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Growth, Development And Regeneration Of The Tomato Leaf

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GROWTH, DEVELOPMENT AND REGENERATION OF THE TOMATO LEAF

by

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ABSTRACT

An analysis of growth, development and regeneration of tomato (Lycopersicon esculentum Mill. cv. 'Farthest North') leaves was carried out in order to provide a basis from which the regulatory roles of gibberellins in the developmental processes of root regeneration could be examined.

The compound tomato leaf exhibited a basipetal sequence of leaflet initiation and development which could be consistently related to quantitative growth aspects as defined by the leaf plastochron index (LPI). A formerly undescribed marginal vein differentiated basipetally from the leaflet tips and interconnected with derivatives of the secondary and minor veins. Discrete ontogenetic stages were recognized in minor veins as they developed from the upper spongy mesophyll layer in continuity with previously differentiated veins. Vein ending formation was a random process and appeared to depend upon the maturation stage of the surrounding veins. Histological evidence was uncovered to support the concept of "morphological fields" in terms of a close interplay between planes of mesophyll cell division and subsequent minor vein development.

Closely related to the leaf's developmental state was its ability to regenerate organs when isolated from the plant. Two regenerating systems were developed during this investigation: 1) the tomato leaf cutting (relatively autonomous with regard to root initiation); and, 2) the aseptically cultured tomato leaf disc (totally dependent on exogenous nutrients and

hormones). Application of the LPI allowed the consistent description of root regenerative capacity in both systems.

In cuttings, gibberellic acid (GA_3) inhibited root regeneration regardless of LPI_3, provided that GA_3 was applied prior to or during initiation of primordia. Depending on application time and concentration, abscisic acid and various auxins were capable of reversing GA_3-induced inhibition. Rooting inhibition by GA_3 could be correlated with a concomitant inhibition of localized starch synthesis in the incipient root primordia regions, and not with a GA_3-induced hydrolysis of starch in these areas.

A more complex pattern emerged in tomato leaf discs cultured on defined media. Auxin-induced root primordia could originate from two tissue types simultaneously over a seven day period, i.e. type I primordia from phloem parenchyma of the primary and secondary veins, and type II primordia from the sheath parenchyma of minor veins. The use of the leaf disc system revealed multiple roles of GA_3 in the inhibition of rooting: 1) an early inhibitory effect (i.e. during the initial 48 hours of culture) which could be related to a GA_3-induced failure to rapidly accumulate starch within the leaf tissue; 2) a late inhibitory effect which could be related to a GA_3-mediated destruction of late forming primordia by a parenchymatization process which had its origin in an earlier GA_3 effect on growth stimulation of adjacent cortical parenchyma; and 3) an inhibition of meristemoid formation from sheath parenchyma derivatives

surrounding minor veins. This inhibition would insure an absence of type II primordia formation.

Further work with cultured leaf discs indicated a GA_3 stimulation of rooting in continuous darkness which could be related to the specific indole-3-acetic acid (IAA) precursors, tryptophan (TPP), tryptamine (TNH_2) and indole-3-lactic acid (ILA). A study of possible GA_3 effects on endogenous IAA synthesis through continuous feeding of TNH_2-2-^{14}C over a 24 hour period revealed no significant effects on label incorporation although IAA synthesis was detected within the first 6 hours. ILA was observed to be a highly active compound in the induction of rooting in leaf discs and GA_3 increased the activity. It is hypothesized that GA_3 either prevents ILA breakdown leading to increased ILA levels or enhances the synthesis of a highly active indole auxin from ILA.

Results indicated that quantitative and qualitative features of tomato leaf growth, development and regeneration could be consistently defined in terms of the LPI. Furthermore, it appeared that gibberellins could affect root regeneration in tomato by two independent methods: 1) inhibition of rooting through a localized inhibition of starch synthesis and, 2) stimulation of rooting through a GA_3-mediated increase in endogenous auxin levels.

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TABLE OF CONTENTS

LIST OF PLATES

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ABA	Abscisic acid
AMO-1618	2-isopropyl-4-dimethylamino-5-methyl phenyl-1-piperdine carboxylate methyl chloride
CCC	(2-chloroethyl) trimethyl ammonium chloride
cv	Cultivar
2,4-D	2,4-dichlorophenoxyacetic acid
DIECA	Sodium diethyldithiocarbamate
FPA	Formalin-propionic acid-alcohol
GA_3	Gibberellic acid
IAA	Indole-3-acetic acid
IAAld	Indole-3-acetaldehyde
Iald	Indole-3-aldehyde
IBA	Indole-3-butyric acid
ILA	Indole-3-lactic acid
IPA	Indole-3-propionic acid
IPyA	Indole-3-pyruvic acid
LPI	Leaf plastochron index
NAA	Naphthaleneacetic acid
PCIB	4-chlorophenoxy-isobutyric acid
PI	Plastochron Index
TIBA	2,3,5-triiodiobenzoic acid
TLC	Thin layer chromatography
TNH_2	Tryptamine
TOL	Tryptophol (indole-3-ethanol)
TPP	Tryptophan

CHAPTER 1

INTRODUCTION

The most important unsolved problem of biology is that presented by development and differentiation in higher plants and animals (Stebbins, 1964). A dramatic and elusive aspect of this challenge is embodied in morphogenesis or the origin of organic form. In attempting to explore this problem, Sinnott (1960) has suggested that one of the most productive approaches has been to observe the consequences which follow the experimental disturbance of developmental events. With Sinnott's suggested approach as a starting point, I undertook the following study with a view to elucidate the developmental processes of root regeneration in Lycopersicon esculentum Mill. cultivar 'Farthest North'.

Regeneration or "the tendency shown by a developing organism to restore any part of it which has been removed or physiologically isolated and thus to produce a complete whole" (p. 231; Sinnott, 1960) is a reproductive process where the totipotency of individual cells and tissues is expressed in a highly regulated, morphogenetic event. While the arguments concerning the "normal" versus "abnormal" features of regeneration as a developmental event appear

circuitous and unproductive, it is readily apparent that regeneration is unique in certain distinct aspects. By definition, regeneration involves an organ forming at a site usually considered unlikely in view of the normal ontogenetic sequence found in the intact plant. In the early stages of regeneration mature, differentiated cells abruptly leave their mitotically quiescent state and, by a process of rapid and highly localized cell divisions, give rise to small and intensely metabolically active derivatives. This dedifferentiation phase (Buvat, 1944) is followed by the formation of an organ precursor or "meristemoid" (Torrey, 1966) which subsequently develops into a discrete organ primordium. This developmental sequence (i.e. dedifferentiation → meristemoid → organ primordium) is unique to the regeneration process. Another distinct feature of regeneration can be found in the possibility that the existence of an individual may be extended indefinitely (i.e. an eternal life form) through cloning or vegetative propagation of the somatic tissues (Sax, 1962). Nevertheless, regeneration does not appear to be essentially different from the normal features of organ ontogeny in terms of organ type or apparent self-regulation during the development of the regenerating organ. Consequently a study of experimentally induced rooting (i.e. roots formed in response to wounding, detachment, chemical or physical treatments; Haissig, 1969) should provide a direct means of studying the developmental processes

of organogenesis.

While profound advances have been made in the "micro-circuity" of the gene (eg. see Watson, 1970; Wainwright, 1972; Dickson, Abelson, Barnes and Reznikoff, 1975), regeneration studies are still enmeshed in relatively simple, empirical observations due to the complexity of the regenerating systems and the attendant technical problems. As a consequence, this dilemma has brought forth a plethora of generalizations concerning the nature of regenerating factors (eg. "morphogens" such as "rhizocauline" or "factor X": Bouillenne, 1961; Libbert 1956/57) with more recent attempts to link these hypothetical substances to the currently fashionable hypotheses of gene regulation.

If one accepts the dictum of S. J. Holmes (1948, as cited in Bonner, 1963) that a 'true theory of development must be primarily a theory of regulation', then a valid initial approach to the regeneration problem should entail: a) creation of an experimental system which would respond to chemically defined exogenous stimuli (known to occur naturally in that organism) by producing a specific organ type which would, in turn, be defined spatially and temporally; and b) modifying that regeneration response by physical or chemical means with a view to linking (i.e. correlating) the morphological result with a specific biochemical event or pathway within the organism. These concepts form the basis of the present study on regeneration.

The regeneration of roots from detached leaves or leaf

fragments is a potentially useful system for studying the developmental processes of organogenesis due to the relative simplicity of the morphological unit which is regenerated and the ease of experimental induction. Root initiation, whether induced or of a "preformed" nature (i.e. formed during the natural process of branch or stem formation; Haissig, 1969) is a highly regulated and orderly event requiring cell division and enlargement in various dimensions at precise moments in the development of that organ. Since all of the naturally occurring plant hormones (i.e. auxins, cytokinins, gibberellins, ethylene, and the naturally occurring inhibitors such as abscisic acid) have been found to modify cell division and enlargement in a variety of plant systems, it is reasonable to suspect that they have a direct involvement in root initiation. However, the cellular and biochemical events modified or controlled by the major classes of hormones are, in most cases, undefined and hence their roles in root regeneration are unknown.

The present study centres on the delineation of one aspect of the hormonal control of root regeneration: the regulatory roles of the gibberellins. The study attempts to define these roles from a physiological standpoint and to study gibberellin interactions with other plant growth substances and nutrient substrates. Two regenerating systems were developed for this purpose: i) the tomato leaf cutting which is relatively autonomous with regard to root initiation

and, ii) the tomato leaf disc cultured aseptically *in vitro* which is totally dependent on a variety of external hormonal and nutritional stimuli.

During the initial course of this work on organ regeneration, however, a survey of the botanical literature revealed a virtual absence of information concerning the structure and ontogeny of the tomato leaf which would adequately serve as a reference point for subsequent regeneration studies. Specific areas of investigation which necessitated a thorough knowledge of the tomato leaf's developmental history included:

i) *in vivo* aging and regenerative capacity; and

ii) anatomical origins of the regenerated organ.

Furthermore, there was a need for a suitable developmental scale or index in order to accurately describe shoot development and minimize environmentally induced fluctuations in leaf growth and regeneration responses.

Consequently the regeneration studies are based upon an ontogenetic study of the tomato leaf with emphasis placed on the differentiation of the major and minor venation as it relates to the leaf plastochron index (Erickson and Michelini, 1957).

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The following review is, of necessity, rather broad in scope and no attempt has been made to examine the literature in its entirety. For quantitative studies and environmental aspects of leaf development the reader is referred to the reviews of Milthorpe (1956) and Maksymowych (1973) while general histological summaries of leaf structure and ontogeny occur in Foster (1936), Esau (1965a; 1965b) and Cutter (1971). Selected aspects of organ regeneration from leaves are provided by such individuals as Swingle (1940; 1952), Libbert (1956/57), Champagnat (1961), Dore (1965), Audus (1972), Hartman and Kester (1968), Haissig (1965; 1969), Girouard (1971a, 1971b) and Murashige (1974).

2.2 Organ Development: The Leaf

Although there is a wealth of information concerning the comparative morphology of mature leaves (e.g. Von Ettinghausen, 1861, as cited by Pray, 1954; Wylie, 1946; Philpott, 1953), relatively few detailed developmental studies of monocotyledonous and dicotyledonous leaves have

been carried out. The earliest (albeit superficial) studies of leaf development appear to be those of Malphighi in the seventeenth century (Goebel, 1905). However, it was not until the extensive studies by Wolf in the 18th century and Nageli, Trecul, Eichler, Goebel and Prantl in the 19th century (as cited by Goebel, 1905) that the basic features of leaf initiation and development were revealed. These studies indicated that:

i) leaf primordia arise from a vegetative point or apex as a lateral outgrowth from the apical dome;

ii) intercalary cell formation and subsequent cell enlargement may proceed in a basipetal or acropetal direction or may take place equally throughout the developing leaf;

iii) both initiation and early development of leaves from different species may initially appear quite similar histologically; and,

iv) lamina development of foliar organs may be quite variable (e.g. see discussions by Goebel, 1905 and Foster, 1952).

Although these studies established the basic framework of leaf ontogeny, they were of a preliminary nature due to restrictive histological techniques, inadequate sampling, disproportionate emphasis placed upon the mode of leaf origin to the neglect of later phases of development (e.g. ontogeny of the leaf vasculature) and the strong tendency towards phylogenetic interpretations.

Avery's (1933) classical work on Nicotiana tabacum leaf differentiation and his later attempt (Avery, 1935) to

relate endogenous auxin levels to growth and development of the tobacco leaf, served to initiate a serious reconsideration of leaf differentiation from a developmental/physiological point of view. Although relying heavily on serially sectioned leaf material, he was able to trace the developmental pattern of the major and minor venation and to distinguish among the procambium, phloem and xylem. Furthermore, his careful mapping of regions of intensive leaf growth and correlated studies of endogenous auxin levels suggested a positive relationship between the two parameters. Related work by Goodwin (1935) in Solidago and Humphries and Wheeler (1964) in Phaseolus support the concept of high endogenous auxin levels coupled with maximal rates of leaf growth in dicotyledonous plants.

Foster's (1935) leaf ontogeny study of black hickory (Carya Buckleyi var. 'Arkansana') and his subsequent review (Foster, 1936) brought together his own extensive research on leaf development with the varied and widely scattered literature of the past one hundred years. Foster pursued Avery's desire to acquire a more detailed and concrete knowledge concerning the manner in which the successive stages of leaf development (i.e. leaf initiation, origin and differentiation of the procambium, petiole, midrib, and lamina) were interrelated and, ultimately, to derive valid generalizations concerning leaf ontogeny in the angiosperms.

Although a number of diverse types of marginal growth and lamina differentiation had been recognized by the time

of Foster's (1936) review, Hara (1957) was the first individual to attempt a complete classification based on the relative roles played by the marginal and submarginal initials in delineating the lamina. Considerable variability in the marginal growth features of most dicot leaves probably limits the usefulness of this classification (Cutter, 1971).

Foster subsequently emphasized the paucity of knowledge concerning the histogenesis of leaf venation and elaborated the specific questions and techniques necessary for probing venation ontogeny (Foster, 1952). In his view, it was evident "that the present limited knowledge of the histogenetic development of venation patterns represents a serious gap in our understanding of vascularization processes in plants and that it also prevents a full insight into the morphology of the leaf in angiosperms" (p. 752). Foster believed that the use of paradermal sections was of key importance in any study of the origin and interrelationships of the procambial network in the young lamina and proceeded to elucidate these features in his accompanying study of Quiina pteridophylla - the first such study to appear in scientific literature.

