported into any of the PF leaves but 8% was found in the roots indicating that a significant basipetal movement of ABA had occurred. When ABA was applied at the I-1 stage, import into the apical bud reduced to 1% and import into the NF leaves to 5% also. Most of the movement was basipetal with 21% in the shoot below, 27% in the PF_1 leaves and about the same as before in the roots (9%). When applied at the I-2 stage the proportion of exported radioactivity found in the bud increased to 12% whilst that in the stem above the donor leaf was reduced and little radioactivity was found in the NF leaves. Most of the radioactivity was found below the donor leaf, especially in the roots and PF_1 leaves where the proportion of radioactivity was increased to 18% and 30% respectively.

Fig. 26. The percentage distribution of radioactivity after uptake of ^{14}C-ABA by a NF leaf at F-2 (a), I-1 (b) and I-2 (c). R, roots; PF_2, second previous flushes; SB, stem below donor leaf; PF_1, first previous flush; NF, new flush; SA, stem above donor leaf; B, apical bud.

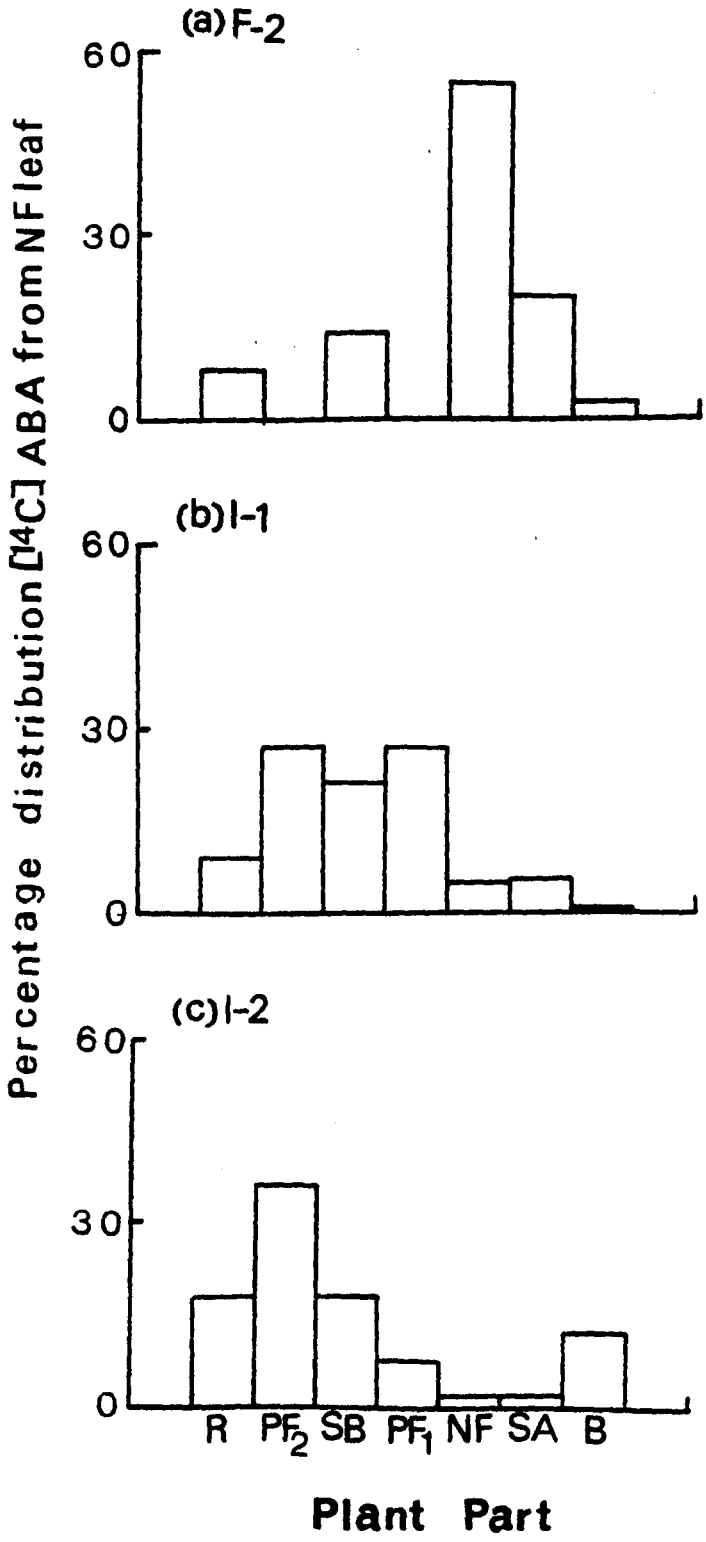
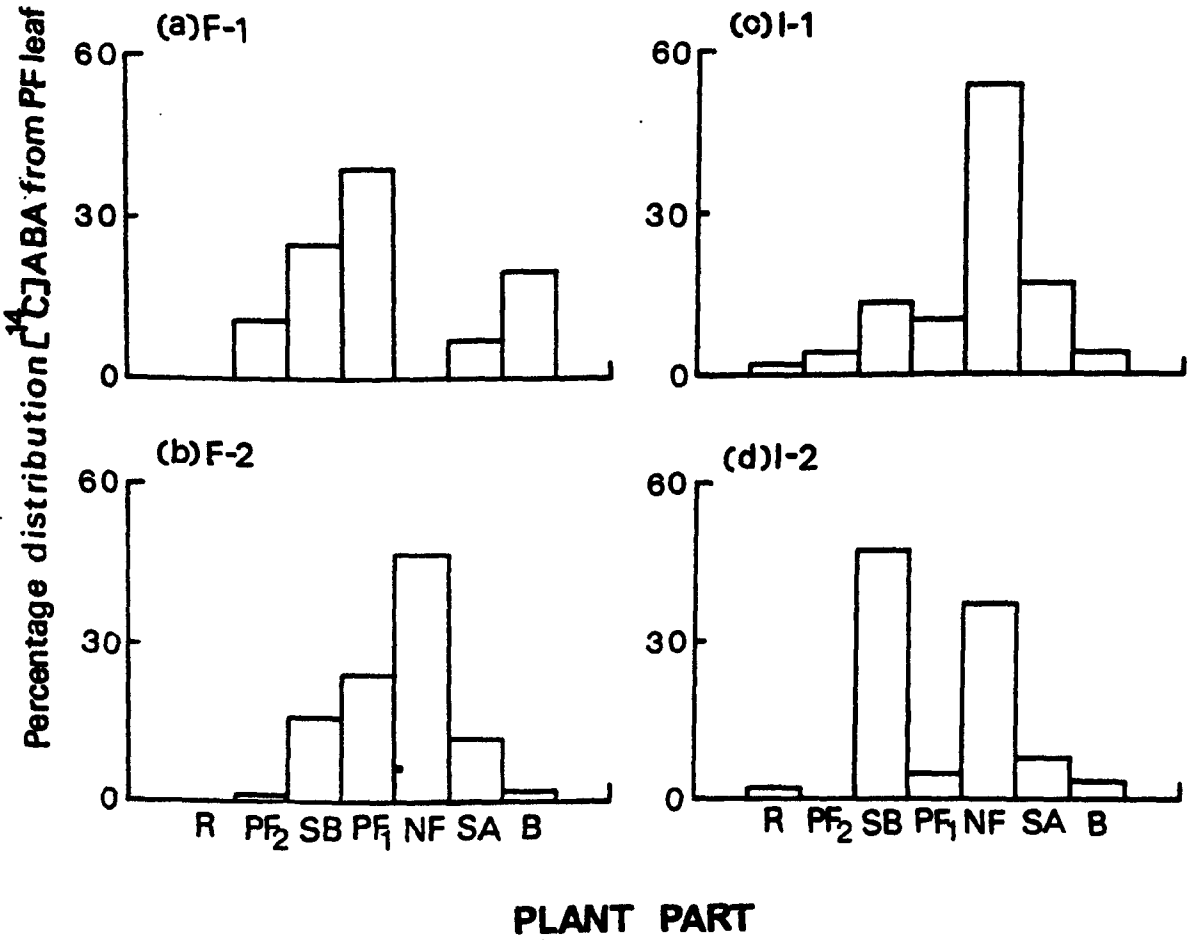

(a) F-2
60
30
0
(b) I-1
60
30
0
(c) I-2
60
30
0
R
PF2
SB
PF1
NF
SA
B
Percentage distribution [14C] ABA from NF leaf
Plant Part

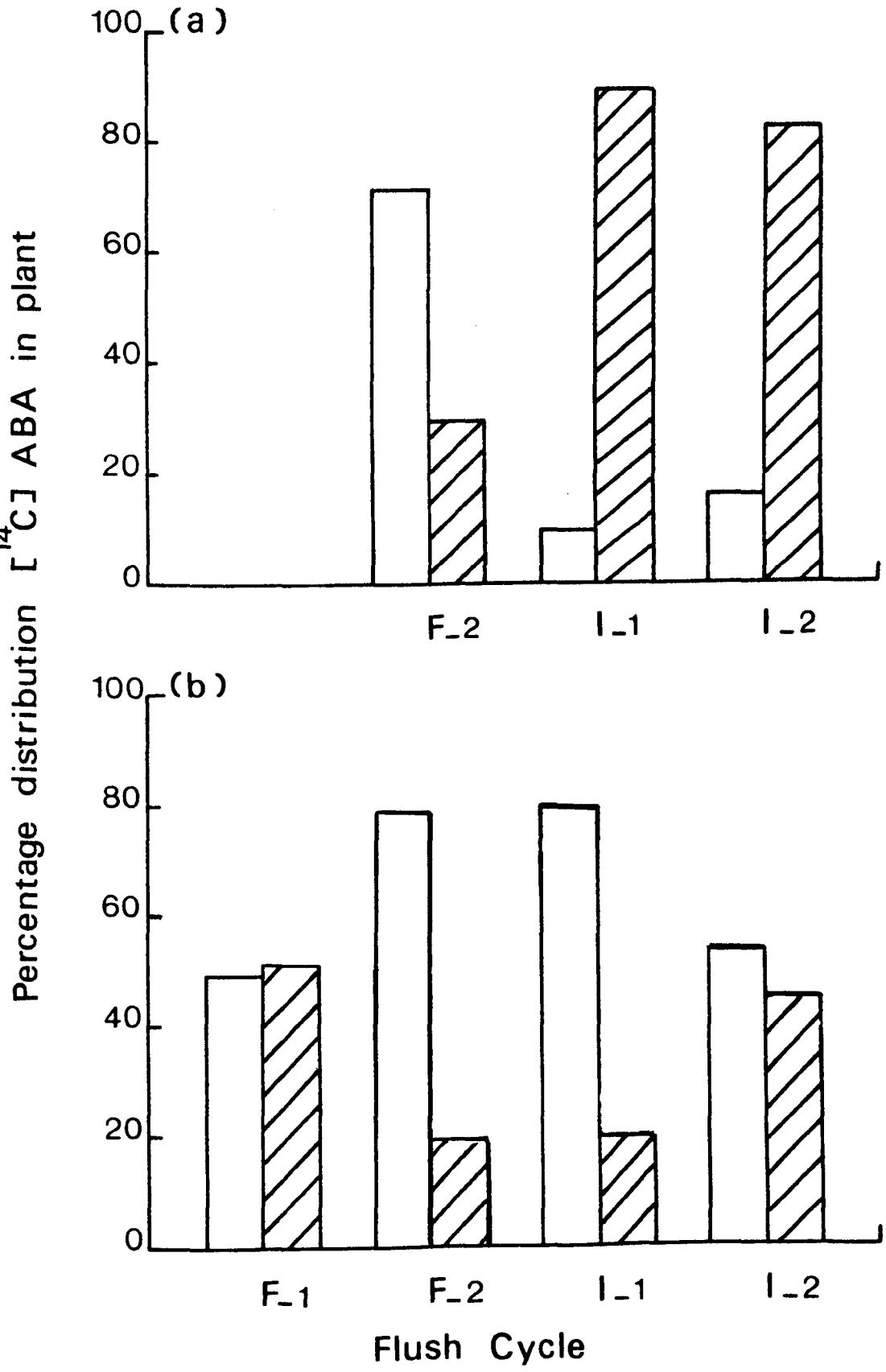
Fig. 27. The percentage distribution of radioactivity after uptake of ^{14}C-ABA by a PF leaf at F-1 (a), F-2 (b), I-1 (c) and I-2 (d). R, roots; PF_2, second previous flushes; SB, stem below donor leaf; PF_1, first previous flush; NF, new flush; SA, stem above donor leaf; B, apical bud.


(a) F-1
(c) I-1
(b) F-2
(d) I-2
60
30
0
R
PF2
SB
PF1
NF
SA
B
Percentage distribution of [14C]ABA from PF leaf
PLANT PART

It seems that in general in the later stages of the flush cycle there was a progressive decrease in the proportion of radioactivity exported to the tissues above the donor leaf. The notable exception was the bud which showed a large increase during the later stages.

When a PF leaf was the donor leaf the distribution of radioactivity showed a different pattern (Fig.27). At the F-1 stage the enlarging buds which would include the pre-emergent NF leaves, contained 20% of the exported radioactivity and the stem above the donor leaf, 7%, the PF_1 leaves contained 38%. The stem below contained 24% and PF_2 leaves, 11%, with no radioactivity being detected in the roots. At F-2 the proportion of radioactivity in the bud was reduced to 2% but the expanding NF leaves contained 46% and the stem above contained 12%. The remaining radioactivity was in PF_1 leaves and the stem below. The distribution of radioactivity showed no major change at I-1 and I-2 except for a reduction in the proportion of radioactivity found in the NF leaves at I-2 and an increase in the roots. The pattern of export from the PF donor leaf to the bud shows that at F-1 the proportion of radioactivity transported to the bud is high, but this then declines to a low figure in the subsequent stages of the flush cycle. After F-1 a large but declining proportion of the radioactivity found above the donor leaf was in the NF leaves. Figure 28 shows the percentage of radioactivity exported above (acropetal) or below (basipetal) the source leaf. When the NF leaf was the source leaf at the F-2 stage 70% of the radioactivity was exported acropetally, whereas the situation was reversed at I-1 and I-2 stages when 80 - 90% of the radioactivity was exported basipetally. The distribution of radioactivity in the plant was altered when the PF leaf became the source

Fig. 28. Percentage distribution of ^{14}C-ABA above (□) or below (◪) the donor NF (a) or PF (b) leaf at different stages of the flush cycle (F-1, F-2, I-1 and I-2).


100
(a)
80
60
40
20
0
F_2
I_1
I_2
Percentage distribution [14C] ABA in plant
100
(b)
80
60
40
20
0
F_1
F_2
I_1
I_2
Flush Cycle

leaf. At the F-1 and I-2 stages of the cycle the amount of upward and downward movement of radioactivity was approximately the same while at F-2 and I-1 more of the radioactivity (80 -82%) was exported acropetally.

The following discussion on interpretation of movement of labelled compounds applies to each of chapters 5 and 6.

Only limited information can be obtained from the raw data giving amount of isotope found in a particular tissue (see Figs. 26, 27, 29, 30, 33, 34, 37 & 38). Clearly, differences in amounts of tissue of the various plant fractions would, even if distribution were uniform, show differences in total isotope in the fractions. As mentioned previously (chapter 2.11) it is for this reason that isotope transport/incorporation into tissues are better considered after calculation of Relative Specific Activities (RSA) (Chapter 2.11). As an expression of isotope per unit tissue this will show where there is any preferential transport and accumulation. Care still needs to be taken in interpreting RSA values however. Firstly in numerical terms RSA values will be dependent upon the amount of isotope taken into the system, and although preliminary work showed this variability to be reasonably low ($\pm$ 20%), this will make standard errors calculated for means high, and for this reason it is felt better to compare RSA values on the basis of 'trends' rather than to compare strict statistical differences. Secondly, as regards interpreting movement patterns, one must be aware that if the isotope were to move passively with an assimilate (or transpiration) stream then accumulation of isotope would merely reflect the movement of the latter. Movement or accumulation of isotope against a known, or expected, major flow pattern will be better evidence of directed transport.