Foster's (1952) work with Quiina and related genera initiated a renewed interest in leaf ontogeny. His student, Thomas Pray, carried out extensive research into the morphology and vascular ontogeny of the monocotyledon, Hosta, the dicotyledon, Liriodendron tulipifera (Pray, 1954; 1955a,

b, c) and the leptosporangiate ferns Nephrolepis and Regnellidium (Pray, 1960; 1962). By examining serially sectioned leaf material as well as whole mounts of cleared leaves at different developmental stages, he was able to outline the differentiation of entire vascular systems in Liriodendron and Hosta. In Liriodendron, his observations included the following:

i) the secondary veins, which are multiseriate in their origin (i.e. when viewed in paradermal section) from the upper and middle spongy mesophyll layer of the developing lamina, start to differentiate soon after lamina initiation and develop progressively toward the margin.

ii) while all intersecondary veins (i.e. the tertiary and higher order veins of uniseriate origin) differentiate simultaneously and continuously between existing procambial strands until the ultimate areoles are formed, vein endings develop progressively from the strand delineating the areoles.

iii) phloem maturation precedes the xylem and is continous while xylem development is discontinuous in all vein categories except the vein endings.

Similar studies by Slade (1957; 1959). De Sloover (1958), Ramji (1961a; 1961b; 1967), Hara (1958; 1962), Arnott (1962), Lersten (1965) Olson, Tibbetts and Struckmeyer (1969) and Herbst (1972) on a number of different angiosperm species showing distinctive leaf venation patterns, have essentially substantiated Pray's conclusions. However, some controversy

has arisen over the nature of vein ending formation.

Primarily on the basis of her work with Liriodendron tulipifera, Slade (1957; 1959; 1961) hypothesized that vein endings may originate by two means:

i) a progressive differentiation of the vein into the areole. The works of Pray (1955a) and Arnott (1962) demonstrate this ontogenetic feature most clearly.

ii) a separation of an intact minor vein due to tensions developed in the expanding lamina.

In the light of Slade's suggestion for vein endings arising by separation of previously intact minor veins in Liriodendron, Pray (1963) exhaustively re-examined his own work as well as Slade's and conclusively demonstrated that - in this species at least - no evidence could be found to substantiate the vein separation idea. No further support for this method of vein ending formation has been presented.

Evidence has been presented by Hara (1962), however, which suggests that in the reduced spring foliage leaves of Daphne, vein endings may be produced through the maturation of potential procambial strand portions (i.e. "extension cells" beyond the endings) into mesophyll parenchyma. Lersten (1965) believes this idea to be applicable to the histogenesis of vein endings in Trifolium wormskioldii although Pray (1963) could find no evidence for such a possibility in Liriodendron, Smilax or Aucuba. A subsequent examination of vein ending and disjunct veinlet formation in Euphorbia forbesii by Herbst (1972) has also supported Hara's

concept. Hara's hypothesis is quite similar to Goebel's idea that vein endings are incomplete connecting veins (Goebel, 1932 as cited in Foster, 1952).

While specific histological problems of leaf ontogeny were being explored by such individuals as Foster, Pray, Hara, and Lersten, attempts to describe leaf growth and the development of the vasculature in reproducible quantitative terms in Coleus shoots were being carried out by Jacobs and his co-workers (Jacobs and Morrow, 1957; 1958; 1967). Extensive studies of serially sectioned Coleus shoots revealed a close quantitative relationship between the leaf length and the particular stage of xylem and phloem development. Furthermore, stem loci of phloem and xylem initiation were discovered for the first time.

The problems assocaited with "objectively" describing the morphological status of the vegetative shoot apex and subtending leaves demanded an adequate developmental scale. Such a scale was produced for Xanthium by Erickson and Michelini (1957) which they termed the Plastochron Index (P.I.). The criteria for P.I. application included:

i) leaves grow logarithmically and exhibit parallel growth curves during at least part of their development,

ii) successive leaves appear at regular intervals. As a consequence, the P.I. is linearly related to time and serves to specify the developmental status of a shoot.

When the above criteria were fulfilled for a given shoot, Euclidean geometry allowed the derivation of a mathematical

expression describing the developmental status of that shoot (see Erickson and Michelini, 1957):

$$P.I. = n + \frac{\log L_n - \log 10}{\log L_n - \log L_{(n+1)}}$$

where 'n' is the serial number of that leaf (counting from the first true leaf above the cotyledons) which first exceeds 10 mm in length; '$\log L_n$' is the log length (mm) of leaf n; '$\log L_{(n+1)}$' is the log length (mm) of leaf n + 1; and 'log 10' is the log reference length (i.e. 10 mm) which is selected on the basis of the criteria mentioned above.

For studying leaf development, the Leaf Plastochron Index (L.P.I.) is a convenient modification of the P.I.:

$$LPI_a = P.I. - a$$

where 'a' is the serial number for any given leaf on the shoot apex.

Utilizing this morphological index, Erickson and Maksymowych extensively studied the quantitative cellular aspects of petiole and lamina differentiation in Xanthium (Maksymowych, 1959, 1962, 1963; Maksymowych and Erickson, 1960; Maksymowych and Blum, 1966a, 1966b; Maksymowych and Kettrick, 1970; Maksymowych and Wochok, 1969), culminating in Maksymowych's (1973) review book, Analysis of Leaf Development. Similar quantitative studies centering on problems of dicot leaf development were also carried out by Sunderland (1960) on Lupinus albus and Helianthus annuus, Denne (1967) on Trifolium repens, and Williams on

Trifolium subterraneum (Williams and Bouma, 1970; Williams and Rijven, 1970). On the basis of his own studies and a reevaluation of exponential leaf growth data in Xanthium (Erickson and Michelini, 1957) and Chenopodium (Thomas, 1961), Williams noted a typical progressive decline in the exponent or rate of leaf growth with increasingly older leaves (Williams and Bouma, 1970). As a consequence, Williams suggested that the value of the plastochron index "as a biological time scale for the system as a whole is limited because it obscures valid properties of the system"(p. 145, Williams and Bouma, 1970). Nevertheless, it is apparent that - given the declining exponent values for successive leaf growth - the P.I. is a valid criterion for leaf growth and development studies, provided the growth behavior of the entire shoot system has been previously defined and shown to be reproducible.

The validity of the P.I. as a basis for the physiological/biochemical analysis of leaf development has been demonstrated by a limited number of researchers (e.g. Michelini, 1958; Holowinsky, Moore and Torrey, 1965; Lowenberg, Chen, Towill and Lowenberg, 1970; Isebrands and Larson, 1973).

The quantitative studies contributed greatly to an understanding of leaf development in dicotyledonous plants by: a) providing a relatively objective morphological index (the Plastochron Index) which is superior to a time scale in allowing precise descriptions of developmental events within the leaf, and b) allowing diverse features of leaf ontogeny

(eg. lamina differentiation and expansion) to be described in quantitative cellular terms. Perhaps the single most important concept to emerge, however, is the idea that the endless variety of leaf forms observed may be due to the duration and relative activity of the five interrelated, meristematic regions of the developing leaf (i.e. the apical, intercalary, adaxial, marginal and plate meristems as outlined by Cutter, 1971). The use of single gene mutants affecting leaf shape may prove to be an important experimental approach to this problem. Initial work in this direction with single gene mutants (eg. Hammond, 1941; Whaley and Whaley, 1942) or distinct plant populations (Kaplan, 1970) has supported the idea of form modification via altered activity of specific meristematic regions within the developing leaf primordia.

2.3 Organ Regeneration: Roots from Leaves

2.3.1 General Considerations

As Knox and Evans (1966) point out, one of the most challenging biological problems concerns the question of how a specific hormonal stimulus can initiate and control the development of a three dimensional structure. As a prelude to answering such a question, however, one must know the ontogenetic events at the tissue and cellular levels accompanying this phenomenon.

The descriptive aspects of root regeneration have been

studied with varying degrees of thoroughness by a large number of researchers (e.g. see reviews by Swingle, 1940, 1952; Esau, 1965a; Haissig, 1969). In general, most root primordia have their origins near developing vascular tissues (Esau, 1965a) although "induced" primordia have been initiated in virtually all tissues capable of re-differentiation (Haissig, 1969). However, there is a strong tendency on the part of researchers to work predominantly with large morphological units (e.g. the plant shoot) rather than stem pieces, whole leaves or leaf fragments. Developmental studies of root regeneration from leaf cuttings or in vitro cultured leaf fragments at the anatomical level are virtually non-existent. Furthermore, the use of leaf cuttings or in vitro cultured leaf fragments has generally not been directed towards regeneration studies. Instead, their use has been directed towards plant pathology problems and the physiological aspects of senescence (eg. Yarwood, 1946; Simon, 1967; Spencer and Titus, 1973).

As Table 2.1 indicates, the few studies of adventitious root initiation in stem cuttings of tomato appear evenly divided as to the roles played by the vascular parenchyma, adjacent pericycle and endodermis. In IAA treated tomato stem cuttings, Borthwick, Hammer and Parker (1937) observed that the proliferating derivatives of the external phloem parenchyma formed the external and adventitious roots while the internal phloem and adjoining pith cells form internal adventitious roots. According to Borthwick, the endodermal

Table 2.1. Histological origins of induced root primordia in tomato.

Organ studied	Inducing stimulus	Initiating tissue	Contributing tissue	References
shoot	wounding, IAA	phloem parenchyma	endodermis	Borthwick et al. (1937)
shoot	wounding, IAA	phloem parenchyma (also interfascicular cambium)	pericycle	Dorn (1938)
shoot	wounding	phloem parenchyma pericycle		Hayward (1938)
shoot	wounding, α-naphthoxyacetic acid	pericycle	phloem parenchyma endodermis	Bausor et al. (1940)
shoot	wounding	pericycle	endodermis, phloem parenchyma, differentiating sieve tubes, companion cells	Buvat (1944)
shoot	wounding	pericycle	endodermis	Bynre and Aung (1974)

cells proliferated and formed a "covering" (root cap?) over the adventitious roots while the pericycle, which would normally mature as fiber cells, played no role in regeneration. A similar study by Dorn (1938) reached essentially the same conclusions.

Bausor, Reinhart and Tice (1939) studied α-naphthoxyactic acid effects on tomato stem cuttings. They found that the small-celled derivatives of the pericycle formed the conical tip of the primordium while the endodermal cells formed the root cap. The phloem parenchyma proliferated with the derivatives forming the inner structure of the primordium next to the cambium. Similar results were presented by Bynre and Aung (1974).

The discrepancy with regard to the role played by the pericycle in primordium formation appears to be one of terminology. As Bausor notes, no sharp distinction can be made between the pericycle and phloem parenchyma in tomato. Metcalfe and Chalk (1950) point out that the term "pericycle" probably refers, in many dicotyledonous plants, to the outer part of the phloem. Furthermore, Esau (1965a) notes that in many stems the so-called pericycle is, by origin, partly primary phloem and partly interfascicular parenchyma between primary phloem strands.

Subsequent vascularization of the developing root primordium has not been investigated except in a cursory manner by Bausor et al. (1939) who noted that the procambium of the developing root primordium differentiates xylem which

subsequently becomes continuous with the secondary wood (basipetal maturation).

Buvat's (1944) study agreed with Bausor's work with regard to the role played by the pericycle as the initiating tissue of the primordium. Buvat also extended Bausor's observations by noting that derivatives of the differentiating stem sieve tubes and companion cells form vascular connections between the phloem tissue of the stem and the developing procambium of the immature root (i.e. acropetal maturation).

2.3.2 Histochemical Studies

Histochemical studies of tissues during root initiation are few although their valuable contributions towards an initial understanding of the basic metabolic events accompanying regeneration are apparent. In stem cuttings of Hydrangea macrophylla, Molnar and La Croix (1972b) found that an increased concentration of protein preceded a similar increase in DNA content and subsequent cell division in preformed adventitious root initials. They speculated that, in this case at least, root initiation may be blocked by inhibition of DNA replication with a prior requirement for protein synthesis to overcome the blockage. Galle (1965, as cited in Hartmann and Kester, 1968), on the other hand, found an increase in RNA content of the basal tissues of cauliflower leaf cuttings during root initiation but found no increase in DNA content.

Specific protein groups and enzymes have been observed to change their distribution and amount prior to and during root regeneration. In Hydrangea stem cuttings, peroxidase was the first enzyme to show an increase at potential primordia sites prior to any noticeable change in anatomy (Molnar and La Croix, 1972a). Similar changes in peroxidase activity prior to organogenetic events have also been described by Van Fleet (1959) although the significance of these observations is unknown. Van Fleet has hypothesized that the cellular units of peroxidase may constitute a continuous system between primordia and adult tissue and function as catalysts for the reduction of hydrogen acceptors essential to cell division and initiation of primordia. Similar changes were observed by Kaminski (1959) in hypocotyl sections of Impatiens balsamina L.. He found that polyphenol oxidase increased in potential root primordia sites prior to any recognizable anatomical change. The significance of this observation is also unknown although this work was cited as evidence to support the hypothetical rhizocauline concept of Bouillenne and his co-workers (see section 2.4 for a discussion of the rhizocauline hypothesis).

The localized synthesis and deposition of high starch concentrations prior to and during the formation of organ primordia appears to be a necessary prerequisite for successful completion of organ initiation and subsequent development. In IAA treated tomato stem cuttings, Borthwick and his co-workers (Borthwick et al., 1939) noted that

starch grains disappeared from endodermal and pith cells which became meristematic but remained in non-dividing cells. Starch subsequently accumulated around these meristematic regions as they developed into root primordia. Changes in starch content could be correlated with various stages of root primordia development in Hydrangea as well as the number of roots subsequently formed (Molnar and La Croix, 1972a). In particular, starch was found to disappear from the region immediately surrounding the developing root primordium. Similar localized starch changes during bud regeneration (Thorpe and Murashige, 1968; 1970), floral induction (Nakata and Watanabe, 1966; Sadik and Ozbun, 1967; 1968) and embryogenesis (Jensen, 1963) support the original suggestion (Thorpe and Murashige, 1968) that starch accumulation may play a critical role in the initiation of organized plant structures. In general, the few histochemical studies of root regeneration have suggested that localized and specific metabolic changes take place prior to and during organ initiation.

2.3.3 Biochemical Studies

Attempts have been made to determine the biochemical events which occur during root regeneration. However, a number of limiting conditions impose themselves upon any conclusions to be drawn from such studies:

a) tissue complexity of the regenerating organ with its attendant biochemical diversity;

b) differential biochemical responses of the various tissues during the regeneration process;

c) the relatively small part of tissue in terms of total volume, mass and metabolic activity involved in organ formation; and

d) the multiple and asynchronous nature of the regeneration response in most regenerating systems.

These limiting conditions combine to restrict the usefulness of the "net" biochemical changes observed. Correlated histochemical and cytochemical studies would greatly aid the biochemically based interpretation.

As Jalouzot (1971) notes, most biochemical studies of animal and plant regeneration have shown an enhanced synthesis of DNA, RNA and protein. These events precede initial cell divisions of the dedifferentiation phase which is obligatory for subsequent primordium initial formation and development. One experimental approach for elucidating the metabolic events of regeneration has been through the use of metabolic inhibitors. Root formation can be blocked by substances which interfere with or modify DNA, RNA, or protein synthesis (Höhm, 1955; Fellenberg, 1965, 1966; Guillot, 1965, 1968; Knypl, 1966; Kaminek, 1967). However, variable results are often observed. Fellenberg (1966) noted that the number of root initials in pea cuttings was unaffected by either actinomycin D or chloramphenicol treatments although the number of roots produced per cutting fell drastically. Kaminek (1967), on the other hand, found that chloramphenicol

treatment of pea cuttings reduced rooting by inhibiting root initial cell development. Mitsuhashi, Shibaoka and Shimokoriyama (1969b) observed that actinomycin D significantly promoted root regeneration in Azukia stem cuttings when applied during the initial 24 hours after excision. During the 24-48 hour period, no effect was apparent.

Similar diverse observations have been made with various purine and pyrimidine analogues which are presumed to be incorporated into, and subsequently produce, defective DNA and RNA which, in turn, causes the synthesis of defective proteins. Fellenberg (1966) observed that 8-azaguanine inhibited rooting in pea cuttings and he was unable to reverse the inhibition by guanine treatment. However, root initial formation was stimulated. Ginzburg (1966) noted a stimulation of root initiation by both 8-azaguanine and 8-azadenine. Inhbition of root formation by 2-thiouracil was not overcome by uracil (Guillot, 1965; Fellenberg, 1966; Knypl, 1966) except in two cases (Höhn, 1955; Mitsukashi, Shibaoka and Shimokoriyana, 1969b). Mitsukashi et al. (1969b) observed that 2-thiouracil inhibited IAA induced rooting in Azukia shoot cuttings when applied during the 24-48 hour period but not during the initial 24 hours. This inhibition was completely reversed by uracil. 2,6-diaminopurine gave similar results while a complete reversal could be brought about by adenine. Interestingly, the same authors reported a promotion of root regeneration in Phaseolus cuttings by 2-thiouracil and fluorouracil (Shibaoka, Anzai, Mitsuhashi

and Shimokoriyama, 1967).

While most of the research in this area using purine and pyrimidine analogues has proved unsuccessful due to apparently overriding secondary effects as well as pronounced differential responses among the various regenerating systems, a few studies done with 5-bromouracil (5-BU) have provided more positive results. Both Melichor (1964, as cited by Kaminek, 1967) and Fellenberg (1965; 1966; 1967) have demonstrated an apparent stimulation of root initiation by 5-BU. In the latter case, Fellenberg observed that stimulation of root regeneration in pea cuttings occurred if 5-BU was applied at the start of an experiment or after 36 hours (Fellenberg, 1967). Application of the mutagen during the 12 to 24 hour period inhibited root regeneration and this effect was reversed by simultaneous thymidine application (Fellenberg, 1966). Fellenberg interpreted these observations as indicative of the necessity of RNA and protein synthesis during the early period (i.e. 12 to 24 hours after initiation of the experiment) of IAA induced root initiation.

Although work with antimetabolites has indicated temporally specific syntheses of important macromolecular groups, a second, and more direct, approach to biochemical changes during root regeneration has been the extraction, separation and quantitative measurement of various molecular species coupled with estimates of the endogenous synthesis of these molecules.

A RNA fraction rapidly labelled with ^{32}P was found in IAA treated leaf cuttings of a Euphorbia species (Böttger and Lüdemann, 1964). This fraction was interpreted to be mRNA and was shown to increase in specific radioactivity until the root primordia began to elongate rapidly and subsequently penetrated the overlying tissues. While no subsequent studies have been pursued, it is known that IAA treatment can lead to enhanced mRNA synthesis in non-regenerating systems (Key, 1969; Davies, 1973).

Brown and Mangat (1971) examined RNA and free nucleotide changes during root initiation in detached leaves of Phaseolus vulgaris L. They found that the free nucleotide pools initially increased and then decreased (with the exception of UTP) prior to root emergence. Release of nucleotides from existing RNA did not appear to affect these pools either qualitatively or quantitatively. The authors suggested that synthesis of new nucleotides occurred from non-nucleotide precursors. UTP and UDP-glucose increased continually during the rooting response and were believed to be involved in the increased synthesis of polysaccharides (especially cellulose) necessary for rapid root cell wall formation. Increased RNA levels within the leaf cuttings were attributed primarily to ribosomal RNA synthesis. Slight decreases in sRNA and low molecular weight fractions were observed during the rooting period. However, Nanda and Bhattacharya (1973) found two new low molecular weight RNAs within 48-72 hours after IAA treatment of etiolated

stem segments of Populus nigra and suggested that these new RNA molecules may be involved in the production of adventitious roots. Since the RNA fractions were characterized on the basis of electrophoretic separation on acrylamide gels and subsequent staining with Toluidine blue, it is questionable whether the two fast migrating bands were new in the sense of de novo or enhanced synthesis.

During adventitious root initiation in isolated hypocotyl sections of chickpea (Cicer ariatinum), Jalouzot (1971) found two periods of intense RNA synthesis preceding mitosis. The first period occurred almost immediatly after cutting the hypocotyls (i.e. within 4-6 hours). The newly synthesized RNA was suspected of being capable of forming a stable factor which maintained the cutting in an active metabolic state. A subsequent increase in protein synthesis occurred which lasted until 12 to 18 hours after cutting. Protein synthesis then declined. Accompanying this decline, enhanced DNA synthesis occurred from 14 to 20 hours and was accompanied by a second period of intense RNA synthesis. Declines in the DNA and protein synthetic rates preceded the subsequent mitotic activity. However, subsequent work by Jalouzot (1975) on regenerating Vicia shoot cuttings indicates that an enhanced synthesis of protein (possibly an acidic fraction) associated with the chromatin takes place within the first 1 1/2 hours and may be important in subsequent DNA transcription and/or replication.

Enzyme changes during root formation have been explored

by Werner and Gogolin (1971), Brown and Mangat (1970), Simola (1973), Haissig (personal communication, 1971), Guillot (1968), Nanda, Bhattacharya and Kaur (1973) and Frenkel and Hess (1974) (see Table 2.2). In general, the results have demonstrated quantitative changes in enzyme activity accompanying organ differentiation which could be related to enhanced or modified metabolic patterns. Qualitative changes (eg. de novo synthesis of unique enzymatic proteins or isoenzymes) have yet to be observed.

2.4 Hormonal Control of Organ Regeneration

The first published proposal for the existence of a specific root forming substance appeared in 1880 (Sachs, 1880, 1982; as cited in Thimann, 1972). Termed 'rhizocauline', this formative substance was hypothesized to be produced in the leaves and transported basipetally to the cutting's base where root initiation occurred. Bouillenne and Went (1933) developed this hypothesis further and, on the basis of their experiments with root regeneration in Impatiens hypocotyls, suggested that rhizocauline was a thermostable, non-nutritive, specific root promoting hormone formed in the leaves by light and often stored in the cotyledons. However, Went and Thimann (1937) pointed out that at least one of the hormones necessary for root formation was identical to an auxin. Undeterred, Bouillenne and his co-workers further elaborated the theory into a two factor system (cf. Champagnat, 1961) and later, a three factor one (Bouillenne, 1961) on the basis

Table 2.2. Enzyme changes during root formation.

Species	Organ(s) studied	Inducing stimulus	Enzyme studied	Activity change	References
L. esculentum	etiolated seedlings	wounding	peroxidase	increase during rooting	Guillot (1968)
			polyphenol oxidase	increase during rooting	
Daucus carota	root callus cultures	IAA	glutamate dehydrogenase	increase prior to rooting	Werner and Gogolin (1970)
			aspartate amino transferase	slight increase	
			isocitrate dehydrogenase	slight increase	
			acid phosphatase	slight increase	
Phaseolus vulgaris	detached leaves	wounding	RNA phosphodiesterase	0-24 hr. 4x increase then decrease as primordium initiated	Brown and Mangat (1970)

Table 2.2 (cont'd.)

Species	Organ(s) studied	Inducing stimulus	Enzyme studied	Activity change	References
Phaseolus vulgaris	seedlings	wounding	glucose-6-phosphate dehydrogenase	slight decrease	Haissig, (personal communication 1971)
			glyceraldehyde-3-phosphate dehydrogenase (NADP)	10 X increase during rooting	
			glyceraldehyde-3-phosphate dehydrogenase (NAD)		
			citrate synthase	no change	
Atropa belladona	suspension cultures of root callus	removal of exogenous IAA or addition of α-naphthoxyacetic acid	aldose	?	Simola (1973)
			glutamate: oxatoacetate transaminase	decrease	
			alanine aminopeptidase	decrease	
			peroxidase	increase during rooting	

Table 2.2 (cont'd.)

Species	Organ(s) studied	Inducing stimulus	Enzyme studied	Activity change	References
			acid phosphatase	increase during rooting	
			ribonuclease	increase during rooting	
Populus nigra L.	etiolated stem segments	wounding and IAA	IAA oxidase isoenzymes	increased number of isoenzymes	Nanda, Bhattacharya and Kaur (1973)
Phaseolus aureus Roxb.	stem cuttings	wounding, IAA and catechol	IAA oxidase isoenzymes		Frenkel and Hess (1974)
			polyphenol oxidase isoenzymes peroxidase isoenzymes	increased number of isoenzymes	

of what must be considered very indirect and circumstantial evidence. Bouillenne concluded that two different types of substances (more specifically, phenol derivatives or flavones termed "rhizocauline mobile" and auxins) were synthesized in the leaves and subsequently translocated into the cutting base where they would react with each other in certain competent cells containing a polyphenolase enzyme and form the specific root forming substance "rhizocaline fixée". This theory has been criticized by Libbert (1956/57) and Champagnat (1961) who suggest instead that adventitious root formation depends on a complex of endogenous factors. In their view, root regeneration would not be due to a specific factor; rather, a specific group (composed of hormonal and nutritional factors) may initiate the process.

Auxins are the only class of applied hormones which have consistently enhanced root initiation in plants capable of spontaneous regeneration (Dore, 1965; Haissig, 1965; 1969). Whether the auxin enhancement of root initiation depends upon a direct effect at the regenerating site or is due to the mobilization of root-promoting substances from the leaves is unknown. However, the initial differentiation of a root meristem is determined, at least partially, by a localized high auxin concentration (Pearse, 1948 as cited by Figueroa, 1966; Warmke and Warmke, 1950; Heide, 1967) which is often brought about by a metabolically active (and basipetal) auxin transport system (Leopold, 1961; Goldsmith,

1960, 1966; Audus, 1972; Thimann, 1972; Rubery and Sheldrake, 1974). Consequently, the physiological basis for auxin stimulation of rooting would appear to be in the localized internal levels (native or applied) of this hormone acting in conjunction with other plant constituents such as the cytokinins. Cytokinins are known to enhance cell enlargement, and in the presence of auxin, cell division (Letham, 1967, 1969; Srivastava, 1967). However, information on the effects of the synthetic cytokinins, kinetin and benzylaminopurine, suggest that this class of plant hormones prevent or, at least, do not stimulate root initiation (Plummer and Leopold, 1957; Humphries, 1960; Heide, 1965, 1969; Haissig, 1965; Letham, 1969) although exceptions have occasionally been observed (Allsop and Szweykowska, 1960; Miller, 1961; Bachelard and Stowe, 1963). Certainly, the promotive effects of cytokinins in bud regeneration (Skoog and Miller, 1957; Letham, 1969) and lateral root formation (Goldacre, 1959; Torrey, 1963; Bonnett and Torrey, 1965; Letham, 1967) indicate a direct morphogenetic role for this class of hormones in at least some forms of organ regeneration.

Gibberellins enhance cell division and cell enlargement in a number of diverse experimental plant systems (Paleg, 1965; Sachs, 1965; Osborne, 1965). Although organ regeneration may be viewed as a highly regulated process requiring cell division and enlargement, and defined by apparently stringent temporal and spatial controls, applied gibberellins have often inhibited, or at least not promoted

root or shoot regeneration (Brian, Hemming and Radley, 1955; Brian, 1957; Chailakhian and Nekrasova, 1958; Kato, 1958; Kribben and Reisener, 1958; Viana, 1958 as-cited by Urban and Libbert, 1967; Gundersen, 1958; Chailakhian and Butenko, 1959; Miller, 1959; Schraudolf and Reinert; 1959; Brian, Hemming and Lowe, 1960; Dostal, 1960; Chailakhian, Turetskaya and Stowe, 1963; Turetskaya, Kefeli and Kof, 1963; Murashige, 1964; Julliard, 1964; Libbert and Krelle, 1966; Jansen, 1967; Bastin, 1976; Urban and Libbert, 1967; Nanda, Purohit and Mahrotra, 1968; Bigot and Nitsch, 1968; Gautheret, 1969; Thorpe and Murashige, 1968, 1970; Besemer, Harden and Reinert, 1969; Krille and Libbert, 1969; Heide, 1969; Mitsuhaski, Shibaoka and Shimorkoriyama, 1969a; Chin, Meyer and Beevers, 1969; Haissig, 1969, 1972; Kefford, 1973). The inhibitory action of GA_3 on root regeneration has been found to be non-reversible by auxin treatment in some cases (Brian et al. 1960; Heide, 1969; Haissig, 1972). However, other researchers have obtained an apparent partial or complete reversal (Schraudolf and Reinert, 1959; Appelgren and Heide, 1972). Abscisic acid has also been observed to partially reverse (Chin, Meyer and Beever, 1969; Thorpe and Meier, 1973) to have no effect (Heide, 1969) or to enhance (Krelle and Libbert, 1969) GA_3 inhibition of organ regeneration.