When the radioactivity data were expressed as RSA's (see Chapter 2.11 for details of RSA calculation and significance), it showed that irrespective of whether the donor leaf was an NF or a PF leaf, the RSA of the bud was very high compared with the other parts of the plant (Table 3). During the different stages of the flush cycle the RSA of the bud showed some variation. At F-1 the bud contains the emerging NF leaves hence the high value for the RSA of the bud may be due to the presence of radioactivity imported into the young NF leaves, rather than to the meristem. At F-2, I-1 and I-2, the bud is dormant at all these three stages, the RSA of the bud is always lower at F-2 than I-1 or I-2. The difference in the RSA values indicates that the bud accumulated more ABA at stages I-1 and I-2 than at the earlier F-2 stage.

The ^{14}C-ABA fed to an NF or PF leaf at different stages of the flush cycle became distributed to various parts of the plant, including both meristematic bud tissue and mature NF and PF leaves. The radioactivity recovered declined with distance from the source leaf. Previous investigations in various species have explained the movement of ABA as being polar (Ingersoll and Smith, 1970); by passive diffusion (Davenport, Jordan and Morgan, 1977); associated with assimilate movement of the phloem (Hocking *et al.* 1972; Bellandi and Dörffling, 1974) and with water movement in the xylem (Hoad, 1975). To account for the distribution of ^{14}C-ABA in cocoa it is suggested that the ABA was exported out of the leaf in the phloem and towards areas of low ABA concentration such as the bud, based on the source sink relationship described by

TABLE 3. RSA values for each part of three replicate cocoa plants after uptake of ^{14}C-ABA by either a NF or a PF leaf at different stages of the flush cycle. (Radioactivity in flushes older than PF_2 never exceeded an RSA value of 1.0 and hence were not included).

Plant part	RSA values at different stages of the flush cycle						
	Donor leaf NF			Donor leaf PF			
	F-2	I-1	I-2	F-1	F-2	I-1	I-2
Bud	± 37.2 ± 9.9	± 75.0 ± 20.0	± 130.6 ± 46.9	± 99.6 ± 52.9	± 42.5 ± 4.6	± 127.7 ± 70.7	± 108.3 ± 41.1
SA	± 52.7 ± 1.7	± 9.9 ± 1.4	± 1.8 ± 0.8	± 9.8 ± 3.4	± 38.8 ± 3.4	± 42.3 ± 27.5	± 12.5 ± 3.9
NF	± 13.6 ± 0.1	± 0.6 ± 0.3	± 0.2 ± 0.1	± -	± 13.3 ± 7.9	± 6.4 ± 3.8	± 3.5 ± 1.5
PF_1	± 1.6 ± 0.2	± 3.2 ± 1.3	± 1.9 ± 1.1	± 6.6 ± 1.3	-	± 1.3 ± 0.6	± 3.2 ± 0.2
PF_2	± 0.2 ± 0.1	± 1.7 ± 0.2	± 7.7 ± 4.9	± -	± -	± 0.2 ± 0.1	-
SB	± 0.3 ± 0.1	± 0.5 ± 0.3	± 0.2 ± 0.1	± 0.8 ± 0.3	± 7.0 ± 2.7	± 0.3 ± 0.2	± 1.3 ± 0.3
Root	± 0.3 ± 0.1	± 0.6 ± 0.1	± 0.7 ± 0.4	± -	-	± 0.2 ± 0.1	± 0.2 ± 0.1

Shindy, Asmundson, Smith and Kumamoto (1973) and Hoad (1973). The appearance of radioactivity in the mature leaves may be due to lateral movement of ^{14}C-ABA into the xylem then the transpiration stream. The presence of ABA in both xylem and phloem of other species provides some support for this suggestion (King, 1976). Alternatively, the ^{14}C-ABA could move out of the donor leaf by diffusion in the phloem and not active flow (Canny and Askham, 1967). The ^{14}C-ABA would then appear in all parts of the plant which would explain the presence of radioactivity in mature tissues. According to Canny and Askham (1967) any accumulation of radioactivity in one part of the plant would require an active process of unloading and uptake. On this basis and from calculation of RSA values, the mature leaves which have a very low RSA show minimal uptake of ^{14}C-ABA whereas the bud with a high RSA would seem to be actively accumulating the ^{14}C-ABA. If it is assumed that the movement, and particularly the accumulation of ^{14}C-ABA, is a reflection of the transport of the endogenous ABA then the bud is capable of accumulating ABA much more than other parts of the plant. The ability of the bud to accumulate ABA seems to vary during the flush cycle; it is lower during the initial stages of dormancy, F-2, than during the later dormant stage of 1-2. It would seem that dormancy at the F-2 phase in the seedlings may thus be initiated by nutrient competition between the apex and the developing flush leaves and it is only the second phase (known previously as the irreversible phase), i.e. dormancy at I-1 and I-2, that is predominantly controlled by ABA transported to the apex from the mature leaves. The role of ABA would then appear to be to maintain rather than initiate

dormancy in the apical bud. This function of ABA in cocoa would therefore accord with its role in temperate perennials where it is also thought to maintain bud dormancy rather than initiate the state (Hocking and Hillman, 1975; Wright, 1975).

The movement of ABA would seem to play an important role in the control of the flush cycle. A detailed examination of the movement of promoters about the plant during the growth cycle has to be followed as well, this will be the subject of chapter 6.

CHAPTER 6

Movement of Plant Growth Promoters in cocoa seedlings throughout a Flush Growth Cycle

6.1 Introduction

It is known that the pattern of growth and development in plants is mediated by growth substances (Thimann, 1974; Wareing and Phillips, 1970; Phillips, 1971). Previous studies have shown that the endogenous levels of various growth substances in plant organs change during growth and development. For instance, there often appears to be a close relationship between the endogenous levels of a growth substance and the control of a growth process; for example, a positive correlation between levels of GA_3, IAA and cytokinins and the rate of growth of cherry tomato fruits (Abdel-Rahman et al., 1975); of IAA level and vegetation growth in Pinus silvestris (Alden, 1971); in cytokinin level and the breaking of dormancy of birch and poplar (Domanski and Kozlowski, 1968), in the involvement of gibberellins in the development of french beans (Wheeler, 1960); in cytokinin level and the rate of growth of the apical region and the young expanding leaves in Lupinus albus (Davey and Van Staden, 1978).

During the flush cycle of cocoa the plant undergoes a number of morphological changes such as bud burst and dormancy, leaf and stem expansion and leaf greening. Such a range of growth processes are likely to be subject to a complex system of control which may involve all the range of growth substances acting concurrently in some way, e.g. interaction between indole acetic acid, gibberellins in stem growth and movement of metabolites (Hill, 1973). Past research on the growth substances in cocoa has revealed how complex the system of control may be. For instance

in the flush leaves, levels of both auxins (Nichols, 1957) and gibberellins (Krekule, 1971) vary during the flush cycle, and abscisic acid and cytokinins have been implicated in the control of apical activity (Alvim et al., 1974). However recently Orchard et al. (1980) using cocoa seedlings grown under glass house conditions in the absence of any environmental stress, found during a flush cycle of shoot growth (stages F-1 to F-2) very low levels of free and bound ABA in young expanding flush leaves. At full expansion (end of F-2 stage) both forms increased, particularly the bound ABA, then declined again during the dormant period (I-1 to I-2) to reach a low level at the start of the new cycle. In the leaves of the previous flush, the early stage of the cycle (F-2) was marked by a high level of free ABA which thereafter declined rapidly and remained low throughout the rest of the cycle, whereas the bound form increased at the end of F-2 and only declined at the end of the cycle. They suggested that both the mature leaves of the current flush and the leaves of the previous flush act as sources of ABA which maintain the bud in a state of dormancy during the latter half of the growth cycle.

In another study by Orchard et al. (1981), they found both auxins and cytokinins to vary in the flush and previous flush leaves during the flush growth cycle. Auxin levels in the flush leaves of seedling plants under constant conditions were correlated with leaf expansion and may therefore form part of a hormone based control mechanism of flush leaf growth. Cytokinins were also at high levels during the early stages of leaf development (F-2), but by full expansion no cytokinins could be detected. However, in the

final stages of the flush cycle (late I-2) significant levels of cytokinins were again detected in the new flush leaves and also at the start of the next flush cycle in the fully matured and now previous flush leaves. The reduced levels of cytokinins in cocoa leaves at I-1 and early I-2 could be due to a reduced synthesis in the main site of synthesis, the roots. Alvim et al. (1974) suggested that in field grown cocoa increased root growth may occur during the late stage of the flush cycle (equivalent to late I-2) and lead to a corresponding increase in the synthesis of cytokinins. Presumably the raised level of cytokinins in the leaves and therefore probably buds at late I-2 and F-1 must overcome the inhibitory effects of high levels of ABA found in the shoot at these stages (Orchard et al. 1980). It is suggested that with the change in balance from high ABA:low cytokinin to high ABA:high cytokinin at the shoot, dormancy of the bud is broken and a new flush leaf growth begins. At the time of active flush growth, and because of a redirection of photoassimilate into the new expanding flush leaves (Sleigh et al. 1979), the root system and the shoot apex may experience a shortage of photoassimilate with subsequent root growth and cytokinin production much reduced. This could result in the marked decline in cytokinin export to the shoot and as a consequence further primordia formation and leaf unfolding could cease.

It becomes clear from the work of Orchard et al. (1981) that the transport of auxins and cytokinins throughout the plant could play an important role in the control of the flush cycle in cocoa.

Most of the major groups of plant growth regulators have been detected in T. cacao : auxins (Nichols, 1957; Orchard et al. 1981),

gibberellins (Krekule, 1971), abscisic acid (Alvim et al. 1974; Orchard et al. 1980), cytokinins (Alvim et al. 1974; Orchard et al. 1981). However, there is no information about the movement of growth regulators in T. cacao. To provide information on the pattern of movement of the growth regulators at stages throughout the flush cycle and how movement may be related to the control of leaf production and other metabolic processes in the plant, ^{14}C-IAA, ^{14}C-GA_3 and ^{14}C-BAP were applied separately to a leaf at different stages of the flush growth cycle, and then the distribution into the rest of the plant followed.

The work is presented in three separate sub-chapters dealing in order with ^{14}C-IAA, 8-^{14}C-benzyl amino purine and ^{14}C gibberellic acid. Both transport throughout plants and possible metabolism of tracer are followed. Information on ABA movement was presented in Chapter 5.

6.2 Transport and Metabolism of ^{14}C-IAA in cocoa seedlings throughout a flush growth cycle

6.2.1 Introduction

Auxin is synthesized in relatively large amounts in only a few localised sites, but it is transported through all the living tissues of the plant. Shoot tips, including the young leaves, are the site of most abundant auxin synthesis in the vegetative seed plant. Other rich sources are enlarging leaves, flowers, fruits and seeds (Moore, 1979). Auxins are correlated with certain developmental stages in plant life. For instance, in cocoa leaves auxin levels are highest at leaf expansion (Nichols, 1957 and Orchard et al. 1981).

In woody plants, the termination of dormancy is accompanied by appearance of natural auxin in the cambium. This is the period of vigorous radial growth of the shoots (Kefeli, 1978). Auxins are well known to direct transport of metabolites of some species (Patrick and Wareing, 1970, 1973, 1978). The transport of auxin is complicated since auxin synthesized in the shoots is transported basipetally through the petioles and the stem and moves acropetally through the roots toward the tip (Pilet, 1964; Kirk and Jacobs, 1968; Scott and Wilkins, 1968; Morris et al. 1969). The polar basipetal movement of auxins has a velocity independent of donor concentration and length of section (McCready, 1963; Pilet, 1965) and depends on metabolism (Goldsmith, 1968; Reiff and von Güttenberg, 1961).

The basipetal movement of auxin has been explained by either that (a) polarity of movement, even with isolated sections, is the result of a polar distribution of growth (Zaerr and Mitchell, 1967). Polar movement of auxin might be driven by the conversion of the auxin in the basal cells to another form. This could produce movement from donor to receiver block against a concentration gradient only if the accumulated auxin were subsequently released and diffused to receivers. Alternatively that (b) polarity is achieved by a specific transport of auxin during basipetal movement (Goldsmith and Thimann, 1962; Leopold, 1963).

Auxins have been found in both xylem sap (Sheldrake, 1973; Hall and Medlow, 1974) and in phloem sap (Hall and Baker, 1972; Hall and Medlow, 1974). This implies some physiological significance of their vascular transport.

The physiological significance of auxins, their pattern of movement and transport from leaves of cocoa and their metabolism were investigated in the flush cycle of cocoa by foliar application of ^{14}C-IAA to cocoa seedlings at different stages of their development. Three replicate plants were used for each treatment. Details of the methods of application of the hormone, extraction and separation are given in chapter 2.

6.2.2 Results

6.2.2.1 Metabolism of ^{14}C-IAA:

Before the distribution of radioactivity in the various parts of the plant was interpreted it was important to establish whether the radioactivity was present as ^{14}C-IAA, or its metabolites. The ethanolic extract of SB after application of ^{14}C-IAA to a PF (I-1) donor leaf was treated in the same way as described in chapter 2, then aliquots of authentic compounds of standard indoles were co-chromatographed by ascending paper chromatography in isopropanol:ammonia:water (10:1:1). The IAA and metabolites were located by examination under 254 and 360 nm and by spraying with ninhydrin or Ehrlich reagent, then the radioactivity of each determined in the same way as the plant extract. Of the total radioactivity in the purified sample, 18% corresponded in position to indole acetyl aspartate (R_f = 0.1-0.2), 21% to IAA (R_f = 0.3-0.4), 11% to indole aldehyde (R_f = 0.6-0.7), 25% as indole acetaldehyde (R_f = 0.7-0.8) and 4% as indole acetonitrile (R_f = 0.8-0.88). After hydrolysis of the aqueous soluble extract by NaOH, the product contained IAA. The conjugate was thought to be IAA-glucose which represents 15% of the total activity in the analysed extract of SB. The

IAA was also conjugated with protein since the residue which remained after the ethanolic extraction when hydrolysed for 16hr with 2M NaOH the hydrolysate showed the presence of IAA. The amount of bound IAA-protein was approximately 5% of the total radioactivity. Most of the ^{14}C-IAA was thus metabolised into a range of IAA derivatives namely of an IAA-protein conjugate, IAAsp or IAA-ald. The metabolism of IAA in cocoa leaves thus seems to be similar to the IAA metabolism of the tissue of other plants (Morris et al.,1969). Because virtually all the ^{14}C-activity isolated from the plant tissue is accounted for by IAA and its derivatives, the pattern of distribution of ^{14}C-activity in cocoa thus shows the transport of IAA and derivatives only.

6.2.2.2 Distribution of radioactivity:

The percentage distribution of radioactivity in all parts of the plant after foliar application of ^{14}C-IAA to either an NF or PF donor leaf is shown in Figs. 29 & 30. When a NF was the donor leaf at the F-2 stage, distribution of radioactivity both in a downward and upward direction had occurred. The upward movement was into the SA (2%), the NF leaves (3%) and bud (8%), while the downward movement was into the PF_1 leaves (5%), the SB (52%), and the roots R (25%). At the I-1 stage the proportion of radioactivity in NF leaves had increased to 12%, 7% was in the bud and 2% was in the SA; while of the downward movement the radioactivity in the PF leaves increased to 46%, the SB and roots were reduced to 26% and 7% respectively. With the end of the maturation phase (I-2), the proportion of radioactivity in the bud had declined to 0.58 % , the NF leaves had increased to 24%

Fig. 29. The percentage distribution of radioactivity after uptake of ^{14}C-IAA by a NF leaf at F-2 (a), I-1 (b) and I-2 (c). MR, main root; LR, lateral roots; SB, stem below donor leaf; PF_s, previous flush leaves; PF_1, first previous flush; NF_s, new flush leaves; SA, stem above donor leaf; B, apical bud.

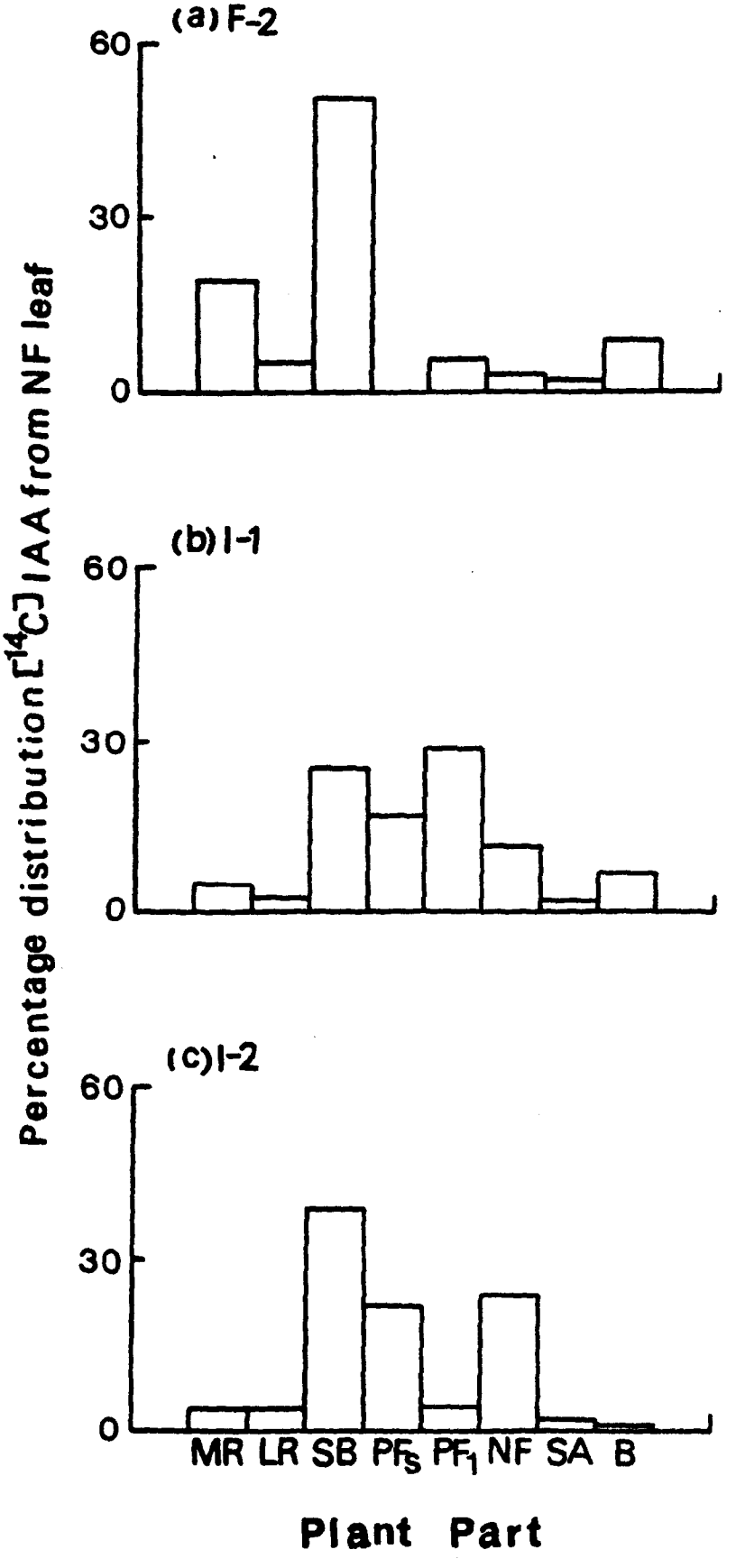
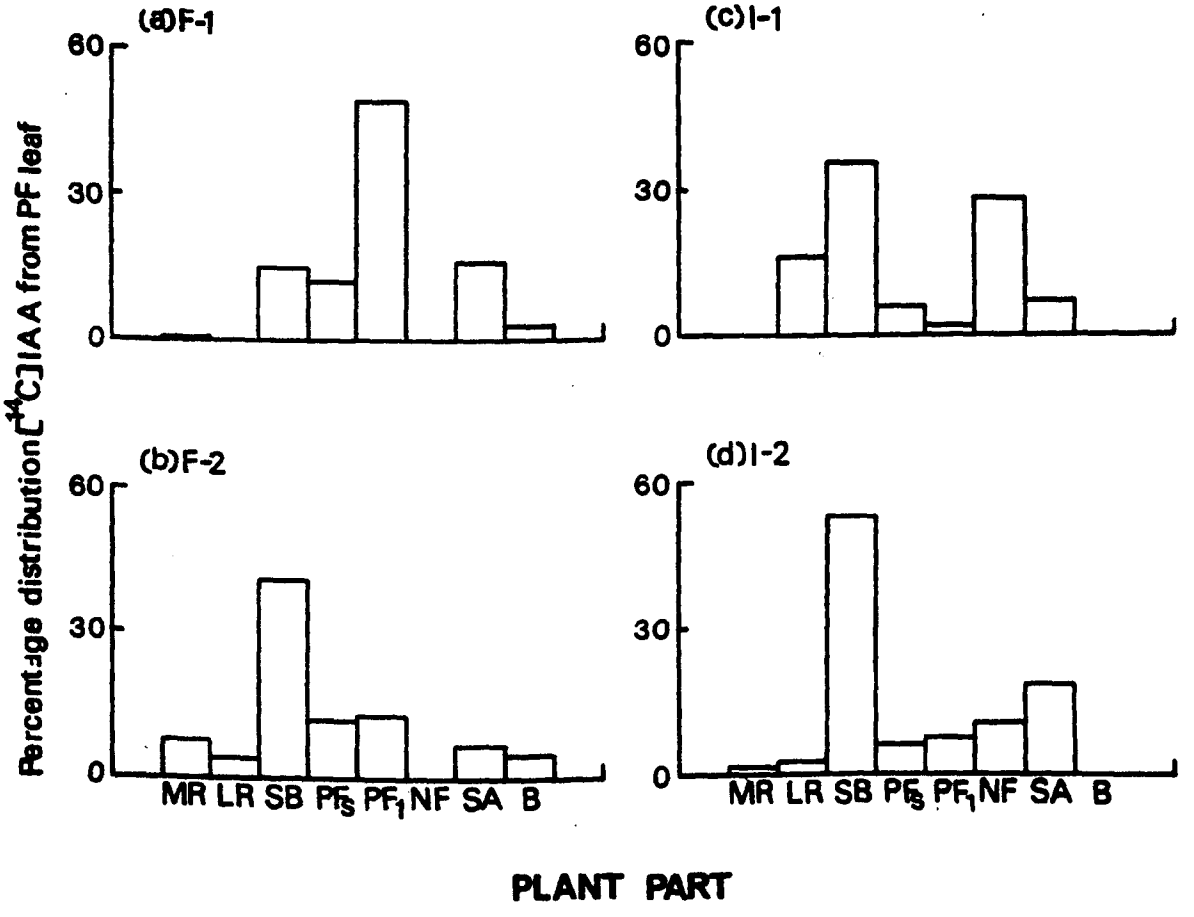

(a) F-2
(b) I-1
(c) I-2
60
30
0
Percentage distribution [^{14}C] IAA from NF leaf
MR LR SB PF_S PF_1 NF SA B
Plant Part

Fig. 30. The percentage distribution of radioactivity after uptake of ^{14}C-IAA by a PF leaf at F-1 (a), F-2 (b), I-1 (c) and I-2 (d). MR, main root; LR, lateral roots; SB, stem below donor leaf; PFs, previous flush leaves; PF_1, first previous flush; NF, new flush leaves; SA, stem above donor leaf; B, apical bud.


(a)F-1
(c)I-1
(b)F-2
(d)I-2
60
30
0
MR LR SB PFs PF1 NF SA B
Percentage distribution [14C] IAA from PF leaf
PLANT PART

and the SA remained the same (2%). At this stage 25% of the radioactivity was in PF leaves, and the SB contained 39% and only a small proportion was in the roots (7.5%).

With a PF leaf as the donor leaf, both upward and downward movement of radioactivity also occurred. The radioactivity in the bud during the F-1 stage was 2.7% , SA (16%-, PF_1 (49%) and the basipetal direction was found in some PF leaves PF_2 and PF_4 (12%), the SB (15%), and the roots (0.65%). During the expantion phase (F-2), the radioactivity in the bud increased to 4.7% and in the SA it decreased (6.7%), no radioactivity in NF leaves, and the mature PF leaves 13%. Basipetal exported ^{14}C gave 25% in the PF leaves, SB (15%) and roots (12%). During the I-1 stage, there was a decline in the radioactivity in the bud (0.6%), the SA showed the same, the NF increased to 28% and the PF_1 reduced to 2%. Basipetal movement occurred to the lower PF leaves (6%), in the SB the radioactivity was still high (36%) and the roots showed an increase (16%). At the I-2 stage there was a very small percentafe of radioactivity in the bud (0.23%), the SA contained 19% , the NF leaves was 6% , the SB 53% and the roots 4%.

From the pattern of distribution of radioactivity, it seems that irrespective of whether the donor leaf is a NF or a PF leaf, during F-2, I-1 and I-2 the IAA is transported predominantly in a basipetal direction (see Fig. 31).

When the radioactivity was calculated on the basis of R.S.A. (Table 4), and when a PF leaf was the donor, a comparison of the RSA of the bud showed an extremely high value at F-1 which then declined

Fig. 31. Percentage distribution of ^{14}C-IAA above (□) or below (■) the donor NF (a) or PF (b) leaf at different stages of the flush cycle (F-1, F-2, I-1 and I-2).

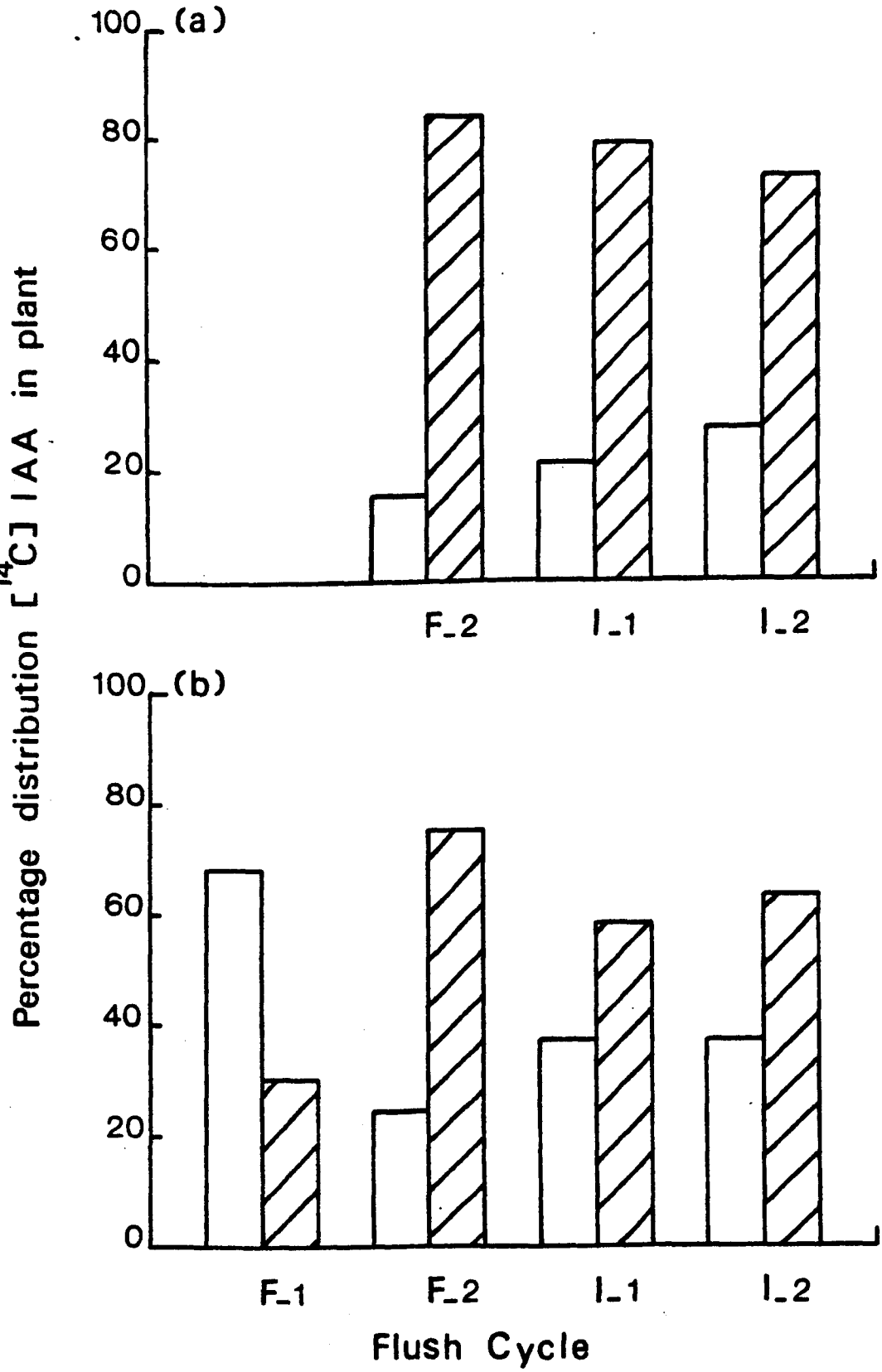

(a)
100
80
60
40
20
0
F_2
I_1
I_2
(b)
100
80
60
40
20
0
F_1
F_2
I_1
I_2
Flush Cycle
Percentage distribution [^{14}C] IAA in plant

TABLE 4. RSA values for each part of three replicate cocoa plants after uptake of ^{14}C-IAA by either a NF or a PF leaf at different stages of the flush cycle.

RSA values at different stages of the flush cycle

Plant part	Donor leaf NF			Donor leaf PF			
	F-2	I-1	I-2	F-1	F-2	I-1	I-2
Bud	61.92 ± 10.03	44.84 ± 8	11.64 ± 5.82	31.43 ± 5.5	20.79 ± 0.75	11.58 ± 4.98	3.25 ± 0.4
SA	1.92 ± 0.3	33.44 ± 20.6	3.45 ± 1.17	39.43 ± 18.02	7.55 ± 0.66	7.7 ± 0.81	7.94 ± 0.81
NF	1.2 ± 0.02	10.35 ± 5.4	3.02 ± 1.03	-	-	12.72 ± 4.84	1.22 ± 0.03
PF_1	0.51 ± 0.05	2.03 ± 0.34	0.37 ± 0.17	5.63 ± 2.39	1.78 ± 0.18	0.35 ± 0.24	1.54 ± 0.01
PF_2	-	1.37 ± 1.08	0.51 ± 0.28	1.0 ± 0.82	2.73 ± 0.02	-	0.68 ± 0.007
PF_3	-	0.19 ± 0.15	0.98 ± 0.33	-	0.31 ± 0.08	0.57 ± 0.28	0.06 ± 0.008
PF_4	-	1.44 ± 1.12	1.49 ± 0.8	0.14 ± 0.11	1.35 ± 0.20	-	-
PF_5	-	-	1.53 ± 0.51	-	-	0.37 ± 0.15	-
SB	1.55 ± 0.34	0.74 ± 0.23	1.29 ± 0.37	0.49 ± 0.16	1.2 ± 0.33	1.11 ± 0.33	1.65 ± 0.002
MR	1.19 ± 0.28	0.43 ± 0.29	0.24 ± 0.03	0.04 ± 0.02	0.51 ± 0.01	-	0.14 ± 0.009
LR	0.75 ± 0.28	0.18 ± 0.13	0.29 ± 0.17	-	0.75 ± 0.09	2.47 ± 1.04	0.20 ± 0.002

through to I-2. When a NF leaf was the donor leaf, the RSA value of the bud was highest at F-2 and declined at I-1 and I-2 stages. With a NF leaf as the donor, the RSA for lateral roots showed a maximum at F-2 with a lower RSA at the I-1 stage. On the other hand, when a PF leaf was the donor, the RSA value for lateral roots was high at F-2 and declined at the I-1 and I-2 stages.