Interestingly, a few reports have demonstrated a stimulation of root initiation by gibberellins (Gray, 1958; Spanjereberg and Gautheret, 1964; Julliard, 1964; Nanda,

Purohit, Bala, 1967; Gautheret, 1969; Erickson, 1971; Tizio et al., 1970; Nanda, Anand and Chibbar, 1972; Anand, Chibbar and Nanda, 1972; Varga and Humphries, 1974; Kochva, Buttan, Spiegel-Roy, Bornman and Kochva, 1974; Hansen, 1975).

Although gibberellins may be important regulators in root regeneration, their role at the cellular and biochemical levels is still unclear. An early hypothesis suggested that gibberellins promoted rapid growth of the regenerating organ and hence diverted essential metabolites from the root initiation region with a consequent inhibition of regeneration (Brian, 1957). This "nutrient diversion hypothesis" was subsequently demonstrated to be incorrect when it was shown that GA_3 inhibited root formation in the absence of GA_3-induced growth (Brian et al., 1960). A second hypothesis was developed by Brian and his co-workers which suggested that GA_3 inhibited root regeneration by preventing organized cell divisions in the tissues that would normally have formed the primordia initials. Murashige (1964) also supported this idea on the basis of his observations of bud regeneration inhibition by GA_3 in tobacco callus cultures.

The basis for this hypothesis of GA_3 inhibition of organ initial formation focused on 3 pertinent observations:

a) necessity of a direct and localized GA_3 application at the potential regeneration site;

b) maximum GA_3 sensitivity occurred prior to primordia formation;

c) GA_3 stimulation of random cell divisions leading to callus proliferation while inhibiting organized divisions leading to shoot or root primordia.

Recently a third hypothesis has been suggested (Haissig, 1969, 1972). After applying GA_3 to the root systems of intact brittle willow plants, Haissig noted that the spontaneously forming (i.e. preformed) nodal root primordia within the shoot system possessed reduced cell numbers. This feature became most evident in the older, more re-established primordia. Haissig suggested that, in brittle willow at least, GA_3 treatment reduced intraprimordium cell division on which continued development of established primordia depended.

Although divergent in their choice of the precise regeneration stage affected, the hypothesis presented by Brian et al. (1960) and Murashige (1964) i.e. direct inhibition of organ initial formation, and by Haissig (1969; 1972) i.e. direct inhibition of organ initial development, both support the general idea that the inhibitory action of GA_3 is maximal at a very early stage of organ formation.

Recently, Thorpe and his co-workers have put forth a biochemically based hypothesis to explain the early inhibitory effects of GA_3 on shoot formation in tobacco callus cultures (Thorpe and Murashige, 1968, 1970; Thorpe and Meier, 1972, 1973, 1974a, 1974b; Ross and Thorpe, 1973; Ross, Thorpe, and Costerton, 1973; Thorpe, 1974).

Based upon the premise that localized starch

accumulation functions in the initiation of organized plant development, Thorpe and his collegues subsequently observed a positive correlation between starch accumulation, shoot formation and respiratory activity in tobacco callus cultures. The starch accumulation occurred prior to any organized development, and both starch accumulation and organ regeneration were suppressed in the presence of GA_3. Although it was suggested that enhanced starch degradation (primarily by increased α-amylase activity) was characteristic of the GA_3 response (Thorpe and Meier, 1974a; 1974b) the data presented showed no pronounced effects of GA_3 on α-amylase activity. Rather, the data suggested that GA_3 treatment repressed the levels of soluble and insoluble starch synthetases as well as increased the level of phosphorylase. The latter enzyme could control the levels of endogenous starch through its reversible role in starch synthesis and breakdown. Instead of producing unphosphorylated sugars via the α-amylase/β-amylase/β-glucosidase route, an energetically more favourable pathway would be found in the production of the glycolytic precursor, glucose-1 phosphate, via the phosphorylase system. However, this possibility at present remains hypothetical with no evidence presented in the case of root regeneration.

The apparent stimulation of root regeneration (but not bud regeneration) by GA_3 has been observed in enough diverse organogenic systems to be considered a valid additional property of the GA_3-mediated regeneration response. Theories

presented to explain this rather paradoxical effect have centred on gibberellin involvement with one or more aspects of auxin metabolism:

a) decreased auxin catabolism;

b) decreased auxin complexing or binding; and/or

c) enhanced auxin synthesis

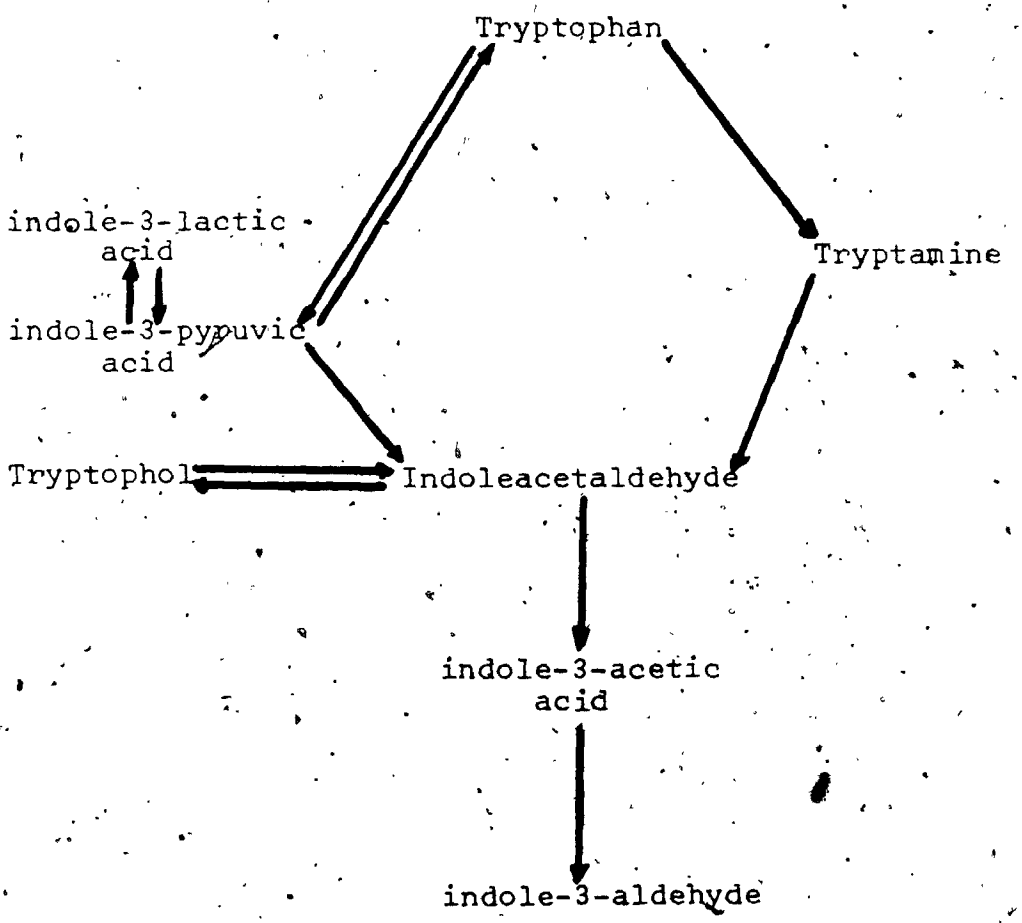
Initial observations on the effects of GA_3 on endogenous IAA levels in non-regenerating systems have often demonstrated increased amounts of diffusible or extractable IAA or IAA-like substances (see reviews by Sachs, 1965; Brian, 1966; Audus, 1972; Thimann, 1972). An early suggestion for this GA_3-mediated effect focused on the hypothesis of decreased auxin catabolism when it was demonstrated that GA_3 treatment could reduce the activity of IAA oxidase *in vivo* (Galston, 1957; Pilet and Wurgler, 1958; Galston and Warburg, 1959; Pilet and Collet, 1960), but not *in vitro* (Kato and Katsumi, 1959). Similar reductions in activity were observed in the related enzyme, peroxidase (McCune and Galston, 1959; Halevy, 1962; Katsumi and Sano, 1968). However, other studies have demonstrated no GA_3 effect on IAA oxidase activity (Kurashi and Muir, 1964; Varga and Balint, 1966) or a GA_3-induced increase (Gaspar and Bouillenne-Walrand, 1966; Bolduc, Cherry and Blair, 1970; Ockerse and Waber, 1970; Lee, 1971). Furthermore GA_3 did not inhibit the decarboxylation of IAA-1-^{14}C in *Coleus* or *Helianthus* stem cuttings (Valdovinos and Ernest, 1966; Valdovinos, Ernest and Henry, 1967).

A hypothesis currently receiving more experimental

support centres on gibberellin enhancement of auxin synthesis (Figure 2.1; for discussions of auxin biosynthesis see Black, 1970; Audus, 1972; Wightman, 1973; Schneider and Wightman, 1974). Muir and his co-workers have demonstrated that GA_3 treatment increases the production of auxin from tryptophan in dwarf pea tissue and in cell-free preparations of Avena coleoptiles (Kuraishi and Muir, 1964; Lantican and Muir, 1967; Muir and Lantican, 1968). Furthermore, GA_3 has been found to enhance the decarboxylation of tryptophan-1-^{14}C in Coleus and Helianthus stem segments and Avena coleoptiles (Valdovinos, Ernest and Perley, 1967; Valdovinos and Ernest, 1967; Valdovinos and Sastry, 1968). The results from both research groups support the concept of a gibberellin promotion of auxin synthesis via the tryptophan → tryptamine → indoleacetaldehyde → IAA pathway.

In root regenerating systems, the available evidence for such a role is meager. Bastin (1967) observed that GA_3 treatment to the shoot bases increased the endogenous levels of IAA-like substances in cuttings of Impatiens balsamina seedlings, while root initiation decreased. Extensive work by Varga has supported the hypothesis of gibberellin stimulation of IAA synthesis from tryptophan using stem tissues and cell free extracts (Varga and Bito, 1967, 1968; Varga, Köves and Sirokmán, 1968; Varga, Köves, Sirokmán and Vito, 1968; and Varga, 1974 as cited in Varga and Humphries, 1974). More recently, Varga and Humphries (1974) demonstrated significantly enhanced rooting of Phaseolus leaf cuttings

Figure 2.1. Probable pathways of IAA biosynthesis from tryptophan in tomato shoots (from Wightman 1973, Biochem. Soc. Symp. 38: 247-275).


Tryptophan
indole-3-lactic acid
indole-3-pyruvic acid
Tryptamine
Tryptophol
Indoleacetaldehyde
indole-3-acetic acid
indole-3-aldehyde

when GA_3 was applied to the lamina in the presence of tryptophan. This result was attributed to an enhanced IAA biosynthesis in the leaf lamina brought about by GA_3 and is contrary to earlier results when GA_3 was applied to the base of Phaseolus stem cuttings and found to inhibit rooting (e.g. Krelle and Libbert, 1969). It is not inconceivable that Bastin could have observed the same phenomena had he treated the leaf laminas with GA_3 instead of the shoot bases where the primordia would subsequently form.

CHAPTER 3

LEAF DEVELOPMENT IN TOMATO

3.1 Introduction

The ontogeny of the determinate organ, the leaf, is a complex, yet highly regulated event which has received relatively little attention from developmental botanists. When the approaches taken towards understanding leaf development are compared between such groups of people as Foster, Pray and Lersten on one hand and Erickson, Maksymowych and Denne on the other (see section 2.2), it is apparent that the ideal study should combine a morphological index of development with the quantitative and qualitative aspects of leaf ontogeny.

The tomato plant, although appreciated as an important vegetable species since the sixteenth century after its introduction from South America (Luckwill, 1943) and presently the basis of a multi-million dollar industry in the United States and Canada, has received virtually no attention from a developmental point of view. Similarly, the tomato plant's extensive utilization in genetical and physiological research with virtually nothing known about its anatomical composition and development has prompted one

noted physiologist dealing with phloem transport to remark that the tomato plant "is as unorthodox in its translocation patterns as in its morphology, but as even that strange confusion of leaves without axillary buds and inflorescences without subtending leaves may be interpreted by a distortion of the ordinary rules of plant architecture, so with a little imagination the distribution of assimilates from tomato leaves can been seen as a twisted version of the usual rules" (p. 66;Canny, 1973). As will be pointed out in this chapter, however, the concept of the tomato plant as a twisted distortion of the ordinary rules of plant architecture as espoused so turgidly by Canny is unrealistic.

While a few studies have explored tomato stem histogenesis (Venning, 1949; Thompson and Heimsch, 1964) with conflicting results, they failed to include a major factor governing stem growth and vascularization - the leaf. Although two studies have been published concerning the origin of the tomato leaf from the shoot apex (Lange, 1927; Hussey, 1971) only one report dealing with the histological development of the tomato leaf has appeared. In his study of the effects of 2,3,5-triiodiobenzoic acid (TIBA) application to tomato (cv. 'Marglobe') shoot apices, Bedesem (1958) included a description of tomato leaf ontogeny which was based solely on serially section material. Bedesem, although he outlined the major developmental features of the tomato leaf, dealt superficially with the histogenesis of the major and minor venation leading to interpretive errors

(e.g. failure to recognize a discrete marginal fimbriate vein). Due to the scarcity of information about the tomato leaf, the present study was undertaken to provide a detailed analysis of tomato leaf growth and development in the cultivar 'Farthest North'.

More specifically, an attempt was made to answer the following questions:

1. Can the growth characteristics and developmental states of tomato leaves be consistently related to the plastochron index as developed by Erickson and Michelini (1957)? Similarly, can various biochemical parameters be related to the developmental states of the tomato leaf?

2. What is the specific ontogenetic sequence of events with regard to:

i) early growth of the leaf primordium and the apparent roles played by the five classes of meristems as outlined by Cutter (1971);

ii) timing and sequence of xylem and phloem differentiation in the major and minor veins; and,

iii) the pattern of cell division activity throughout the leaf during the course of development?

In view of the vascular origins of regenerating roots, it is readily apparent that a thorough knowledge of the tomato leaf's vasculature and its development is a necessary prerequisite for subsequent regeneration studies.

Furthermore, as noted in Chapter 1, there is a need for a developmental index which would be superior to a time-based

description of plant material by virtue of its ability to minimize environmentally and, to a lesser extent, genetically induced fluctuations in growth and regeneration responses.

3.2 Materials and Methods

3.2.1 Growth and Biochemical Studies

3.2.1.1 Seed and Plant Production

Seeds of Lycopersicon esculentum Mill., cv. 'Farthest North' used in this study, were initially obtained from T. and T. Seeds Ltd., Winnipeg, Manitoba. Subsequent seed production from the original stock was carried out by the author. All seeds were washed for 24 hours prior to sowing in order to promote rapid and uniform germination.