6.2.3 Discussion

The movement of ^{14}C-IAA and its metabolites out of the NF or PF donor leaves of the cocoa is predominantly basipetal, which is a pattern of movement in agreement with that of ^{14}C-IAA applied to sections of coleoptile, stem or petiole (McCready, 1966) and also more recently where the ^{14}C-IAA has been applied to the bud and leaves of intact plants (Ballag and Galun, 1966; Morris, Briant and Thompson, 1968; Hollis and Tepper, 1971; Morris and Kadir, 1972; Bonnemain and Bourbouloux, 1973; Goldsmith, Cataldo, Karn, Brenneman and Trip, 1974). The movement of auxin following its application to buds was found to be strictly basipetal, occurring in living tissue but not the phloem (Morris et al. 1969; Morris and Kadir, 1972; Pilet, 1964), whereas auxin applied to the bark or leaves was exported both acropetally as well as basipetally in the direction of the assimilate stream (Morris and Kadir, 1972; Bonnemain and Bourbouloux, 1973; Goldsmith et al. 1974). According to Scott and Briggs (1960) the auxin synthesized in the buds is then translocated basipetally to other parts of the plant, a view which has been confirmed by the more recent ^{14}C data. In the cocoa the application of ^{14}C-IAA to an NF donor

leaf at the F-2 stage of the flush cycle showed that the direction of movement was almost totally basipetal. This direction of flow of auxin occurred despite the fact that the donor NF leaf at F-2 was a major importer of assimilate (Hardwick, Sleigh and Collin, 1981). At the F-2 stage the NF leaf also contains a significant amount of auxin which is not present in the leaf at the later I-1 and I-2 stages (Orchard et al. 1981). Both the data on the distribution of ^{14}C-IAA and the presence of the auxin would suggest that the young cocoa leaf was a source of auxin for the rest of the plant until the stage of I-1 and I-2 when this source ceased to function. After the F-2 stage the donor NF leaf becomes a major exporter of assimilate as is the PF donor leaf at all stages of the cycle. The characteristic feature of ^{14}C-IAA export from the mature leaves is that the export shows a larger acropetal component and that the distribution is largely to mature tissue, e.g. the SB part of the stem, PF and mature NF leaves. The assimilate demand for growth in these tissues will be low yet an accumulation of auxin still occurs. There is evidence to show that the lower stem in cocoa is a storage site for carbohydrates which is then withdrawn during the F-1 and F-2 stages of the flush cycle (Hardwick, Sleigh and Collin, 1981). The mature leaves may have a similar function since, unlike many other species studied, even after maximum size and also as mature PF leaves assimilate is imported into the leaves (Hardwick, Sleigh & Collin, 1981). The fact that mature tissue of cocoa can also accumulate auxin suggests it does so because of a particular function. It has already been established that auxin can control the direction of flow of assimilates and other nutrients (Patrick and Wareing, 1970, 1973, 1978), so it is

possible that the large proportion of auxin present in the mature tissue could be related to their function as storage sites for carbohydrates.

The bud shows a large proportion of radioactivity in the early F-1 and F-2 stages of the flush cycle, which also coincides with the period of highest auxin level in the NF leaves. The ability of the bud to accumulate auxin at these stages is also indicated by the RSA data which shows the highest value at F-1 and F-2. It is suggested that the role of auxin in the control of bud activity and correlated growth in the rest of the plant is twofold. In the stages of active growth of the apex, i.e. F-1 and F-2 the bud and young leaves act as a powerful source of auxin which is transported basipetally to the lower part of the plant. This flow of auxin from the expanding flush growth assists in the transport of assimilate and other nutrients to the expanding leaves. Once the leaves are fully expanded and are able to export assimilate in the same way as the PF leaves, endogenous auxin then controls the direction of assimilate to other less demanding growth areas such as the roots, elongating upper stem and secondary thickening in the lower stem and also to the mature tissue where the carbohydrate is stored.

In view of the importance of cytokinins in the control of development, the movement of a kinin, ^{14}C-BAP was subsequently investigated.

6.3 Transport and Metabolism of 8-^{14}C-BAP (benzylamino purine) in cocoa seedlings during a flush cycle.

6.3.1 Introduction

There is evidence from the work by Alvim *et al.* (1974), Orchard *et al.* (1980 and 1981) and Abo-Hamed *et al.* (1981) that the control of the alternating periods of growth in cocoa is due to a change in the balance of promoters and inhibitors exported from the leaves to the apex. Abscisic acid transported from the mature leaves has been clearly implicated (Alvim *et al.* 1974; Orchard *et al.* 1980; Abo-Hamed *et al.*(1981). Alvim *et al.* (1974) and Orchard *et al.* (1981) have also suggested that a cytokinin(s) is one of the promoters which might affect the shoot apex activity in cocoa. Orchard *et al.* (1981) have shown that the young flush leaves contain high levels of both auxins and cytokinins during leaf initiation at the shoot apex, both declined during the dormant phase and increased again a few days before bud-break occurred again.

The balance of promoters and inhibitors is important in determining the development of the apex, since with the change in balance from high ABA: low cytokinin to high ABA: high cytokinin in the shoot, dormancy in the bud is broken and a new cycle of leaf growth begins(Orchard *et al.*1981). The movement and accumulation of cytokinins thus appear to play an important role in the control of the flush cycle. A detailed examination of the transport of these compounds about the plant during the growth cycle would add to our understanding of the control of the flush cycle. Accordingly ^{14}C-benzylamino purine as a labelled kinin was applied to donor leaves of cocoa at different stages of the flush cycle and its movement to the apex and other parts of the plant was followed. Three replicate plants were used for each treatment.

Details of the methods of application of the hormone, extraction and separation are given in chapter 2.

6.3.2 Results

6.3.2.1 Metabolism of ^{14}C-BAP

Before the distribution of radioactivity in the various parts of the plant could be analysed and interpreted, it was important to establish whether the radioactivity extracted from the plant after a period of transport was present as ^{14}C-BAP, or its metabolites. An aliquot of the extract from SB when a NF leaf at F-2 was the donor leaf, was analysed. It showed 60% of the radioactivity was present as free ^{14}C-BAP at Rf 0.95 following a separation by ascending paper chromatography in isopropanol : ammonia : water (10:1:1:v/v). The remainder of the radioacticity was found at Rf = 0.15. On hydrolysis with alkaline phosphatase this yielded a compound with the same Rf as ^{14}C-BAP (in the chromatographic system described). This means that in cocoa tissues the ^{14}C-BAP was being converted into only one other compound which was thought to be BAP-glucoside. The latter may represent an inactive form of cytokinin with a storage function (Davey and Van Staden,1978a). The metabolism of ^{14}C-BAP in cocoa would seem to be rather different to that in some other tissues, although the time that the tissue is exposed to labelled precursor will influence the number of metabolites formed. For example, soybean tissue incubated with ^{14}C-BAP produced 4 major metabolites (Fox et al.,1973). When 8-^{14}C-zeatin was taken up by excised bean axes it was metabolised into zeatin riboside and zeatin ribotide as well as dihydrozeatin derivatives (Sondheimer & Tzou, 1971). After feeding ^{14}C-BAP to cocoa leaves the main conclusion to be drawn from the pattern of

distribution of radioactivity was that it reflected the distribution of cytokinin in the plant.

6.3.2.2 Distribution of radioactivity

The percentage distribution of radioactivity after feeding ^{14}C-BAP to a NF and a PF leaf is shown in Figs. 33 & 34. With a NF source leaf, export of ^{14}C during the expansion phase (F-2) was mainly to the other NF leaves (52%) and the remainder downward to SB (32%) and roots (main and laterals) 15%. There was no accumulation in the SA, B or PF leaves. At the I-1 stage, the distribution of radioactivity increased to 41% in the SA and 45% in the SB but the roots remained the same at 14%. At the L-2 stage, the proportion of ^{14}C in the SB increased further to 58%, and also in the roots 27%, whereas the SA showed a decline to 13% and the B, 2%.

When a PF leaf was the donor, the ^{14}C activity was high in the roots (35%) during F-1, the SA contained 20%, the SB 16% and the B 28%, but no radioactivity was found in PF leaves. During the expansion phase (F-2), the ^{14}C-activity in the roots was increased to 43%, the SB was approximately the same (20%), the NF were 22% and the SA showed some reduction to 13%, but there was very little in the bud (1%). During the maturation stages of the flush cycle (I-1 and I-2), the proportion of ^{14}C activity found in the roots declined to 25 and 32% at I-1 and I-2 respectively. The ^{14}C activity in the SA increased at I-1 and I-2 to 50 and 40%. The ^{14}C activity also increased in the SB to 25 and 27% at I-1 and I-2, while the B contained only a small proportion (ca 1%).

The results shown in Fig. 32 indicate that the distribution of radioactivity above and below the donor leaf was very similar irrespective of whether the donor was a NF or PF leaf. The only exception was when the donor NF leaf was at the I-1 stage when distribution

Fig. 32. Percentage distribution of ^{14}C-BAP above (□) or below (■) the donor NF (a) or PF (b) leaf at different stages of the flush cycle (F-1, F-2, I-1 and I-2).

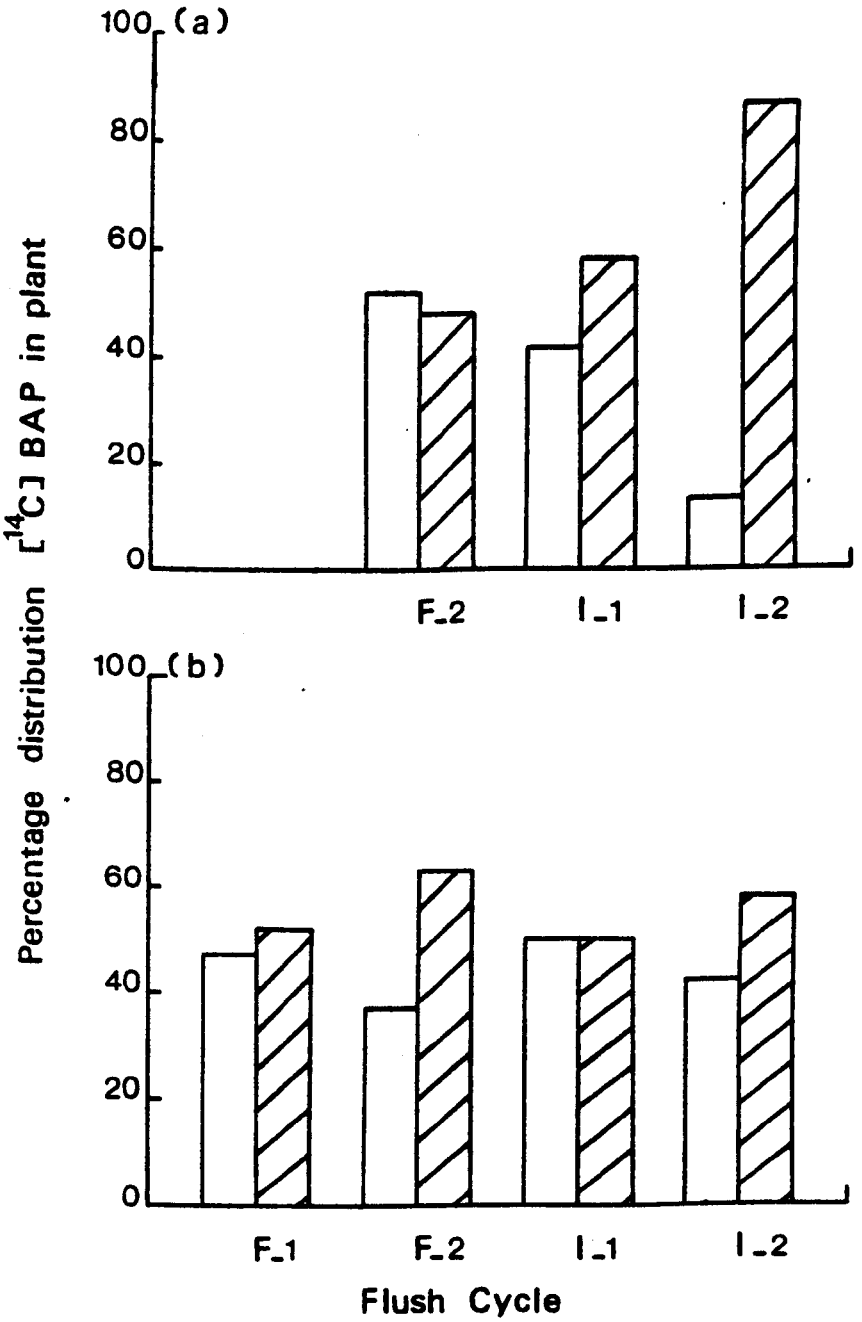

100
(a)
80
60
40
20
0
F_2
I_1
I_2
100
(b)
80
60
40
20
0
F_1
F_2
I_1
I_2
Flush Cycle
Percentage distribution [14C] BAP in plant

Fig. 33. The percentage distribution of radioactivity after uptake of ^{14}C-BAP by a NF leaf at F-2(a), I-1(b) and I-2(c). MR, main root; LR, lateral roots; SB, stem below donor leaf; NF, new flush leaves; SA, stem above donor leaf; B, apical bud.

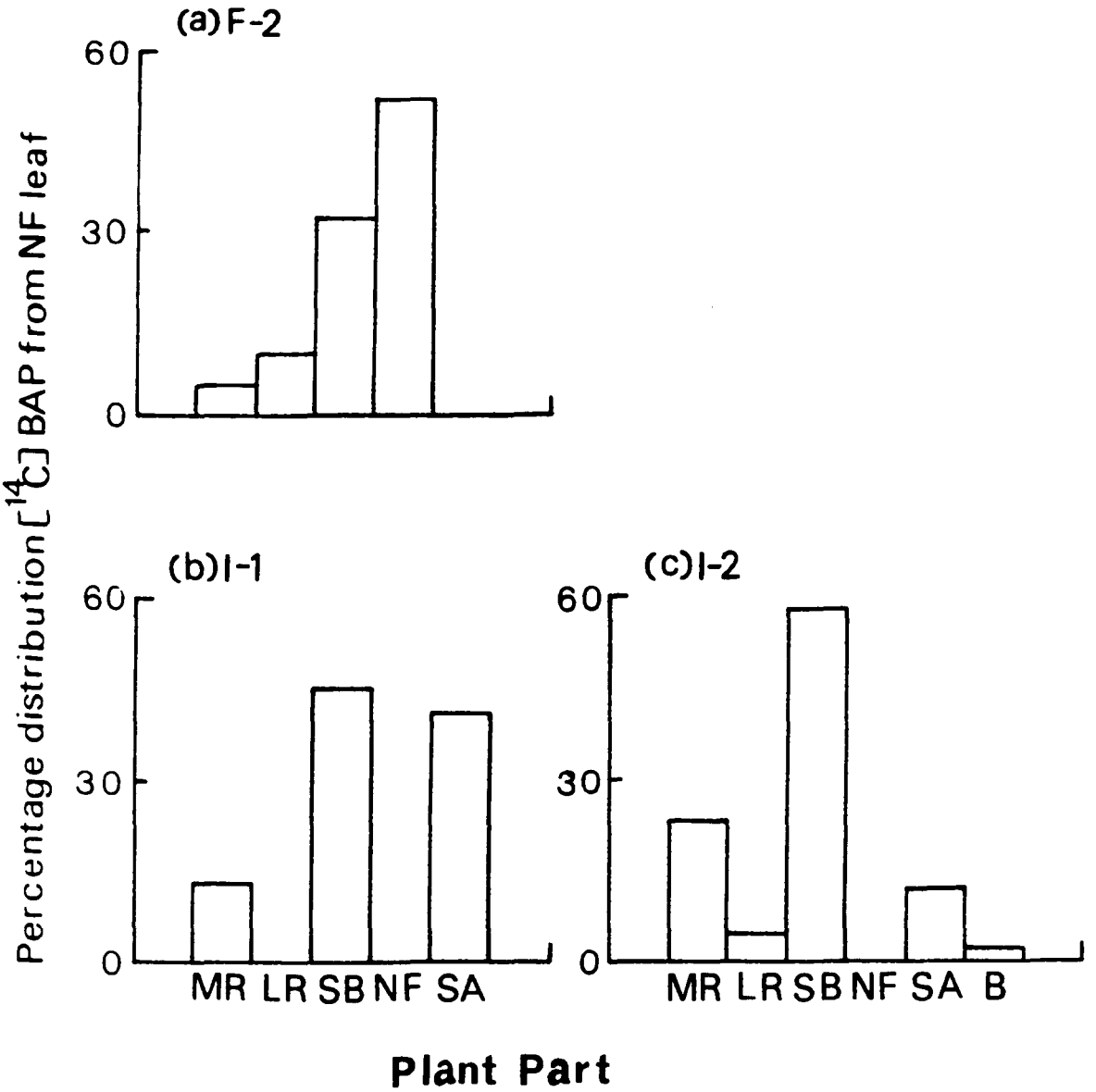
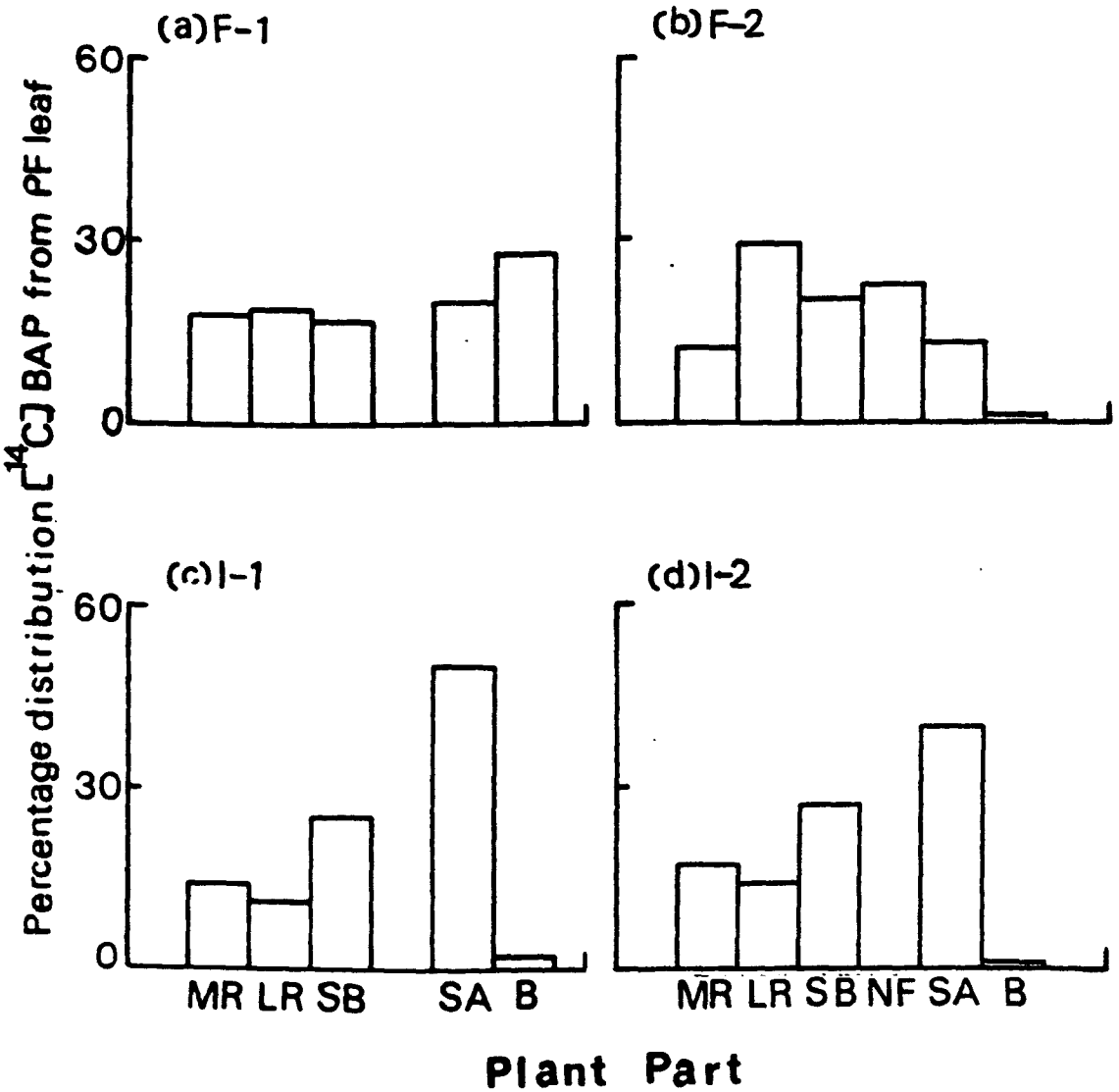

(a) F-2
(b) I-1
(c) I-2
60
30
0
Percentage distribution [^{14}C] BAP from NF leaf
MR LR SB NF SA
MR LR SB NF SA B
Plant Part

Fig. 34. The percentage distribution of radioactivity after uptake of ^{14}C-BAP by a PF leaf at F-1 (a), F-2 (b), I-1 (c) and I-2 (d). MR, main root; LR, lateral roots; SB, stem below donor leaf; PF_s, previous flush leaves; PF_1, first previous flush; NF, new flush leaves; SA, stem above donor leaf; B, apical bud.


(a) F-1
(b) F-2
(c) I-1
(d) I-2
60
30
0
Percentage distribution [^{14}C] BAP from PF leaf
MR LR SB SA B
MR LR SB NF SA B
Plant Part

of radioactivity was predominantly basipetal.

When the radioactivity data were expressed in the basis of the RSA (Table 5), the RSA values differ with the different parts of the plant at one stage, and for the same plant part at different stages of the growth cycle. The bud showed a clear pattern of change when a PF leaf was the donor, with a sequence from the highest to the figure of F-1, F-2, I-1 and I-2. With a NF as the donor leaf the highest RSA for the bud was at I-2 while no radioactivity could be detected at F-2 and I-1. The RSA value for lateral roots when a NF leaf was the donor, was high at F-2 then declined in the later stages of I-1 and I-2. With a PF leaf as the donor, the RSA values were again highest during the earliest stages F-1 and F-2 then declined at I-1 and I-2.

TABLE 5. RSA values for each part of three replicate cocoa plants after uptake of ^{14}C-BAP by either a NF or a PF leaf at different stages of the flush cycle.

Plant part	RSA values at different stages of the flush cycle						
	Donor leaf NF			Donor leaf PF			
	F-2	I-1	I-2	F-1	F-2	I-1	I-2
Bud	0	0	5.54 ± 2.90	235.46 ± 135	4.23 ± 2.87	5.35 ± 1.79	1.94 ± 0.84
SA	0	7.01 ± 2.35	4.0 ± 1.23	99.82 ± 23	1.22 ± 1.07	14.29 ± 0.62	6.17 ± 2.11
NF	15.59 ± 6.6	0	0	0	4.34 ± 1.53	0	0
SB	1.14 ± 0.36	0.62 ± 0.27	0.68 ± 0.39	0.74 ± 0.34	0.53 ± 0.30	1.09 ± 0.01	0.92 ± 0.47
MR	0.40 ± 0.10	1.64 ± 0.26	4.88 ± 1.78	2.0 ± 1.0	1.17 ± 0.38	1.46 ± 0.35	1.29 ± 0.87
LR	1.50 ± 0.83	0	0.23 ± 0.12	2.40 ± 1.50	3.51 ± 2.0	2.13 ± 0.02	1.29 ± 0.87

6.3.3 Discussion

The detailed distribution of ^{14}C-BAP within the plant showed that the ^{14}C-BAP was only found in tissues which showed some growth and not in either dormant or mature organs of the plant. The highest level of radioactivity was found in the SB and SA where elongation and secondary thickening were occurring, in the lateral roots and main roots at F-2 and also the expanding leaves at F-2.

As already shown by Orchard *et al.* (1981) the expanding NF leaves at F-2 contain high levels of auxins and cytokinins. It seems that from the distribution of ^{14}C-BAP the radioactivity is transported to parts of the plant which are rapidly growing and which contain high auxin. This might support the hypothesis of Phillips (1969) that in an intact plant cytokinins are synthesised in roots, then transported acropetally towards the source of auxin situated in the apex. For instance, in intact pea seedlings ^{14}C-kinetin applied to the roots was transported to the apex (Prochazha *et al.* 1977). They showed that the intact apex can attract cytokinins and this ability of the apex when a plant is decapitated may be simulated by the application of various concentrations of IAA to the apical stump. In cocoa the transport of ^{14}C-BAP to the apex was maximum during F-1 and F-2 when auxins levels in the young shoot are also maximum (Orchard *et al.* 1981).

According to Orchard *et al.* (1981), the level of endogenous free cytokinin in the NF leaf is highest at the F-2 stage, is absent at I-1 and shows a very low level at late I-2. The highest endogenous cytokinin level coincides with the time that these leaves import ^{14}C-BAP. If it is assumed that the ability of the bud to import cytokinin is indicated by the import of ^{14}C-BAP, then the bud appears to be unable to import cytokinin until F-1 when growth has already begun.

However, the RSA values for the B show some interesting trends. The RSA for the B is highest at F-1 then either declines or shows no activity at all until late I-2 when the activity increases again. This increase in RSA at late I-2 indicates that the bud is able to import and accumulate cytokinin at this stage. It confirms the earlier work by Alvim *et al.* (1974) and Orchard *et al.*(1981) that cytokinin imported from the root accumulates in the bud at late I-2 and is likely to help trigger the break in bud dormancy and the transition of the bud to F-1. Some agreement for such a role of cytokinin is provided by Smith and Schwabe (1980) who found the activity of cytokinins in the sap of oak trees was a maximum 20 - 25 days before bud-break. Levels decreased during leaf expansion, rising once again 10 - 15 days before a second shoot growth flush. Also the work of Engelbrecht (1971) showed that the resting bud of maple contained considerable amounts of cytokinin, but later, during bud growth, their concentration increased further, indicating a role for cytokinins in the processes of bud opening.

^{14}C-BAP in cocoa is also transported to the lateral roots with a maximum at F-2, which is the phase of maximum root growth (Sleigh, 1981). Some support for a role for cytokinins in roots is provided by kinetin which was found to enhance the lateral root growth in *Lupinus hartwegii* seedlings (Fries, 1960). It is possible that the accumulation of ^{14}C-BAP in the lateral roots suggest that cytokinins also have a role in controlling root growth.

The movement of ^{14}C-BAP to the base of the shoot (SB) in cocoa seedlings agrees with results of Chvojka *et al.* (1961, 1962 & 1963) and Friedrich *et al.* (1970) with apple trees which showed that

^{14}C-BAP moved towards the base of the shoot and also the work of Pieniazek (1964) on apple seedlings using kinetin and found the kinetin treatment led to the breaking of buds and growth of new shoots during the rest period. It is possible that the movement of ^{14}C-BAP to the basal part of the shoot leads to the renewed production of phloem and xylem tissue along the entire seedlings, the same as it does in the apple seedling (Pieniazek, 1964). Cytokinins may also affect growth by mobilisation and distribution of carbohydrates to the base of the shoot (Wittwer and Dedolph, 1963; McDavid et al. 1974).

In conclusion, cytokinins may influence the growth of cocoa by being involved in breaking bud dormancy, by affecting lateral root growth, by expansion of the new flush leaves, and possibly by mobilisation and distribution of carbohydrates to the basal part of the shoot.

6.4 Gibberellins in cocoa leaves during a flush growth cycle; their endogenous levels, movement and distribution of ^{14}C-GA_3

6.4.1 Introduction

Gibberellins are one of the groups of growth promoting compounds which have a physiological role during the life of plants. Gibberellins affect cell elongation as well as cell division, they can also stimulate cambial activity and in many cases are involved in the differentiation of cambium into xylem and phloem in the veinal regions of leaves (Wareing and Phillips, 1970). In addition to their role in leaf development, gibberellins are also considered to be growth promoters able to overcome bud dormancy (Eagles and Wareing, 1963; Nooden and Weber 1978 and Abo-Hamed et al. 1981). Of particular relevance to this work is the demonstration by Orchard et al. (1979) that GA_3, but neither kinetin nor IAA, would break the dormancy (cause bud-burst) and stimulate leaf expansion from detached shoot apices of cocoa cultured in liquid Linsmaier and Skoog medium. Gibberellins may be of particular importance in controlling the growth of the shoot apex in cocoa.

In French bean, Wheeler (1960) found the levels of gibberellins in young developing leaves were maximum during the expansion period, and declined during leaf maturity. On the other hand, when Krekule (1971) measured the gibberellin levels in cocoa seedlings, he found the young leaves contained much less gibberellins than mature leaves. Krekule's work is the only report of gibberellin assays with cocoa tissue and although he did compare levels in leaves of different ages it is not possible to infer very accurate ages of the leaves used. In order to resolve this confusion and to get some information of any role gibberellins may play in controlling shoot apex activity in intact

plants of cocoa the endogenous levels of gibberellins in the cocoa leaves and movement of exogenous ^{14}C-GA_3 between leaves and other parts of the plant were examined in detail so as to relate the physiological state of the plant and the pattern of accumulation and distribution of gibberellins. To analyse the leaves for gibberellin levels only a single determination was possible for each stage of leaf development due to the large amount (approx. 55g. fresh weight) of plant tissue required for accurate analysis. For the movement and distribution of exogenous ^{14}C-GA_3, triplicate plants were used in each determination and results are expressed as the average of three plants. For details of methods of gibberellin extraction and purification see chapter 2.

6.4.2 Results

6.4.2.1 Change in endogenous levels of gibberellins:

The pattern of change in gibberellin levels for total, bound and free fractions in NF and PF leaves of cocoa plants at certain stages of the flush cycle is given in Figs.35 & 36. In both kinds of leaves (NF and PF) there was the same general pattern of change in the gibberellin levels. Thus during the F-2 stage the gibberellin level reached a maximum which coincided with the end of the expansion phase. After leaf expansion was complete and during the maturation stage I-1, there was a very sharp decline in gibberellins in the leaves; while the late I-1 was typified by low levels in NF leaves and none was detected in PF leaves. Gibberellin levels were still low by mid I-2 in the NF leaves, and again undetected in the PF leaves. Later in the cycle, in late I-2, the levels had begun to rise rapidly especially in the PF leaves. This rise continued into the F-1 phase for the PF leaves. Most of gibberellins

Fig. 35. The change in endogenous gibberellins in NF leaves during a flush cycle.