The plants were raised under greenhouse conditions in wooden flats throughout the year. Natural daylight conditions supplemented with a 16 hour photoperiod of combined fluorescent (Sylvania Gro-Lux) and incandescent lighting were provided. In general, night temperatures of 60-65°F and day temperatures of 75-85°F prevailed. During their development, seedlings were periodically thinned out and all morphological abnormalities (e.g. multiple cotyledons, leaf fusions) were discarded. Emphasis was placed on the growth and development of true leaf number 3 (counting from the first leaf above the cotyledons) in these studies. Unless otherwise noted, all further references are to this

leaf position.

3.2.1.2 Growth Measurements

In order to evaluate the feasibility of applying the P.I. to tomato shoots in the initial studies of leaf and leaflet growth, vernier calipers were used to measure length and width of the leaves and leaflets of 10 randomly selected seedlings to the nearest 0.1 mm at 2 day intervals from January to March 1971. Later studies relied on a clear plastic ruler graduated in mm. Leaf areas were measured using an EEL (Unigalvo 200) photometric leaf area meter (Evans Electroselenium Ltd., Halsted, England) and the readings converted to the nearest cm^2 using a calibration curve provided. This method proved more rapid and gave area values similar to the determinations obtained by a compensating polar planimeter (Keuffel and Esser Co.) from leaf tracings.

Fresh and dry weights of individual leaves were obtained to the nearest 0.1 milligram. In the latter case, leaves were dried at 105°C for a minimum of 48 hours prior to weighing.

3.2.1.3 Chlorophyll Determinations

Chlorophyll a/b levels were determined using a modified version of Bruisma's (1963) method. Instead of homogenizing the leaf samples in a blendor, the individually weighed leaves were ground in a chilled mortar and pestle with a

small amount of purified sand, magnesium carbonate and acetone. All operations were carried out in an ice water bath (2-4°C) in a dimly lit room. The resulting slurry was quantitatively transferred onto a sintered glass filter and the extract filtered into a flask. The residue on the filter was reextracted twice with acetone and the combined extracts made up to volume using acetone and distilled water (80% acetone v/v). The aqueous acetone extracts were stored in foil wrapped volumetric flasks (either 10 ml or 25 ml capacity depending on the initial fresh weight of the particular leaf). Samples of the extracts were measured in 1 cm ground stoppered glass cells against 80% aqueous acetone in a Beckman DB spectrophotometer at 645, 652, and 663 nm. All samples were measured within 2 hours of extraction with a 10 minute period at room temperature in order to avoid condensation on the spectrophotometric cells.

The concentrations of chlorophyll a,b, and a + b, in milligrams per liter, were computed from the following equations:

$$C_a = 17.2\ A_{663} - 2.7\ A_{645} \quad (1)$$

$$C_b = 22.9\ A_{645} - 4.7\ A_{663} \quad (2)$$

$$C_{a+b} = 20.2\ A_{645} + 8.0\ A_{663} = 27.8\ A_{652} \quad (3)$$

As noted by Bruisma (1963), the two calculations of C_{a+b} should lead to similar values. Deviations larger than a few per cent would indicate either inaccurate adjustment of wave lengths or the occurrence of breakdown products. The chlorophyll a/b ratio was also calculated.

3.2.1.4 Total Soluble Carbohydrate Determination

Oven dried leaf samples were ground to a fine powder with mortar and pestle and 10-50 mg weighed out and placed in 15 ml Pyrex centrifuge tubes. A few drops of 80% ethanol was added to prevent clumping followed by 1 ml of distilled deionized water. The suspension was stirred thoroughly, placed in a hot water bath at approximately 70°C and 5 ml of hot 80% ethanol was added. The mixture was stirred continually for 10 minutes and then spun at high speed (4,000 rpm) for 10 minutes in a table top centrifuge (Sorvall General Laboratory Model HL-4 and fixed angle rotor-Type A). The supernatant was removed with a Pasteur pipette and retained (soluble sugar fraction). This extraction was repeated three times. The supernatant fractions were combined and reduced in volume to one or two ml under reduced pressure at 65°C in a Buchler Evapo-mix. The resulting extract was made up to 25 ml in a volumetric flask. Total soluble carbohydrate levels were determined in the tissue extracts using the phenol-sulphuric acid colorimetric method (Dubois, Gilles, Hamilton, Rebers and Smith, 1956). Sucrose was used as a standard over the concentration range of 0 to 125 ugm/ml extract. Three to four determinations were made per sample.

3.2.1.5 Starch-Determination

The resulting tissue pellets prepared as outlined in section 3.2.1.4 were oven dried at 80°C for 48 hours prior to

use. The starch levels were determined using the phenol-sulphuric acid colorimetric method (Dubois et al., 1956) after hot water gelatinization and perchloric acid extraction according to McCready, Guggolz, Silviera and Owens (1950). A glucose standard was used and all values were converted to starch equivalents by multiplying the experimental values by 0.90. Three to four determinations were made per sample.

3.2.2 Leaf Ontogeny Studies

3.2.2.1 Clearing Techniques

Leaf and seedling samples were collected between noon and 2:00 p.m. from greenhouse grown material over approximately a one year period (July 1972 to August 1973) and the P.I. and LPI_3 noted. Over one hundred individual samples were processed by the NaOH-basic fuchsin method of Fuchs (1963) in order to study vascularization (especially the xylem) of the developing leaves. A fast-green counter stain was used as recommended by Fuchs (1960).

Another group of over one hundred samples were fixed in FPA for 24-48 hours and then stored in fresh FPA until the material was completely bleached. Whole leaves and seedlings were washed for 2-3 days in 50% ethanol followed by staining in propriocarmine for 20 minutes at 60°C (Jensen, 1962). Further washing in 50% ethanol was followed by dehydration in a graded ethanol-xylene series. The leaf material was permanently mounted on glass slides using

Permount. This technique proved ideal for studying mitotic activity distribution and major and minor vein development.

3.2.2.2 Histological Technique

Fifteen seedlings were fixed in FPA, dehydrated and embedded in Paraplast. Five μ thick serial sections were prepared from transversely or longitudinally cut material. The sections were dewaxed in xylene and hydrated through a t-butanol-distilled water series. Staining was carried out either in aqueous 0.05% Toluidine blue or tannic acid-ferric chloride-safranin (Jensen, 1962), dehydrated and permanently mounted in Permount. Over fifty leaf primordia from inception to approximately 1500 μ long were studied by this method. Fifty individual leaflets selected at LPI_3 = -2.0 and LPI_3 = 0.0 were also processed by the above methods and sectioned paradermally for a detailed examination of minor vein ontogeny.

3.2.3 Photography

All black and white photography was carried out with a Zeiss Photomicroscope II and 35 mm Plus-X film. Film was developed in D-76 (Kodak) in 1:1 (v/v) solution with tap water for 10 minutes at 20°C. The sectioned material which had been stained with Toluidine blue, was photographed at an ASA 80 rating using a light blue/green filter combination in order to reduce the severe contrast condition which otherwise occurred.

3.2.4 Statistical Analysis

Regression, correlation and Chi-Square analyses were performed according to Woolf (1968) with the aid of a Monroe electronic programmable printing calculator (Model 1766).

3.3 Results

3.3.1 Growth and Biochemical Studies

3.3.1.1 P.I. and L.P.I.: Application to the Tomato Shoot

A plot of log. leaf length versus time (January - March 1971) measurements for 1 plant arbitrarily chosen from the 10 plants studied (Fig. 3.1), revealed that successive leaves of the 'Farthest North' tomato cultivar appeared at regular intervals and grew exponentially for at least part of their development. Similar results were obtained with the nine remaining plants. The choice of a reference length for the calculation of the plastochron index values was broadly restricted within the exponential or the log. phase of leaf growth (i.e. between leaf lengths of appoximately 10 mm to 40 mm). Thirty millimeters was selected due to the ease of handling and the eventual use of this morphological index as a basis for regeneration experiments. A nomogram was constructed according to Erickson (1960) in order to calculate fractional plastochron values more quickly (Fig. 3.2). In order to test for a linear

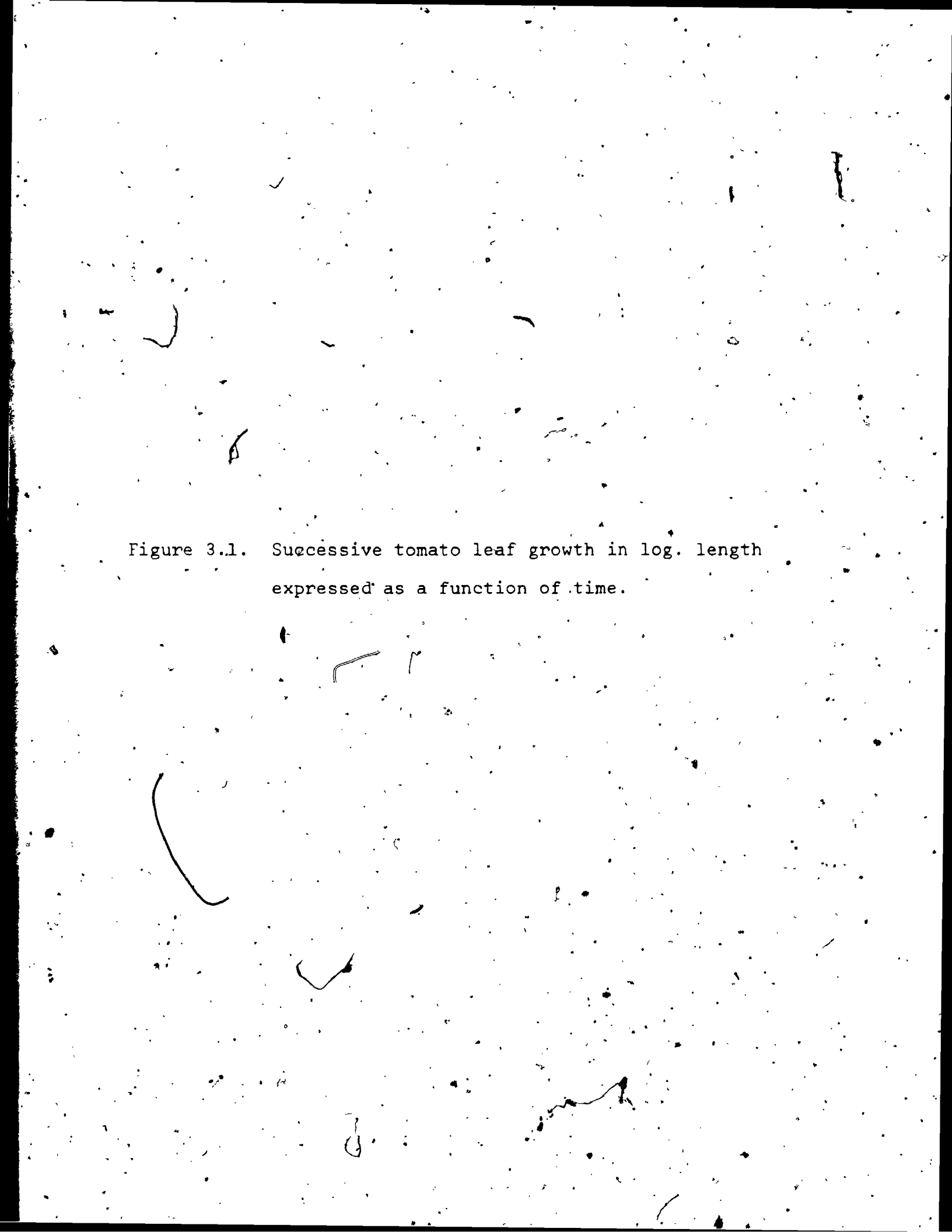


Figure 3.1. Successive tomato leaf growth in log. length expressed as a function of time.

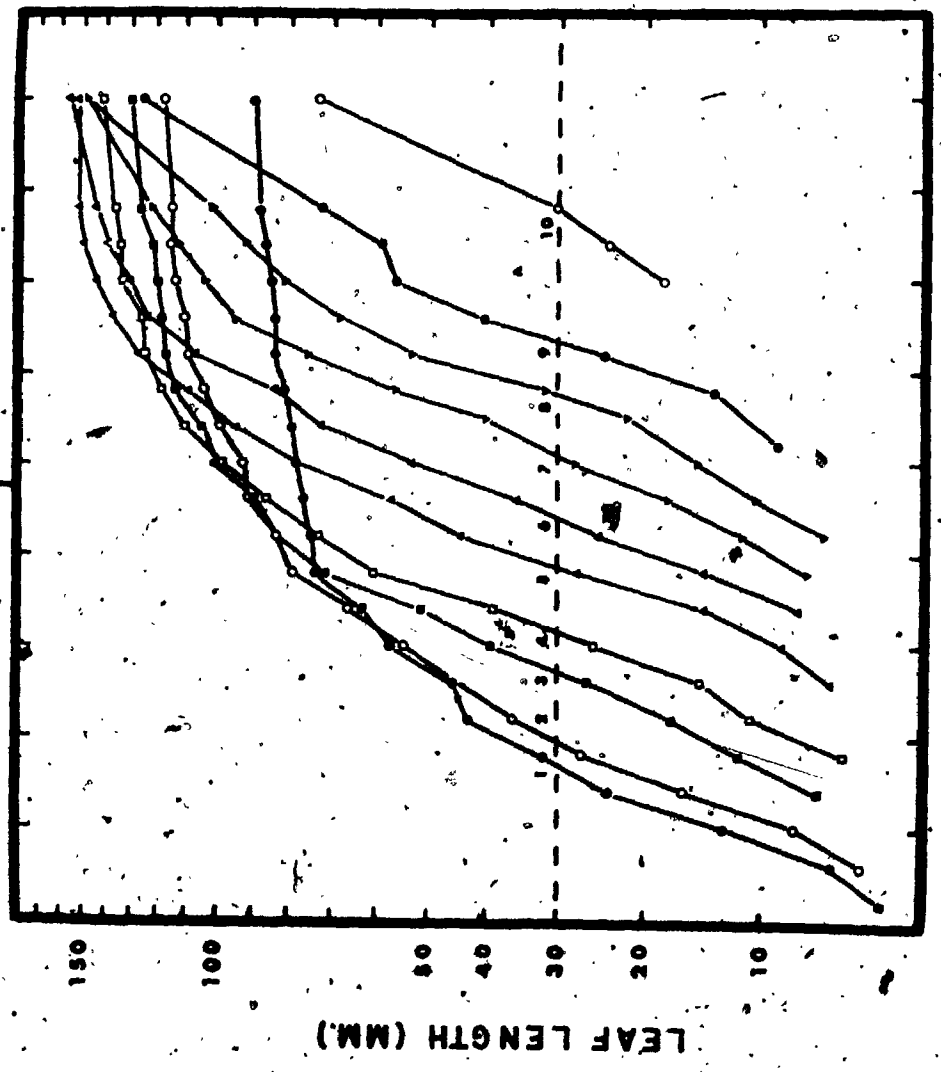
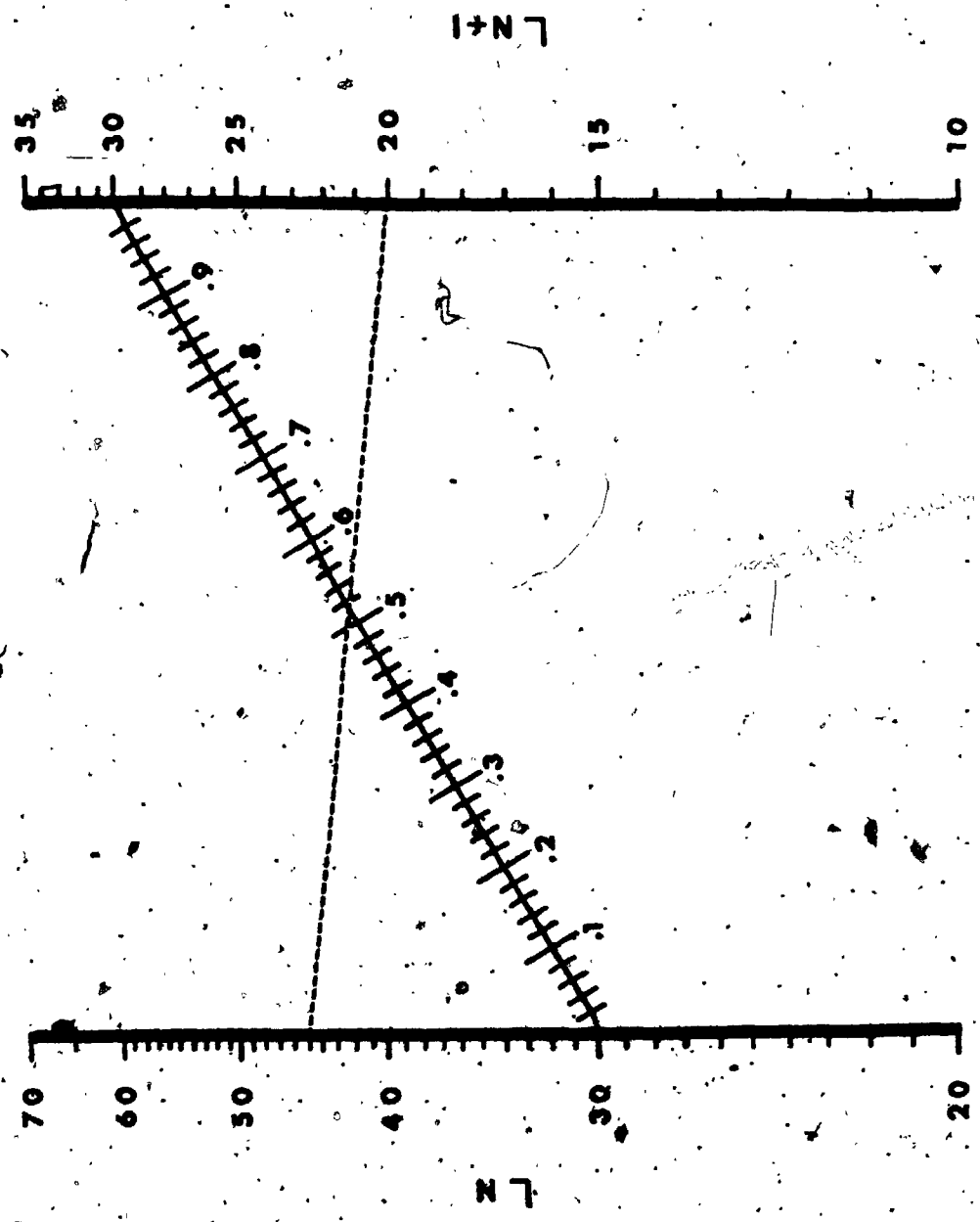

LEAF LENGTH (MM.)
150
100
50
40
30
20
10
JAN.
15
20
25
30
FEB.
4
9
14
19
24
29
TIME (DAYS)

Figure 3.2. Nomogram used to calculate fractional plastochron values. For example, if leaf 6 (i.e. Ln) was 45 mm long and leaf 7 (i.e. Ln + 1) was 20 mm long, joining the two values by a straight edge (dashed line) would give a fractional plastochron value of .52 and, consequently, the PI value for the tomato shoot would be 6.52.


L N+1
35
30
25
20
15
10
9
8
7
6
5
4
3
2
1
70
60
50
40
30
20
L N

relationship between time and the P.I., data from an arbitrarily selected plant was plotted against time in days (Fig. 3.3). It is apparent that a straight line results for most of the growth period studied. Regression analysis of the data up to P.I. 8.7 revealed that the slope of the line (i.e. the plastochron interval/day) was .405 and, consequently, the average duration of one plastochron was 2.5 days or 59.3 hours (Table 3.1). Calculation of the 95% confidence intervals for the true regression coefficient (Woolf, 1968) revealed that the calculated P.I. duration is accurate within a 5 hour period. Since the L.P.I. is of more direct importance for this leaf development study, an examination of the growth in length of alternate, successive leaves against L.P.I. was carried out (Fig. 3.4). The leaf growth curves indicated that leaves 1, 3, 5 and 7 were very similar in the early log. phase of growth. They differed, however, in the L.P.I. at which they leave the exponential, or phase 1 growth as well as in the subsequent growth rates and final lengths attained during phase 2 growth (i.e. "mature" phase).

3.3.1.2 Seasonal Aspects

Shoot material sampled at 1 month intervals over a 16 month period from four week old plants revealed considerable fluctuations in various growth features (Fig. 3.5). However, correlation analysis revealed statistically significant positive relationships (at the 5% level) between the

Figure 3.3. P.I. data for an individual plant plotted against time in days.

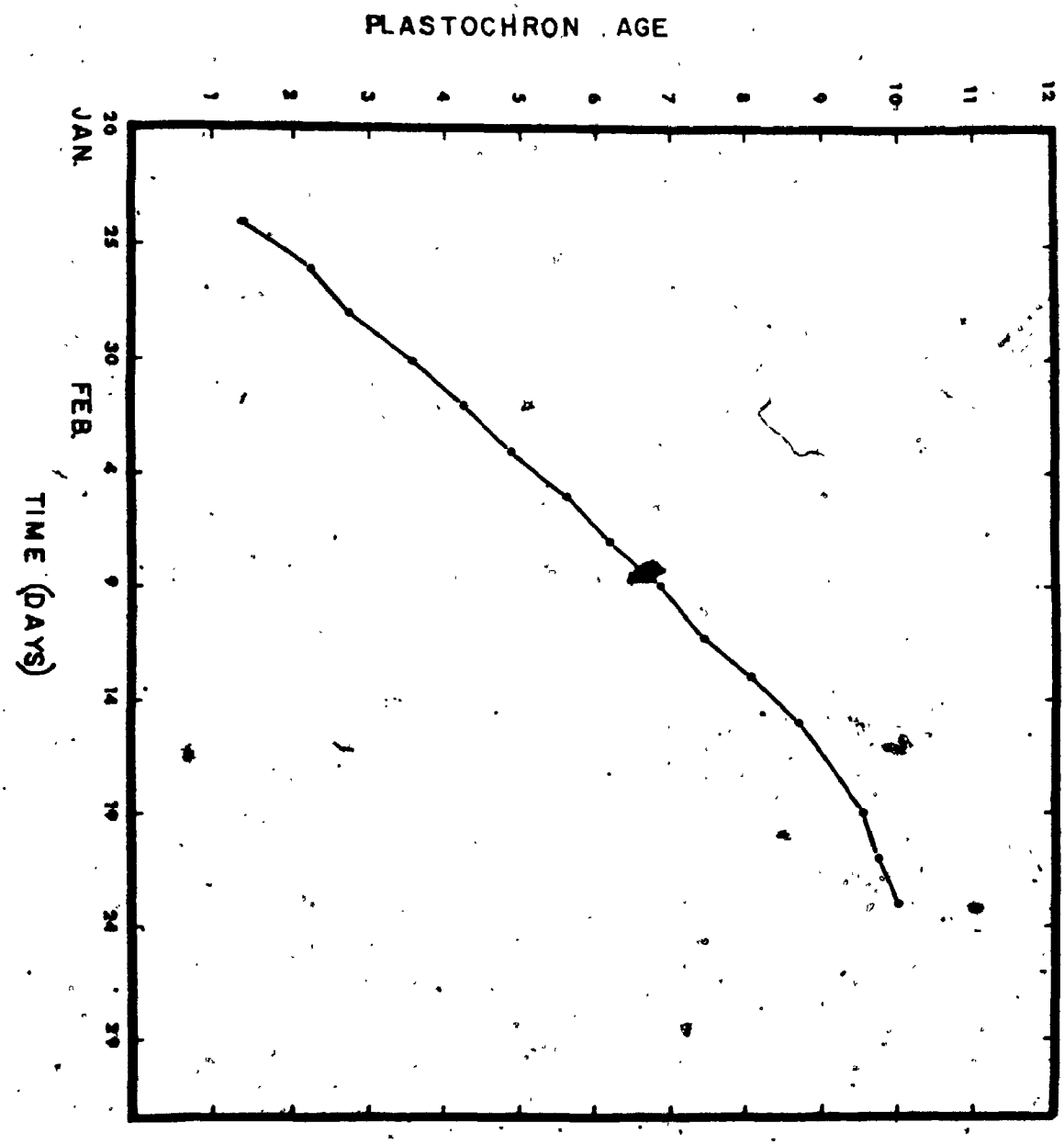
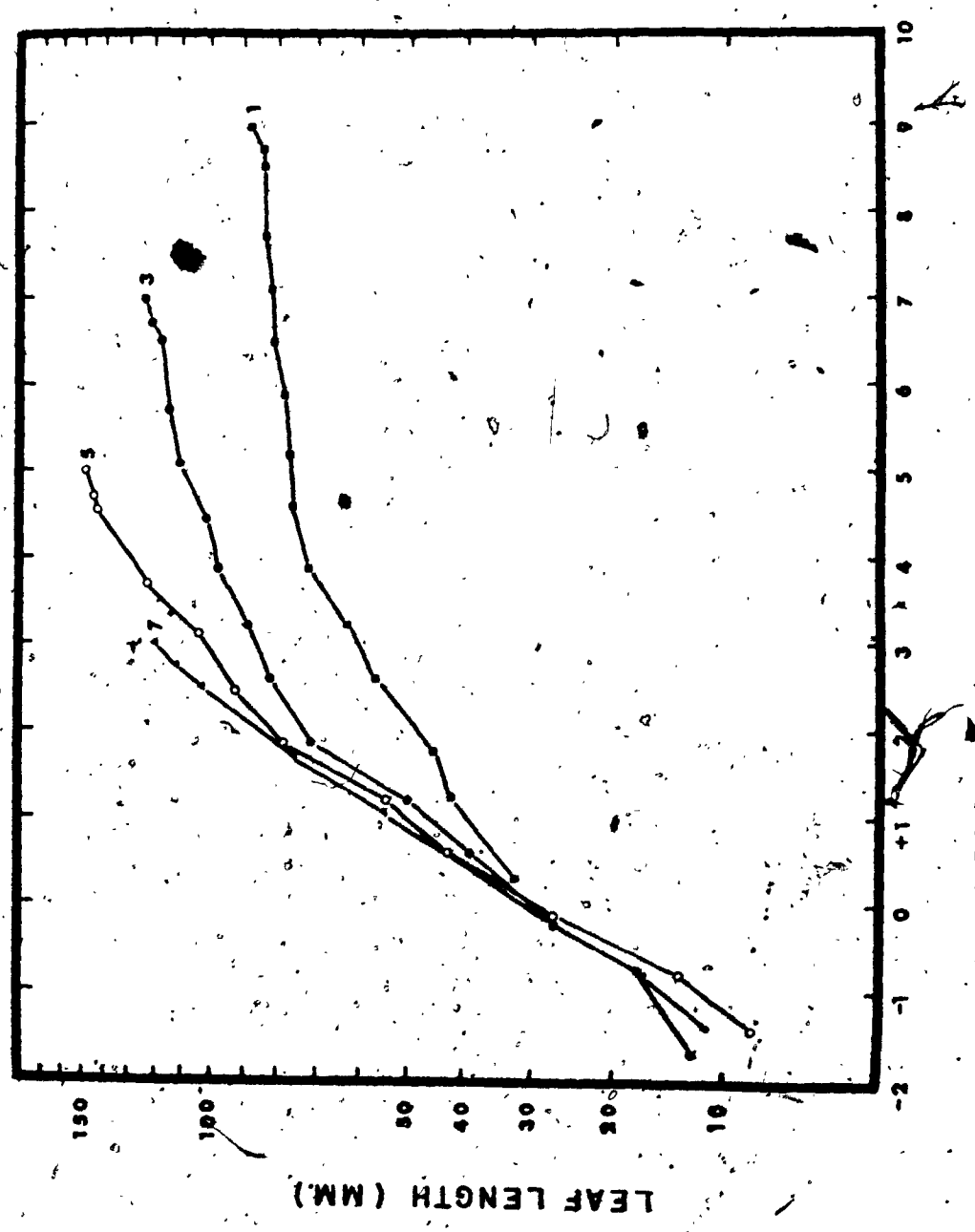

PLASTOCHRON AGE
1
2
3
4
5
6
7
8
9
10
11
12
TIME (DAYS)
JAN.
20
25
30
FEB.
4
9
14
19
24
29

Table 3.1. Regression Analysis of Plastochron Index versus Time (days).

Regression Formula	Regression Coefficient	Calculated PI Duration
$Y = .30 + .405\ X$	$.405 \pm .0309^a$	2.47 days ($\pm$ approx. 5 hours)

a = 95% confidence limits

Figure 3.4. Growth in length of alternate, successive leaves expressed as a function of L.P.I.