Total gibberellins
Free "
Bound "

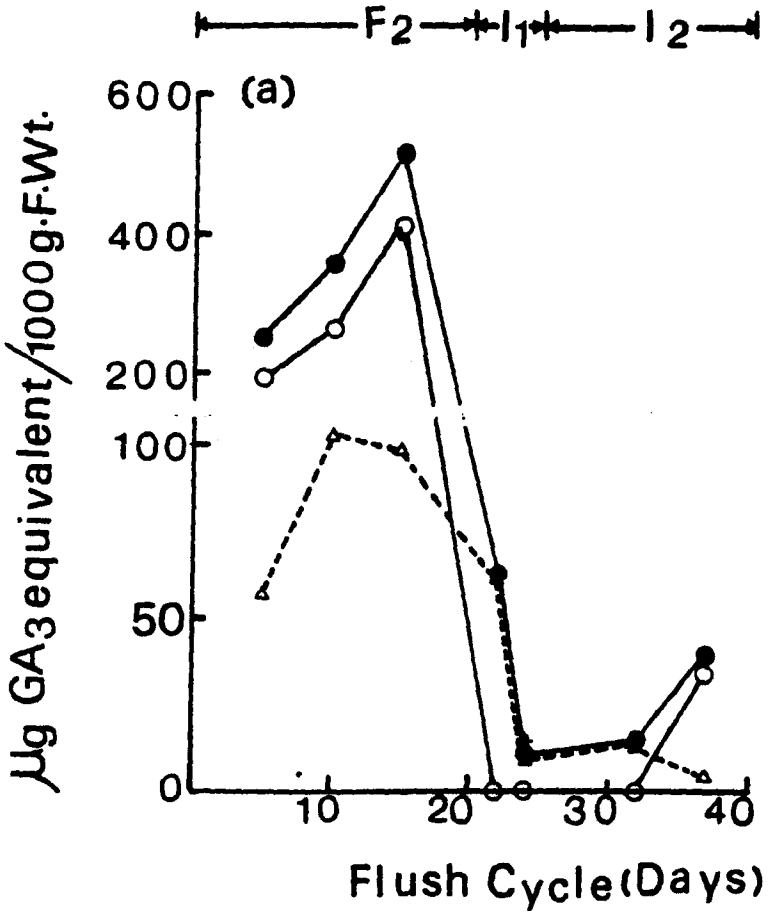
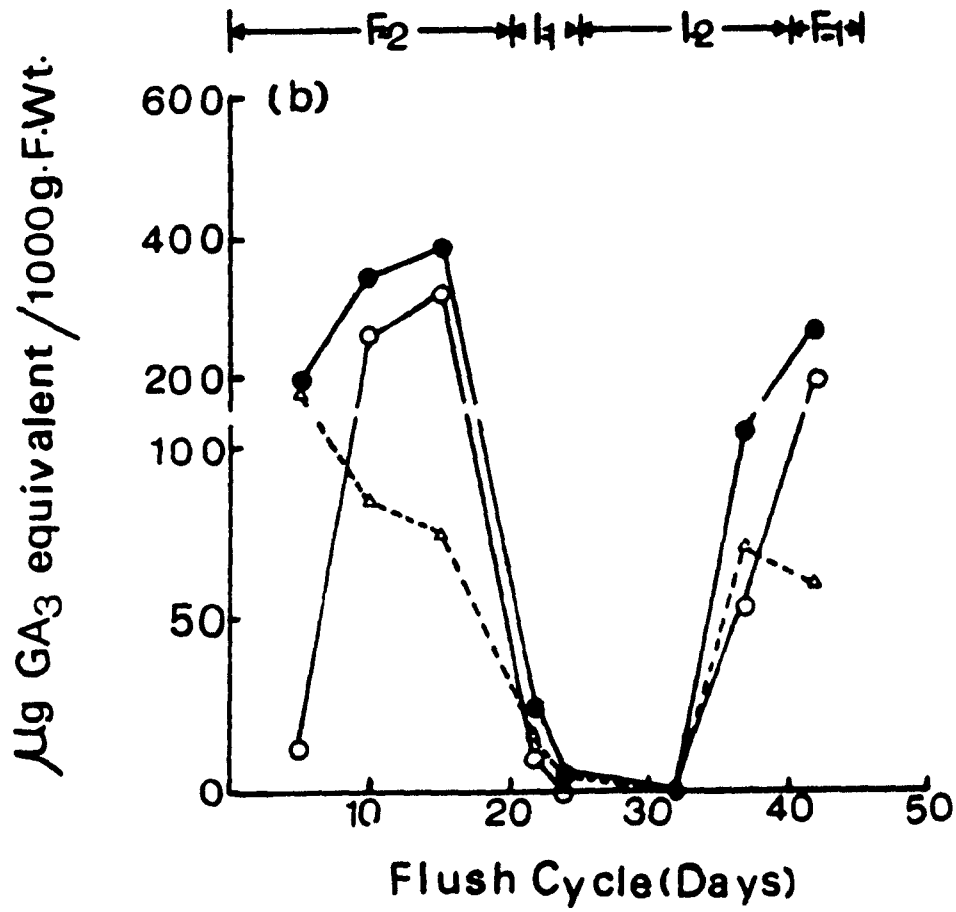

F2
I1
I2
(a)
600
400
200
100
50
0
10
20
30
40
µg GA3 equivalent/1000g.F.Wt.
Flush Cycle(Days)

Fig. 36. The change in endogenous gibberellins in PF leaves during a flush cycle.

Total gibberellins
Free "
Bound "


F2
I1
I2
F1
(b)
600
400
200
100
50
0
10
20
30
40
50
µg GA3 equivalent /1000g.F.Wt.
Flush Cycle(Days)

are as the free form when the total gibberellin level is high. The one exception is early F-2 when in the PF leaves the bound form is the major component. The main reason for the increase in total gibberellins from early F-1 through to late F-2 is thus the rise in free gibberellins.

6.4.2.2 Distribution of ^{14}C-GA_3

Before the distribution of radioactivity in the various parts of the plant was examined and interpreted it was important to establish whether the radioactivity extracted from the plant after a period of transport was present as ^{14}C-GA_3, or its metabolites. When an aliquot of extract from MR was analysed with a PF leaf at the I-1 stage as the donor, the results of analysis showed that 90% of the radioactivity was present as free GA_3, 5% as non-polar esters and 5% as GA-glucosides. No radioactivity was detected in any other metabolites. From another check analysis when the PF leaf at the I-2 stage was the donor, and PF_1 leaves were extracted and assayed, the results showed a very similar pattern, with 88% of the radioactivity remaining as free GA_3, 5.5% as non-polar esters and 6.5% as GA-glucosides and again no radioactivity in other metabolites. This indicates that when ^{14}C-GA_3 is applied to cocoa leaves at these stages and left for a period of 48 hours transport, most of the radioactivity remained as free GA_3 and very little is metabolised. Because most of the radioactivity extracted from the plant remained as free GA_3, it is safe to infer from the distribution of radioactivity that this represents predominantly the movement of gibberellin only.

It seems that cocoa is unusual in its GA_3 metabolism since the application of ^{14}C-GA_3 to many plant species normally results in the rapid formation of new metabolites (Wample et al. 1975; Durley et al. 1974; Barendese and Klerk, 1975 and Yamane et al. 1975 & 1977). Reasons for this difference remain to be investigated.

The percentage distribution of radioactivity into various parts of the plant after feeding ^{14}C-GA_3 to an NF or PF leaf at certain stages of the flush cycle is shown in Figs. 37 & 38). With both NF and PF leaves as the donors, export of ^{14}C during all the phases shows both a downward and upward movement of the hormone. The major portion of the radioactivity was distributed basipetally with one exception when a PF leaf at I-2 was the donor leaf, when the movement of ^{14}C-GA_3 was predominantly acropetal (Fig. 39).

Looking now at the more detailed distribution pattern of ^{14}C into the plant parts (Fig. 37) when NF leaves are donors, it is seen that at the F-2 stage 16% was to other NF leaves, 3% to the bud, while the downward movement included 53% in all PF leaves, 13% in SB and 17% in the roots (main and lateral combined). At the I-1 stage 28% of the radioactivity was exported to the NF leaves, 5% to SA and 1% to the B, while the downward movement to PF_1 leaves was 66% and no radioactivity was detected in other older PF leaves or the SB or roots. By the end of the maturation phase (1-2), there was a decline in export of ^{14}C to the bud (0.58%), to the NF leaves (2%) and the SA (6%), but the radioactivity in all PF leaves was still high (30%) and there was an increased amount in the SB (25%) and in the roots (34%).

When a PF leaf was the donor, the ^{14}C distribution at the F-1 stage was 5% to the bud, 5% to the SA and 26% to other PF_1 leaves. The downward movement resulted in the presence of radioactivity in all PF leaves at 28%, 16% in the SB and 20% in the roots. During the expansion phase, F-2, the ^{14}C-activity in the roots remained very constant (18%), while in the bud it declined to 1% and the NF leaves to 6%.

Fig. 37 The percentage distribution of radioactivity after uptake of ^{14}C-GA_3 by a NF leaf at F-2 (a), I-1 (b) and I-2 (c). MR, main root; LR, lateral roots; SB, stem below donor leaf; PF_s, previous flush leaves; PF_1, first previous flush; NF, new flush leaves; SA, stem above donor leaf; B, apical bud.

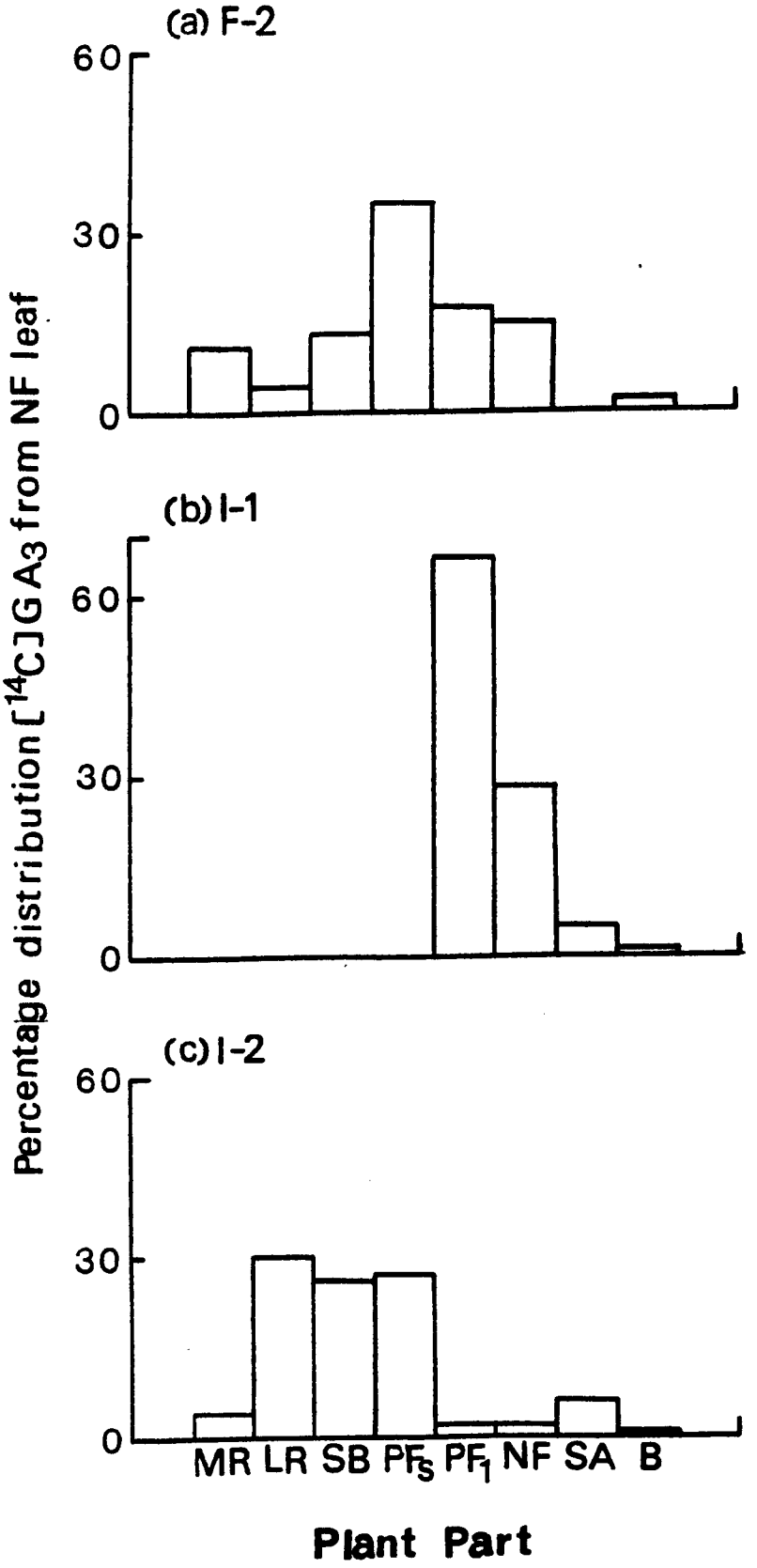

(a) F-2
60
30
0
(b) I-1
60
30
0
(c) I-2
60
30
0
Percentage distribution [^{14}C] GA$_3$ from NF leaf
MR LR SB PF$_S$ PF$_1$ NF SA B
Plant Part

Fig. 38. The percentage distribution of radioactivity after uptake of ^{14}C-GA_3 by a PF leaf at F-1 (a), F-2 (b), I-1 (c) and I-2 (d). MR, main root; LR, lateral roots; SB, stem below donor leaf; PF_s, previous flush leaves; PF_1, first previous flush; NF, new flush leaves; SA, stem above donor leaf; B, apical bud.