LEAF LENGTH (MM)
150
100
80
60
40
30
20
10
LEAF PLASTOCHRON INDEX
10
9
8
7
6
5
4
3
2
+1
0
-1
-2
1
3
5
7

2 6

OF/DE

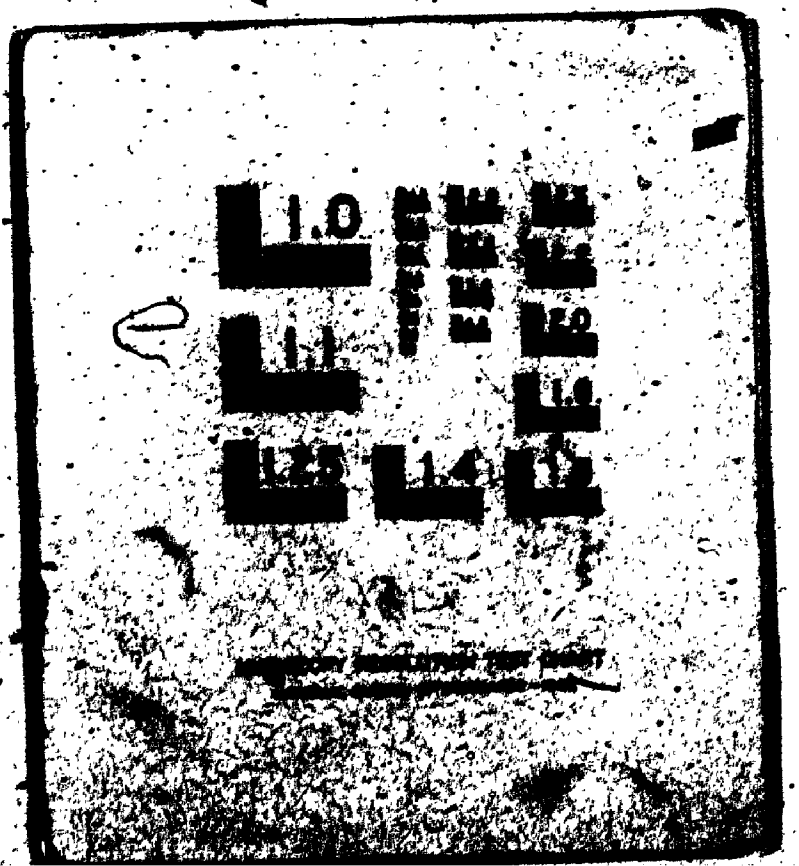
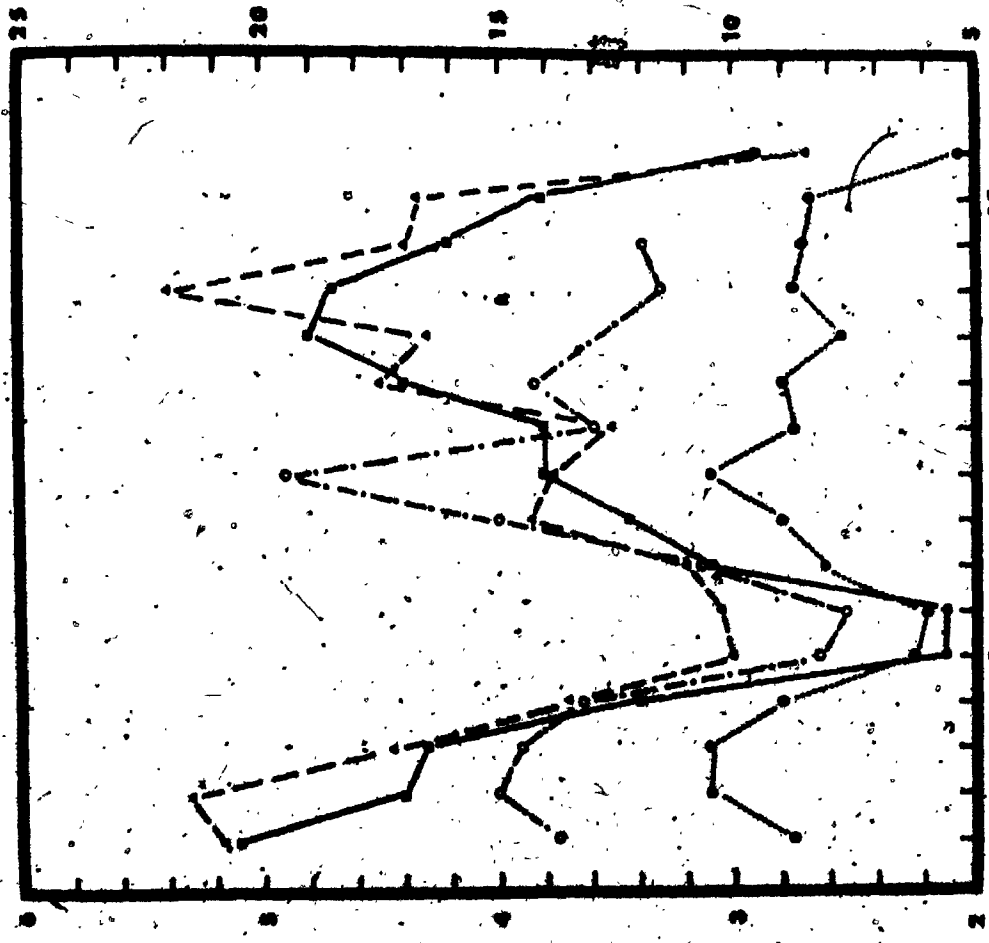


Figure 3.5. Seasonal fluctuations in various growth parameters of 4 week old tomato shoots.. Closed squares, mean LPI_3; closed circles, mean leaf length in cm.; closed triangles, mean plant height in cm.; open circles, mean leaf area in cm^2.

GROWTH PARAMETER



developmental status of the tomato shoots and $L.P.I._3$ (Table 3.2). A plot of 50-60 individual lengths sampled at one time during the summer and a separate group sampled during the winter reinforced the validity of the L.P.I. as a useful, constant morphological criterion for evaluating leaf development (Fig. 3.6).

3.3.1.3 Growth Features of a Tomato Leaf

As noted previously, leaf three was chosen for a detailed examination due to its mature growth form and its use in associated regeneration studies. An examination of whole leaf, petiole and rachis (including terminal leaflet no. 3) elongation revealed a basipetal trend in the termination of phase 1-log. growth (Fig. 3.7). While rachis elongation (including the terminal leaflet) ceased at approximately $LPI_3 = +2.0$, petiole elongation continued until $LPI_3 = +4.0$. Growth in leaf area was significantly and positively correlated ($r = .998$) with leaf length.

Fresh and dry weights of individual leaves over a LPI_3 range of -1.0 to +7.0 revealed growth curves similar to those of leaf length (Fig. 3.8). At approximately LPI_3 2.5, a declining rate of increase in dry weight relative to fresh weight caused a gradual increase in the fresh/dry weight ratio. This feature became more prominent after entrance of the leaf into mature (phase 2) growth around LPI_3 4.0.

The total soluble carbohydrate fraction appeared to

Table 3.2. Correlation analysis between LPI_3 and different tomato growth parameters.

Growth parameter	Correlative coefficient (r)
mean leaf area	.60 *
mean leaf length	.57 *
mean plant height	.87 ***

* significant at 5% level

*** significant at .1% level

Figure 3.6. Tomato leaf (no. 3) growth during the summer (o — o) and winter (● — ●).

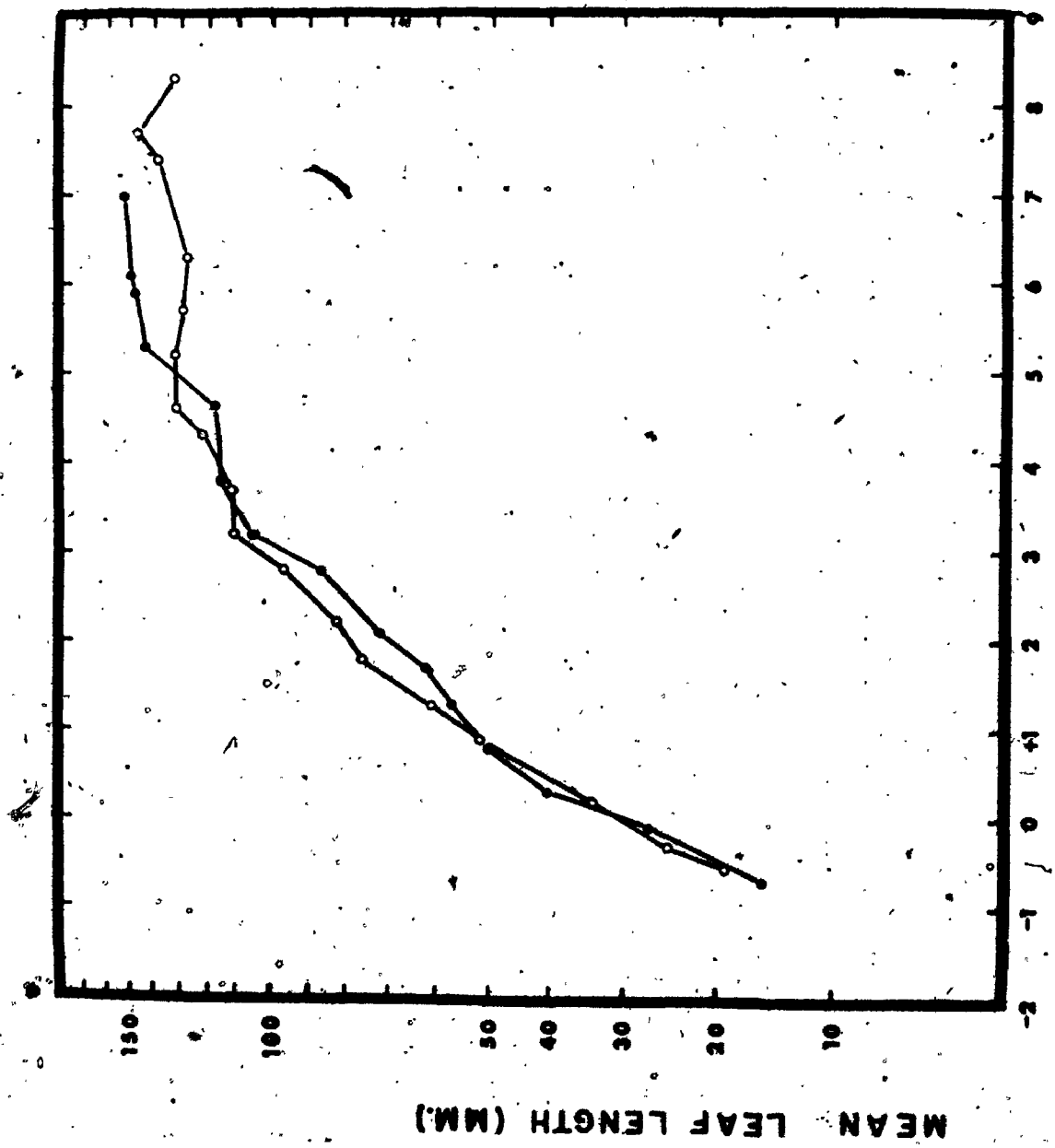

MEAN L.P.I.$_3$
MEAN LEAF LENGTH (MM.)
150
100
50
40
30
20
10
-2
-1
0
1
2
3
4
5
6
7
8

Figure 3.7. Growth in length and area of the tomato leaf. Closed circles, mean leaf length; open circles, mean length of the rachis (including terminal leaflet no. 3); closed squares, mean petiole length; open squares, mean lamina area.