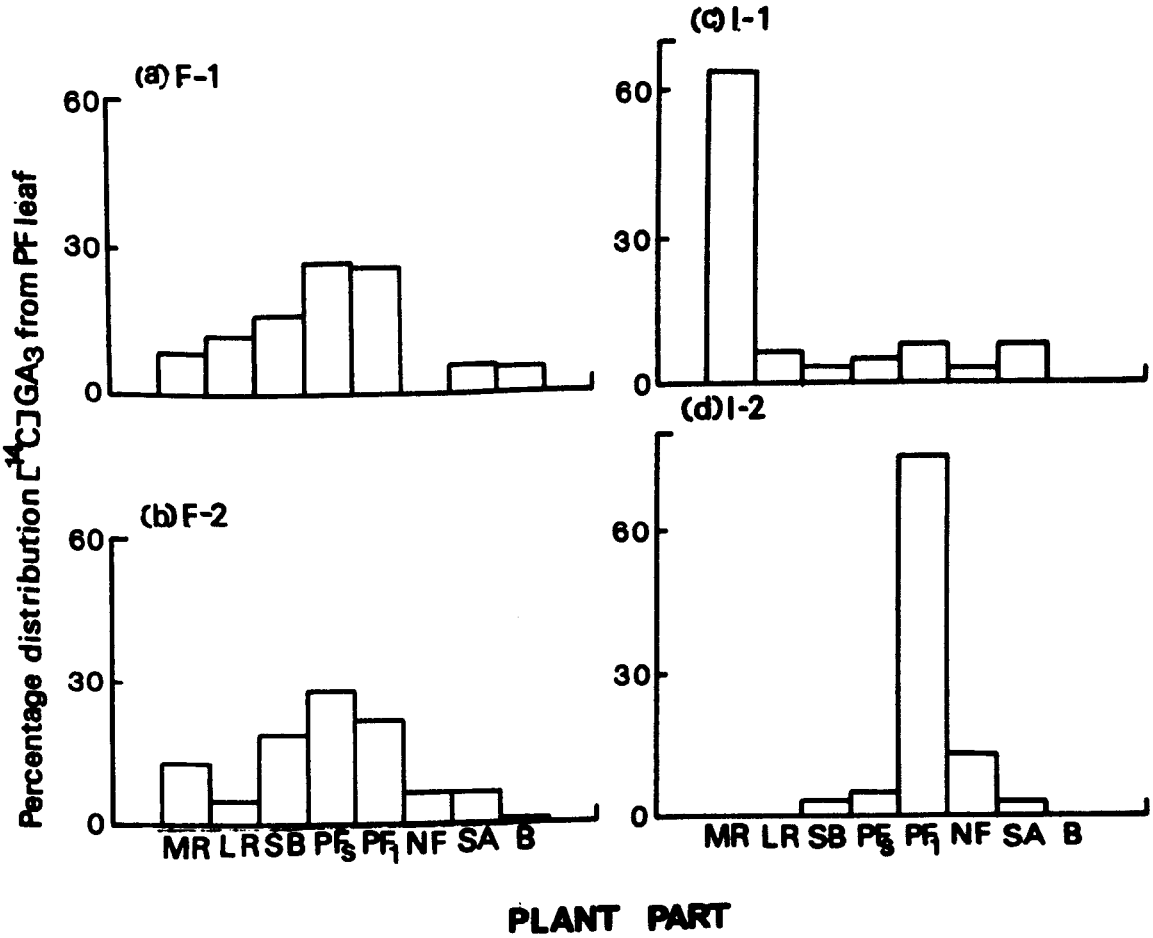
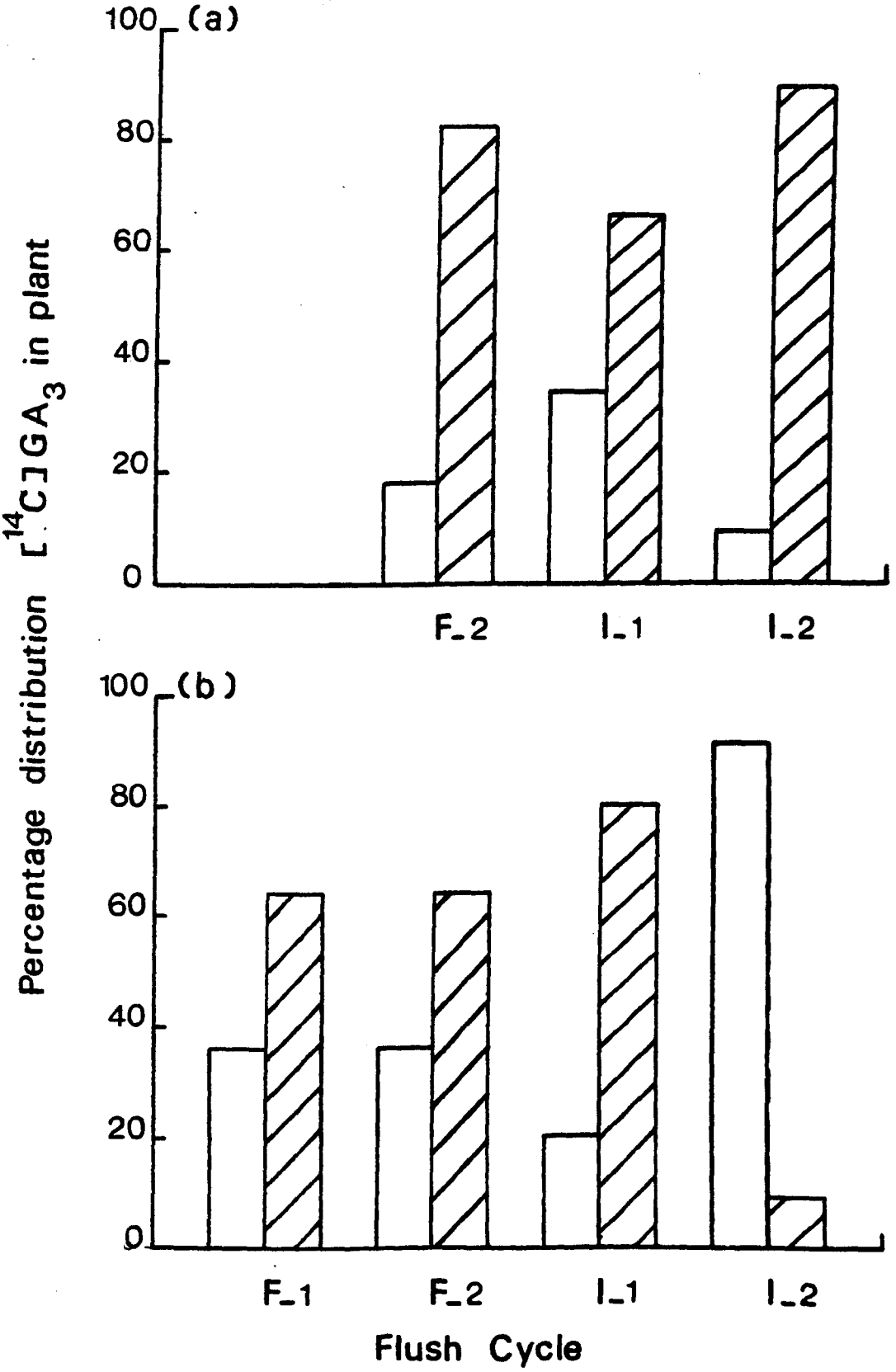

(a) F-1
(b) F-2
(c) I-1
(d) I-2
60
30
0
MR LR SB PFs PFi NF SA B
Percentage distribution [14C]GA3 from PF leaf
PLANT PART

Fig. 39. Percentage distribution of ^{14}C-GA_3 above (□) or below (▨) the donor NF (a) or PF (b) leaf at different stages of the flush cycle (F-1, F-2, I-1 and I-2).


100
(a)
80
60
40
20
0
F_2
I_1
I_2
Percentage distribution [^{14}C]GA$_3$ in plant
100
(b)
80
60
40
20
0
F_1
F_2
I_1
I_2
Flush Cycle

In the SB it was 19%, SA 6%, the PF_1 leaves 22% and all the other PF leaves 28%. During the I-1 stage, most of the transported radioactivity was detected in the roots (70%), while ^{14}C in the bud declined to 0.22%, NF leaves to 3%, SA to 8%, PF_1 leaves to 8%, all other PF leaves to 6% and the SB to 3%. At the I-2 stage the ^{14}C activity was mostly accumulated in PF_1 leaves (75%), while the activity in the bud continued to decline to a value less than the proportion in previous stages (0.04%), while the other values were approximately the same as at I-1, SA (3%), NF leaves (13%), all PF leaves (5%), SB (3%), except for the roots which now had a very low proportion (0.19%).

When these radioactivity data were expressed on the basis of RSA, Table 6 the bud showed a very high value at F-1 and F-2 stages, then declined through to I-2. In contrast, the lateral roots showed lower RSA values at F-2, I-1 stages with an increase towards I-2 and F-1 stages. The RSA values of the NF leaves showed high values during the expansion phase (F-2), then declined during the maturation stages (I-1 and I-2). At the F-2 stage it is clear that whether the donor leaf is NF or PF, the bud and the NF leaves showed the highest RSA values of all the measured RSA values of the other plant parts. The RSA values of the SA when a NF leaf was a donor at I-1 stage was high then declined at I-2, but could not be calculated at the F-2 stage as no radioactivity was detected. With the PF leaf as the donor, the RSA value of the SA was high at F-1 and remained constant with low values in the later stages (F-2, I-1 and I-2) of the cycle. The MR showed a high RSA value when the PF leafwas a donor at I-1 and declined to low values in other stages.

TABLE 6. RSA values for each part of three replicate cocoa plants after uptake of ^{14}C-GA_3 by either a NF or a PF leaf at different stages of the flush cycle.

Plant part	RSA values at different stages of the flush cycle						
	Donor leaf NF			Donor leaf PF			
	F-2	I-1	I-2	F-1	F-2	I-1	I-2
Bud	39 ± 8.1	14.86 ± 2.12	6.13 ±5	42.77 ±10.5	14.76 ± 6.2	4.21 ±1.35	0.34 ±0.04
SA	-	26.98 ± 4.22	0.99 ±0.81	8.08 ± 2.37	3.26 ± 1.70	4.61 ±0.80	2.35 ±0.30
NF	16.18 ± 3.8	7.08 ± 1.03	6.26 ±2.74	-	3.51 ± 0.38	0.76 ±0.34	1.14 ±0.03
PF_1	1.15 ± 0.23	10.76 ± 2.78	0.13 ±0.10	3.05 ± 1.77	1.88 ± 0.81	0.77 ±0.22	12.37 ± 1.33
PF_2	1.72 ± 1.21	-	0.94 ±0.77	0.41 ± 0.21	0.46 ± 0.39	0.32 ±0.12	0.53 ± 0.04
PF_3	0.72 ± 0.51	-	1.68 ±0.71	0.94 ± 0.42	1.72 ± 0.95	-	-
PF_4	0.68 ± 0.48	-	-	0.40 ± 0.21	1.88 ± 1.08	0.38 ±0.17	0.03 ± 0.004
PF_5	2.11 ± 0.83	-	1.76 ±0.72	2.03 ± 0.89	0.58 ± 0.47	-	-
SB	0.42 ± 0.16	-	0.85 ±0.20	0.49 ± 0.26	1.41 ± 0.89	0.11 ±0.05	0.12 ± 0.002
MR	0.73 ± 0.70	-	0.31 ±0.25	0.54 ± 0.17	0.64 ± 0.03	4.61 ±1.03	0.01 ± 0.001
LR	0.35 ± 0.007	-	2.16 ±0.89	1.13 ± 0.14	0.64 ± 0.35	0.74 ±0.29	-

With a NF leaf as donor at I-1 the RSA of the PF_1 leaves was very high but was lower at F-2 and I-2 stages. The RSA of the older PF leaves was generally lower during all the flush cycle.

6.4.3 Discussion

Gibberellin levels seem to fluctuate in various tissues during plant development. In Picea abies Dunberg (1976) found in the terminal buds and elongating shoots very little gibberellins before bud-break but then a very rapid and conspicuous rise in the content of gibberellins during the period of most rapid shoot elongation. A few days later, when the shoot growth in this species had terminated, gibberellins were again very low. There is thus a great deal of similarity between Picea abies and cocoa. Thus in cocoa leaves the gibberellin levels before bud-break are low then increase rapidly during leaf production and expansion.

When the gibberellin level was high most of the gibberellins detected in the leaves (either NF or PF leaves) were in the free form and the reverse was true when the level of gibberellins was low, most of the detected gibberellins were then in the bound form. This suggests that it might be the free gibberellins which are very active in contributing to the control of leaf production and expansion in cocoa. The bound gibberellins might represent a non-active form, a storage form, which may be hydrolysed at certain times to release the free or active form of gibberellins, although this conversion alone would not be sufficient to account for a large rise in the free gibberellins. There must be either or both new synthesis within the leaves or import from other parts of the plant.

The results for the gibberellin levels in cocoa leaves disagree with those obtained by Krekule (1971) both from the pattern of change and the amounts detected, but the pattern of change in gibberellin levels in cocoa leaves does agree with the results of Wheeler (1960) for French bean leaves and Dunberg (1976) for Picea abies. It is not possible to say why these present data conflict with Krekule's.

Taken together the present results for cocoa and previous ones by other authors for other tissues would seem to indicate a major role for gibberellins in both the breaking of shoot apex dormancy and promotion of leaf expansion.

The pattern of distribution of ^{14}C-GA_3 shows the majority is moved basipetally when either a NF or PF leaf was the donor, with the one exception when a PF leaf was donor at I-2 when the majority of the radioactivity was then distributed acropetally. The reason for this very different distribution pattern at this stage is not clear. Without replication it may have been due to a 'freak' plant, but with the pattern being very similar for three plants, this explanation cannot apply. Some subtle environmental effect may be the reason, but further repeats of the isotope feeding to a PF leaf at I-2 should be undertaken. Although this phase with predominantly acropetal movement is unlike the others, most of the isotope is found in other leaves of the same flush as the fed leaf so there is very little long distance transport at this stage in either direction.

As with other labelled isotopes (chapters 5.4 and 6.2.2) it was a surprise to find so much movement into leaves older than the fed ones. Reasons for this are not apparent either, but Sleigh (1981) with cocoa did also find quite high levels of import of ^{14}C-photoassimilate into

older leaves. For reasons given earlier (see chapter 5) further consideration of isotope movement is best done from the RSA values. Although "Standard Errors" are presented (Table 6) again for reasons already presented it is felt more appropriate to restrict discussion to 'trends' rather than on statistical significance only.

Interpretation of the movement data is thus not easy. However, RSA values do differ in many cases (and significantly too) both; i) within the different parts of the plants at one stage, and ii) for the same plant part at different stages of the growth cycle. Highest RSA values are found for the bud and the magnitude of difference at different stages of the cycle is greatest for this plant part. The bud values show a very clear pattern of change which is consistent for NF and PF fed leaves. The sequence from the highest to the lowest values is F-1, F-2, I-1 and I-2. In general, the pattern is similar for SA and NF, but not for PF or roots which have a low F-2, increasing towards I-1, I-2 and F-1. Levels of transport to the bud and NF are a direct reflection of levels of gibberellins found by bioassay, thus have seems to be a strong correlation with and a specific involvement for, gibberellins in promoting bud and leaf development in intact cocoa plants as has been previously shown for other species (Wheeler, 1960 and Dunberg, 1976) and detached cocoa shoot apices (Orchard *et al.* 1979).

The problem which cannot be evaluated at present is to what extent movement of ^{14}C-GA_3 represents merely passive flow of the exogenously supplied 'hormone' along with the assimilate stream. As discussed in the general discussion (Chapter 7) future work with simultaneous application of hormone and photosynthetic marker could

help to resolve the question. An indication of a difference between the control of transport for hormone and photoassimilate is provided by the RSA values for PF leaves with a PF donor leaf at F-1. This is essentially the stage of bud reactivation and very early leaf growth, when the demand for photosynthate is low. It is at F-2 the major phase of leaf growth, that photoassimilate demand is high, and indeed results of Sleigh (1981) confirm greater photosynthate movement at F-2. The highest RSA for the bud is at F-1 which indicates GA_3 movement and accumulation occurs independently of photoassimilate movement and that GA_3 has a specific role in breaking bud dormancy. Root RSA values are more variable but are in general higher at I-2 and F-1. These are stages when root growth is maximal (Sleigh, 1981) and again could either reflect a role for gibberellins in promotion of root growth or passive movement with assimilate. Perhaps in this case the latter is more likely because gibberellins are not generally thought to play a major role in controlling root growth (Brian et al. 1960; Jansen, 1967; Fullenberg, 1969 and Nanda et al. 1968), rather the roots are a major site of their synthesis (Sitton et al. 1967 and Crozier and Reid, 1970 & 1971).

In conclusion in this chapter it can be said that the data on gibberellin levels and movement pattern of exogenously supplied GA_3 are all consistent with a major role for these compounds in the control of bud activity and leaf development of cocoa.

GENERAL DISCUSSION

Cocoa, like many other tropical trees, does not produce leaves continuously throughout the year, but behaves like an intermittently growing evergreen, exhibiting periods of intensive leaf growth alternating with periods of dormancy. This pattern is maintained even when plants are grown under controlled environmental conditions (Greathouse et al. 1971; Orchard et al. 1980), indicating that under these circumstances at least, the cycle is endogenously controlled. Borchert (1973) produced a computer model of intermittent tree growth, assuming constant environmental conditions, and suggested that this type of growth could result from a feedback interaction between the root and shoot systems. The maintenance of a root system that can support shoot growth probably reflects the basic biological requirement that an organism can only survive, and prosper, if its organs maintain a functional equilibrium. Borchert's model suggested that during the course of shoot growth a large number of leaves are produced. These expand rapidly and at a rate "higher than" the growth of the roots. To restore the balance between root and shoot which existed at the commencement of leaf growth the temporary arrest of shoot development and leaf production becomes necessary. During the period of shoot dormancy root growth continues and restoration of the root/shoot equilibrium is achieved. Although the cocoa shoot has been shown to undergo periods of growth and dormancy Borchert's hypothesis was advanced without any detailed information on root growth.

Vogel (1975) showed there was also some periodicity in root growth in cocoa but the detailed picture of root growth throughout successive flushes of leaf growth has only recently become clear and hence the role roots

may play in determining periodic leaf production can now be evaluated (Sleigh, 1981; Sleigh, Hardwick & Collin, 1979). From these studies marked rhythm of root growth was also found, with a maximum rate of root growth occurring before active leaf expansion (during I-2 early F-1).

The rapid expansion of a flush of several leaves must represent a considerable drain on the carbohydrate balance of the plant, and carbohydrate limitation at this stage could restrict root growth if the developing leaves constituted the stronger sink. Once the new leaves were mature and themselves photosynthetic, the absence of carbohydrate demand for leaf development during shoot apex dormancy would mean an increased supply of carbohydrate for the root system and hence an increase or resumption of root growth is likely. This assumption was tested by allowing leaves to assimilate $^{14}CO_2$ at different stages of the flush cycle (Sleigh, Hardwick & Collin, 1979). Large changes in the pattern of distribution of ^{14}C during the flushing cycle were found, assimilate being preferentially transported to developing leaves during flush expansion and conversely to the lower stem and roots during the time of maximum root growth.

Dormancy of the shoot apex of cocoa was found to be overcome by the removal of the expanding flush leaves of cocoa (Vogel, 1975 and Iserentant, 1976). It has also been observed that more shoot growth could be obtained by transplanting trees to larger containers, presumably facilitating more root growth (Borchert, 1975). This suggests that bud dormancy in the seedlings was due to correlative inhibition rather than a true dormancy. Since the well watered plants

also show periods of dormancy, there is the possibility (as suggested by Borchert 1973) that there may be a water deficit in the plant, resulting from the inability of the roots to supply the shoot with sufficient water during periods of rapid shoot growth.

It has been suggested from studies on other species that an internal water deficit inhibits growth directly by its effect on stomatal aperture, transpiration, photosynthesis, enzymatic activity, and on other physiological processes involved in growth, as well as altering nutrient, mineral and hormonal relationships (Kozlowski, 1968; Plaut, 1971; Livne and Vaadia, 1972; Arad et al. 1973; Da Suva, 1973; Hsiao, 1973; Pasternak and Wilson, 1974). There also seems to be in many species a functional relationship between the plant water status and growth and the levels of various growth substances.

The development of a water deficit usually occurs when a high rate of transpiration is combined with an inability of the root system to absorb sufficient quantities of water to meet the demand. In order to evaluate the significance of plant water status on the control of leaf production in cocoa, the changes in those parameters which might affect the water loss from plants, such as leaf position, surface hairs, stomatal number and development and leaf cuticle thickness were investigated during development of the flush leaf.

Water loss from a leaf surface is determined both by its structure and orientation. For the plant the total leaf surface area - or during development, also the rate of increase in leaf area, will determine the total and rate of increase of water demand the plant will have to satisfy to prevent an internal water stress developing. For reasons which are not

understood, developing leaves of most cocoa varieties, including of course the one used in this study, are much slower at developing photosynthetic ability than they are at increasing leaf area. Apart from the carbohydrate considerations resulting from this mode of development very rapid increase in leaf area could mean high water consumption to support both expansion and (normally) a rapidly increasing water loss through cuticular and stomatal transpiration. It was shown (Chapter 3) that stomata only develop, and transpiration rates rapidly increase from around the time leaves become fully expanded. This could be seen as a way of limiting water demand early in leaf development when consumption in expansion is high. On the other hand slow cuticle development was also shown to be somewhat, although by no means as much as stomatal development, a feature which would mean some potential high water loss early in leaf development. The fact that leaves are i) hairy, this will increase the boundary layer resistance; ii) red pigmented, will reflect heating (long wave) radiation; and iii) hang vertically in early development will all (together with late stomatal development) reduce the water demand on the plant during early leaf development. As such all these modifications could be characteristics evolved to reduce water demand or water stress during leaf development and show such is important in the growth of cocoa. Maximum water loss from leaves occurs from late F-2 when the leaves are fully expanded, horizontally positioned and stomata fully developed. The changes in water loss during the growth cycle was reflected in the uptake of water (chapter 4). The increase in water uptake which occurred with the production of new leaf area was relatively small during the period of leaf expansion of the new flush. The water uptake only showed a large increase at the end of the F-2

and beginning of I-1 stages. It is possible that the large transpiration loss after the late F-2 stage of the flush cycle causes an internal water deficit in the young seedling plant which, in turn, could cause a build up of ABA in the shoot. The correlation between the ABA levels in the shoot and the presence of water deficit in the plant at this stage was thus subsequently investigated.

Cocoa seedlings grown under well-watered conditions showed increases in leaf ABA during the stages of the cycle when the bud was dormant, and then declined at bud break (Orchard et al. 1980). Since there was also a close correlation between high ABA levels and water deficit in the field grown cocoa (Alvim et al. 1974) and also in many other species (Mizrahi et al. 1970; Most, 1971; Zeevart, 1971; Wright, 1972), it is possible that the increase in ABA in the cocoa seedlings is indeed a consequence of water deficit developing in the plant even under optimal irrigation conditions. To establish the role of water status on the control of flush growth in cocoa seedlings, the water status and ABA levels of seedlings were examined at different stages of the flush cycle. To indicate water status of the plants measurements of Relative Water Content (RWC) and leaf water potential (ψ_l) were made on NF leaves starting at the I-1 stage and continued throughout two flush cycles. From both measurements there does appear to be a cyclic change in water status within the plant. Thus, the later stages of F-2, I-1 and the beginning of I-2 are characterised by reduced relative water content and low (more negative) water potentials, whereas the remaining part of I-2, F-1 stage and the beginning of F-2 stage do not show such low values. Relatively speaking then a water deficit does occur in a growth cycle under optimal irrigation conditions, and it coincides with the period when the apex is dormant.

In an attempt to see how sensitive the normal pattern of leaf development was to the water status, experiments were undertaken where plants were water stressed by withholding the supply, and then the RWC, ψ_{leaf} and leaf diffusive resistance followed during leaf development. Droughting of the plants, as suspected, destroyed the rhythmic pattern of change of both the RWC and ψ_{leaf}. The diffusive resistance in the droughted plants increased markedly just prior to leaf wilting. The droughted plants also showed a massive increase in both free and bound ABA. When the effect of drought on the timing of leaf production was examined, it showed that accompanying the decrease in RWC and ψ_{leaf} and large increase in ABA, there was a delay in the initiation of bud break. The I-2 stage was extended in the droughted plants but bud-break did eventually occur even though the plants were heavily water-stressed and wilting. However, the leaves visible at bud break on the droughted plants did not expand until after watering recommenced and even then the initiation of rapid expansion was delayed by four days. These experiments on effects of droughting on apex activity and leaf expansion clearly showed that the initiation of bud-break could still occur during the period of water stress but that leaf expansion could only take place after the water deficit was relieved.

In view of results obtained early it was important to investigate further the site of control factors regulating apex activity. In view of the report of Vogel(1975) that defoliation of the mature leaves at certain times during the flush cycle affected the apex activity, experiments involving leaf defoliation, hormone application and determination of distribution pattern of ^{14}C-ABA were undertaken. Defoliation of either NF or PF leaves of cocoa seedlings reduced the length of the dormant phase of the next flush cycle, thus showing that the mature

leaves were a source of growth inhibition which could affect shoot apical activity. The next problem was to identify the inhibitor. In view of earlier findings, ABA was thought to be a strong candidate. This possibility was strengthened when experiments (Chapter 5) showed that application of ABA to the NF or PF leaves led to an extension of the dormant phase, whereas application of zeatin or gibberellic acid decreased it. Presumably the effect of the growth promoters in reducing the dormant period was to overcome the endogenous inhibitors in the leaves. The fact that these growth promoters stimulated earlier leaf production may also indicate that the control of the timing of leaf production is hormonal rather than due to low carbohydrate availability.

The ^{14}C-ABA fed to a NF or PF leaf at different stages of the flush cycle became distributed to various parts of the plant, including both meristematic bud tissue and mature NF and PF leaves. On the basis of RSA values, the mature leaves which have a very low RSA show minimal uptake of ^{14}C-ABA whereas the bud with a high RSA would seem to be actively accumulating ^{14}C-ABA. The ability of the bud to accumulate ABA seems to vary during the flush cycle so that it is lower during the initial dormant stage of F-2 than during the later dormant stage of I-2. The role of ABA would then appear to be to maintain rather than initiate dormancy in the apical bud.

The importance of the growth promoters in the control of the flush cycle has not had a great deal of investigation up until now, except for the work of Nichols (1957), Krekule (1971), Alvim *et al.* (1974) and Orchard *et al.* (1979). More recently Orchard *et al* (1981) using cocoa seedlings

grown under constant conditions measured the levels of cytokinins and auxins in the NF and PF leaves during the flush cycle. Auxin compounds were present in the flush leaves during leaf expansion (F-2), but immediately after reaching full size and during the period of leaf maturity, no auxins could be detected. The cytokinin levels were also high during leaf expansion, declined in the recently matured NF leaves and then increased again just prior to the renewal of apical bud growth, indicating maybe that cytokinins could be important for stimulating renewed leaf production.

The changes in total leaf cytokinin could be taken as an indication of similar changes in levels reaching the bud, since cytokinins are mainly synthesised in the roots (Van Staden, 1973, 1977). Levels in the leaves (transported from the roots) would thus reflect levels in the whole plant at any one time. Clearly from the work of Orchard *et al.* (1981) cytokinins fluctuated during the flush cycle even though the plants were maintained under constant conditions.

By comparing the work of Orchard *et al.* (1980 and 1981) with the results presented in this work, the following points emerge:

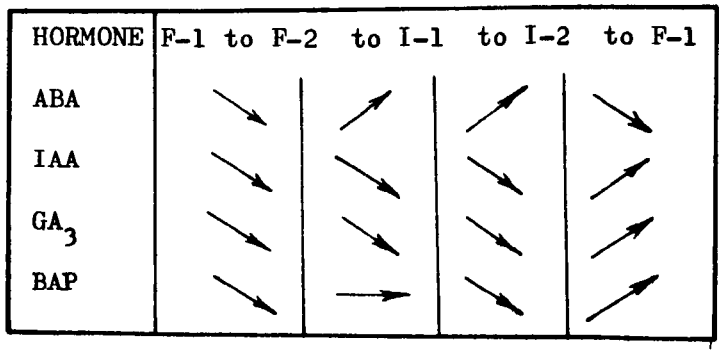
a) the period of water deficit as shown by a low RWC and Ψ_{leaf} during late F-2, I-1 and the beginning of I-2 is accompanied by high ABA and low cytokinin and auxin levels;

b) late I-2 of the flush cycle which was shown here to have relatively high RWC and high (less negative) Ψ_{leaf}, coincided with a decline in ABA levels and increase in cytokinin levels;

c) an inverse relationship between levels of ABA and gibberellins comparable to the relationship between ABA and cytokinin and auxin.

Since the presence of promoters is important in the development of the apex, information about the movement of auxins, cytokinins and giberellins between the leaves and other parts of the cocoa plant at different times of the flush cycle was obtained. The only way this could be investigated was by following the movement of isotopically labelled and exogenously supplied growth substances or analogues. It was appreciated that the results from such experiments must be interpreted with care since for example an increase in level of growth substance within the donor organ as a result of feeding radioactive tracer may produce a rate or export pattern somewhat different to the normal one. However many other workers have adopted very similar approaches to a study of growth regulator movement in relation to developmental studies so such an approach was judged worthwhile (Hocking, 1974; Hocking et al. 1972; Morris et al. 1969; Chovjka et al. 1961; Friedrich et al. 1970). To minimise the problems discussed compounds of high specific activity were used.

The results for the movement of ^{14}C-labelled growth regulators exported from NF or PF leaves can be summarised so as to show the similarities and differences between movement of the four isotopes. This has been described on the basis of trends in RSA values for the buds either as increase ↗ or decrease ↘ between successive stages.



The ^{14}C-growth promoters IAA, kinetin and gibberellic acid, show the same trend of movement to the bud throughout the flush cycle. Thus at the time of bud-break the growth promoters are accumulating in the bud, while the ^{14}C-ABA is low and *vice versa* during the dormancy of the apex when the ^{14}C-ABA accumulates in the apex and the ^{14}C growth promoters decline. The work on the movement of growth regulators shows that the bud changes its ability to accumulate promoters and inhibitors during the flush cycle. It thus establishes quite clearly the important role of the growth regulators in the control of bud dormancy in the cocoa plants.

Conclusions

In order to explain the control of the growth cycle in cocoa it is essential to establish those factors which initiate, prolong and terminate the dormancy period. From the present study it is clear that:

1. Water deficit and the ABA levels do not increase until well after leaf production ceases. This does not agree with Borchert (1973) who suggested that the flush cycle was just controlled by water deficit mediated by changes in the ABA. The results from this project strongly suggest that water stress and resulting ABA maintain but do not initiate dormancy.
2. The basis for the initiation of dormancy may be that only a limited number of leaf primordia develop on the apex and these expand into flush leaves at the same time as the initiation of further primordia. The leaves expanding from the pre-existing primordia create such a strong sink for photoassimilate as to monopolise all photoassimilate exported from mature leaves and prevent any further consecutive primordia

intiation followed by early leaf development. This hypothesis may be supported by work showing that removal of all young cocoa leaves at ≏1.0 cm. long promotes continuous leaf production (Sleigh, 1981). It also supports the suggestion that the initiation of dormancy is carbohydrate controlled.

3. Reactivation of the bud follows the reduction of water stress, ABA levels in leaves and transport from the growth inhibitory leaves to the apex, together with increased levels and transport from leaves to apex of the growth promoters cytokinins and gibberellins. These growth promoters increase before dormancy is broken whereas ABA only declines after dormancy is broken. The dormancy is also broken under an imposed water stress when ABA levels are very high. Thus it is the changing balance of promoters and inhibitors which appears to be more important in causing dormancy break than simply a reduction of inhibitor levels.

Outlines of future work.

1. Most of the growth regulator analyses to date have been carried out on flush leaves since the apex represents such a small amount of extractable tissue. Analysis of the apical buds for their plant growth regulator content during complete flush cycles is now required.
2. To investigate further the effect of plant water status on control of the flush cycle. Experiments to manipulate plant water status are therefore required. Defoliation or reduction of transpiration demand by maintaining leaves / plants in high humidity should reduce water stress in plants and study of consequent timing and rate of development of flush leaves would clarify the importance of water status in growth control.
3. The problem of control of growth regulator movement could be investigated further by simultaneous application of i) combinations of ^{14}C-labelled growth regulators and ii) labelled growth regulators and $^{14}CO_2$. Specificity of control of movement of individual hormones could be then resolved as would the problem to what extent growth regulators move passively with the assimilate stream.
4. The role of the bound forms of the growth regulators in controlling the flush cycle.
5. There is now a growing amount of evidence to show that regulation of plant growth and development by the groups of growth regulators is likely to be dependent on both concentrations of growth substances and sensitivity of sites / receptors within target tissues. This must be borne in mind in future work and experiments to determine the number and sensitivity of receptor sites in the shoot apex throughout growth cycles determined, although it is realised that such a study will be a very difficult one.

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APPENDIX

The psychrometer is widely used as an instrument for measuring plant water potential. However, the instrument does suffer from a number of errors whose sources are described below :

(1) Water loss occurs between cutting the sample and its insertion into the chamber.

(2) Adsorption of water onto chamber walls lengthens equilibrium time.

(3) Excision releases xylem tension, which may cause a rise in water potential.

(4) The solutes released by the cut cells may be actively taken up by the intact cells, followed by the diffusion of the dilute sap into the intact cells hence raising the turgor pressure and water potential. Excised tissue water potential has been shown to be more negative than in situ measurements by 1-3 bars but in some tissues the reverse has been found.

(5) The heat of respiration of the tissue may cause heat gradients affecting the result. Alternatively after an hour the cells may have ceased respiring due to utilisation of cell-available oxygen which can lead to a progressive lowering of water potential to the value of the osmotic potential. (This may be why the results show a steady decline after an hour).

(6) Extraneous sinks for water vapour e.g. salt in the tissue or on the chamber walls may be responsible for long equilibration times. However, salt excretion by the tissue does not seem likely, but removing all traces of salt from a chamber is almost impossible.

(7) In young tissues cells may be enlarging. In the absence of an external water supply the turgor pressure and water potential will decrease until turgor becomes too low for further enlargement. This would cause a lowering of water potential during the equilibration period.

(8) Metabolic changes may occur during equilibration altering osmotic pressure.

(9) Changes in laboratory temperature may affect readings.

(10) The continued decline in water potential with time raises difficulties about the length of equilibration time required in any experiment.