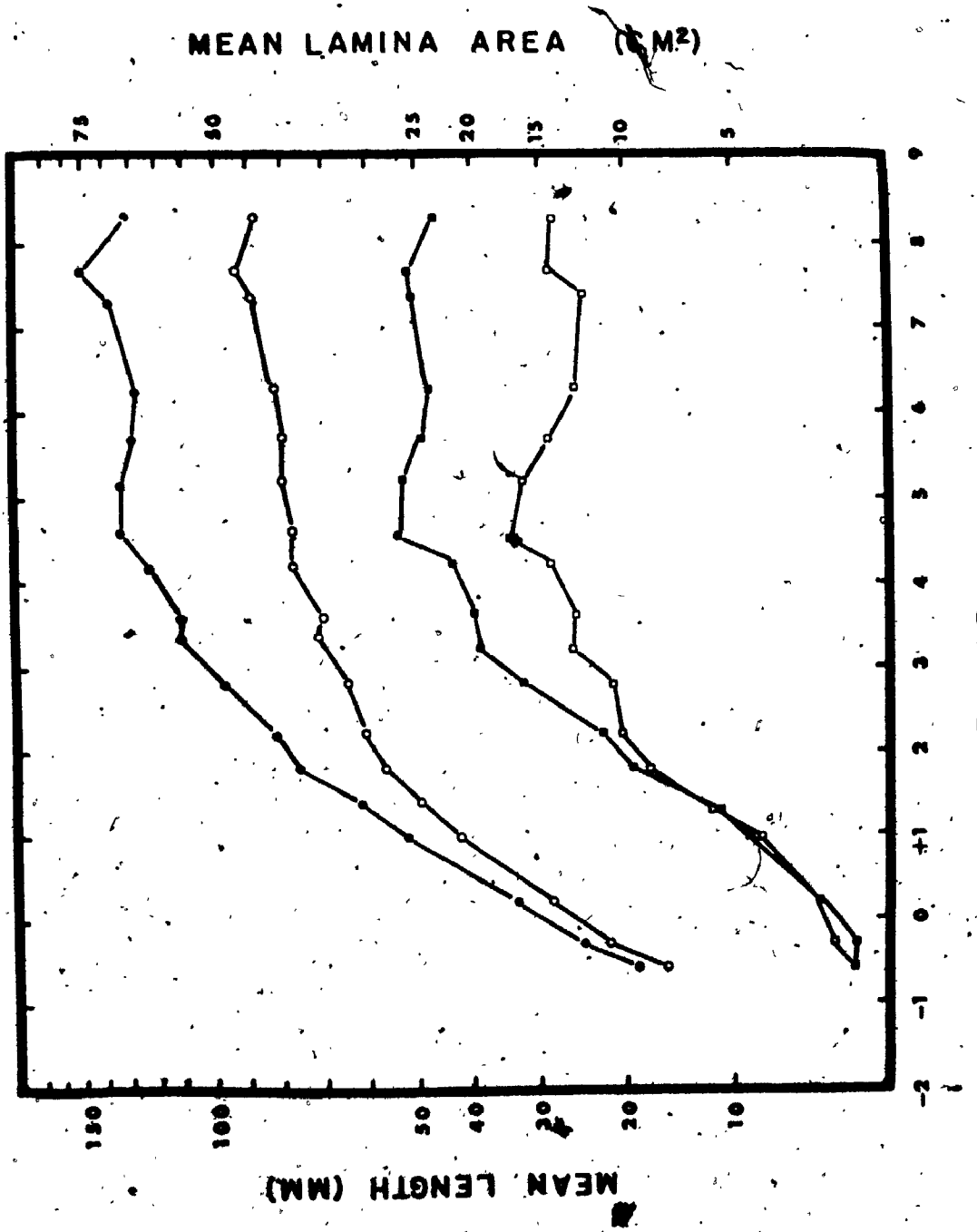
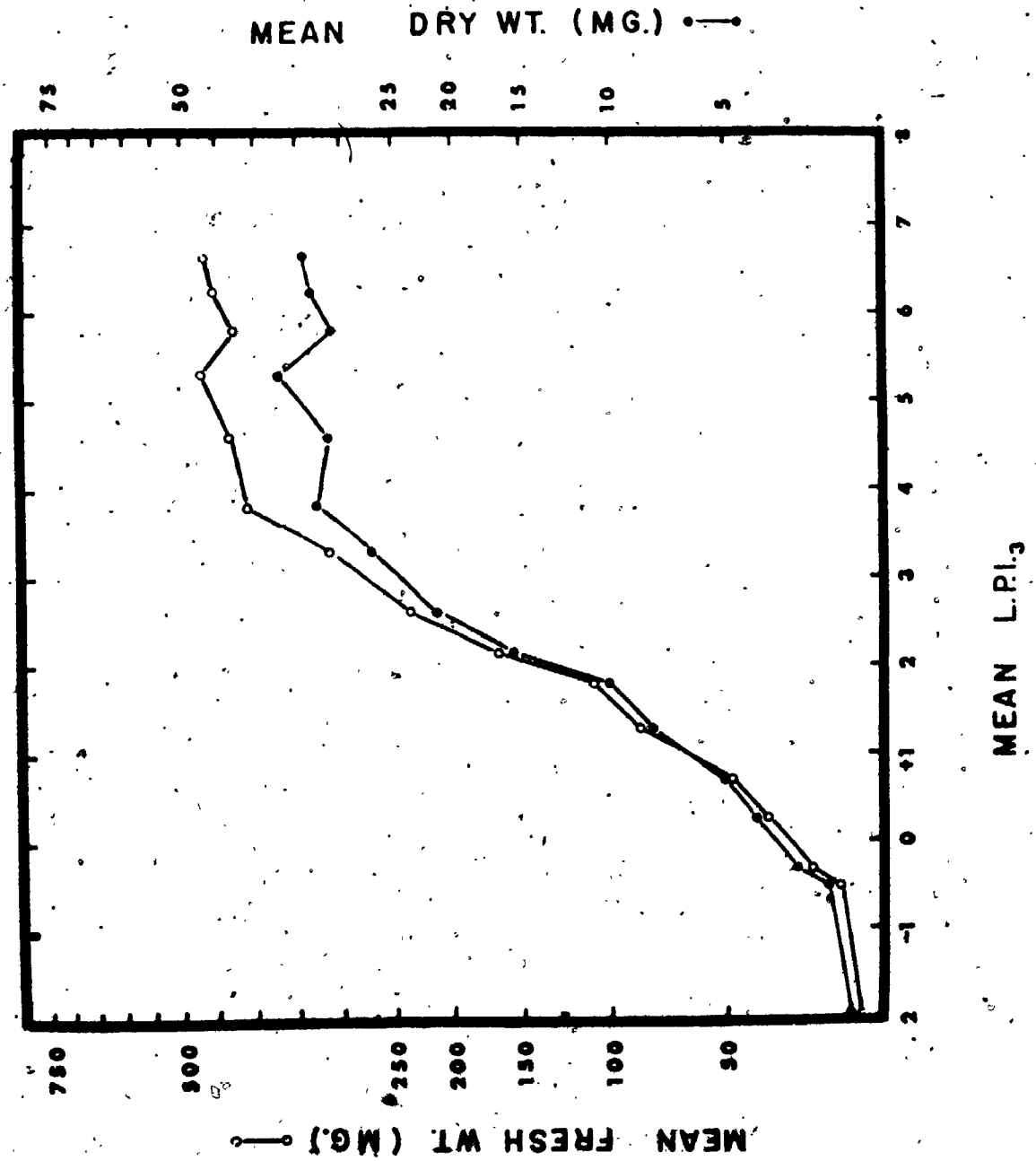

MEAN LAMINA AREA (CM²)
75
50
25
20
15
10
5
0
1
2
3
4
5
6
7
8
MEAN LPI3
-2
-1
0
+1
2
3
4
MEAN LENGTH (MM)
150
100
50
40
30
20
10

Figure 3.8. Fresh and dry weight changes during tomato leaf development.


MEAN DRY WT. (MG.)
75
50
25
20
15
10
5
MEAN L.P.I.3
8
7
6
5
4
3
2
+1
0
-1
-2
750
500
250
200
150
100
50
MEAN FRESH WT (MG.)

decrease slightly until LPI_3 2.5 - 3.0 when a noticeable drop became apparent. This fraction subsequently increased until LPI_3 7.0 (Fig. 3.9). Starch content increased steadily over the LPI_3 range of 0.0 to 7.0. Total chlorophyll per leaf increased in a typical exponential fashion until LPI_3 4.0 when it reached a plateau (Fig. 3.10). Although there was no indication of breakdown products in the samples (as judged by the absorption values), the chlorophyll a/b ratio remained consistently around .75 to .80 over the entire sample range. The reasons for the low chlorophyll ratio (i.e. .75-.80), which deviated from an expected ratio of 2.5 - 3.0, are unknown. However, subsequent growth and development of the tomato plants over the following 6 week period was severely retarded due to a combination of macronutrient deficiencies (nitrogen and phosphorus) and insecticide (whitefly/aphid) spray damage. Consequently, the low chlorophyll a/b ratio may have been due to the early effects of these factors.

3.3.1.4 Leaflet Growth

Growth in length of leaflets 1, 2 and 3 from the 10 plants originally sampled during January to March 1971 was plotted against LPI_3 (Fig. 3.11). Two features are noteworthy: the individual leaflets demonstrate progressively later growth cycles in a basipetal direction and the final length attained by the individual leaflets is progressively smaller in a basipetal manner. On the basis of palisade

Figure 3.9. Starch and total soluble carbohydrate changes during tomato leaf development. Vertical lines ± S.E.

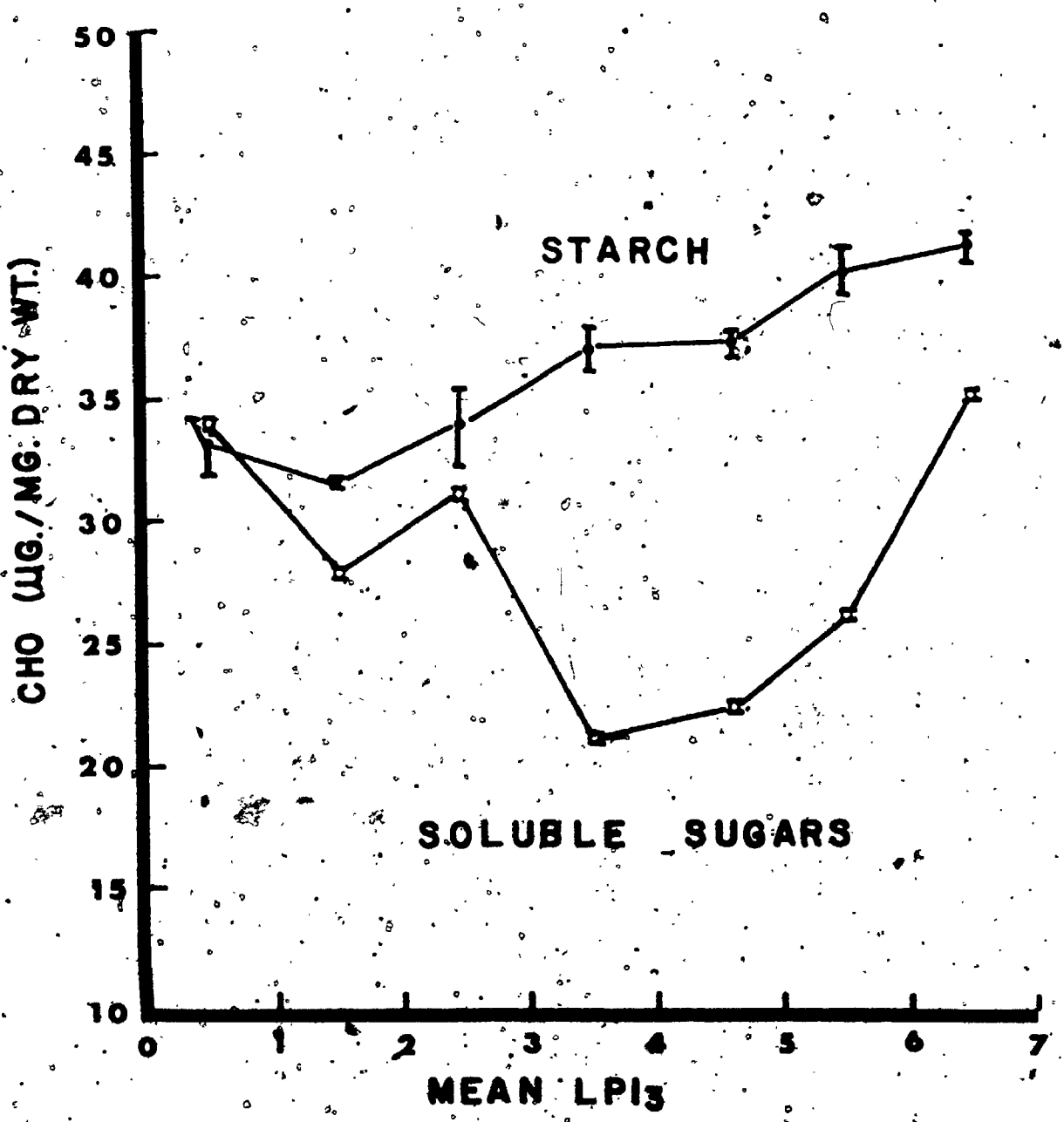

50
45
40
35
30
25
20
15
10
CHO (MG./MG. DRY WT.)
STARCH
SOLUBLE SUGARS
0
1
2
3
4
5
6
7
MEAN LPI3

Figure 3.10. Total chlorophyll changes during tomato leaf development. The dashed line emphasizes the general trend only.

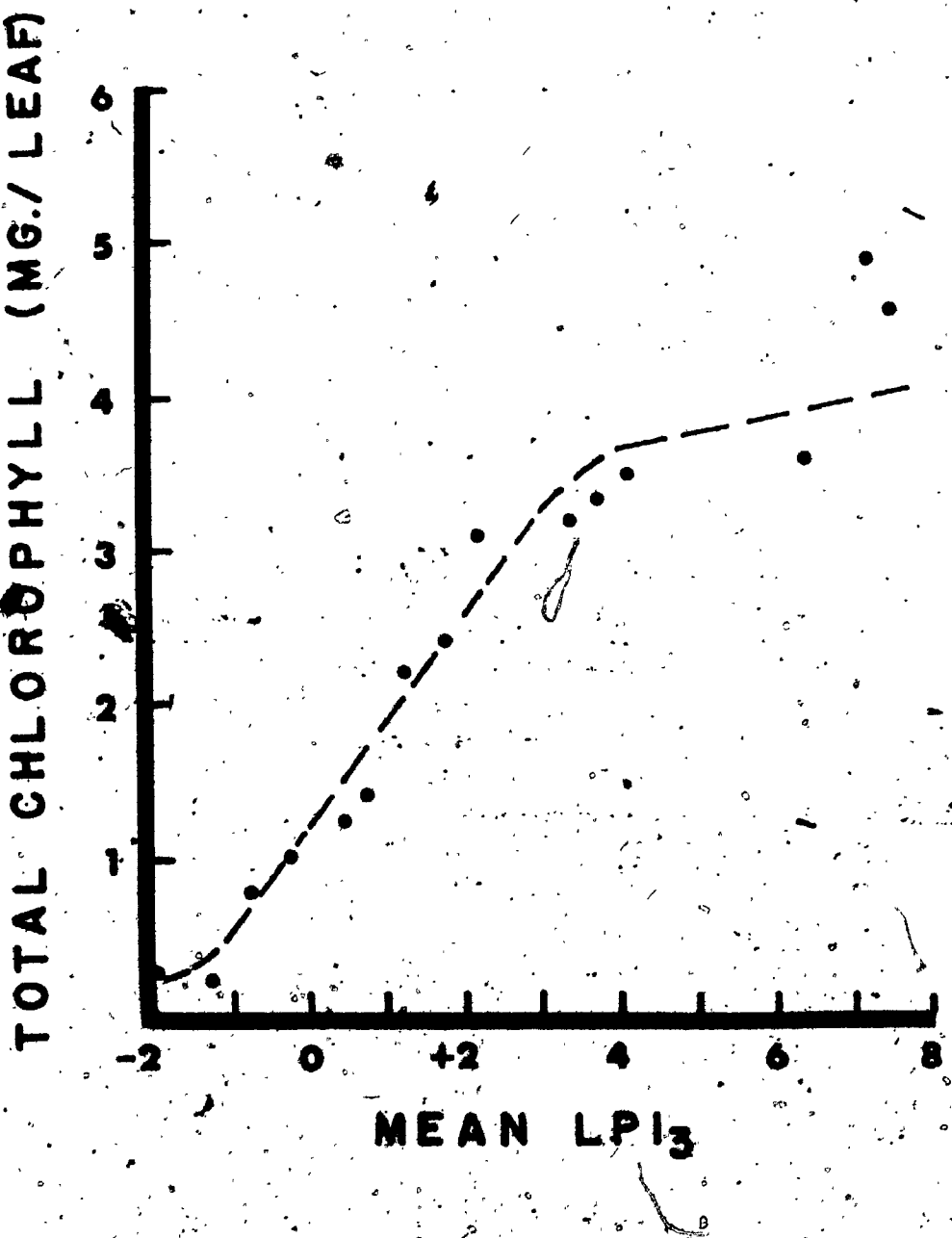
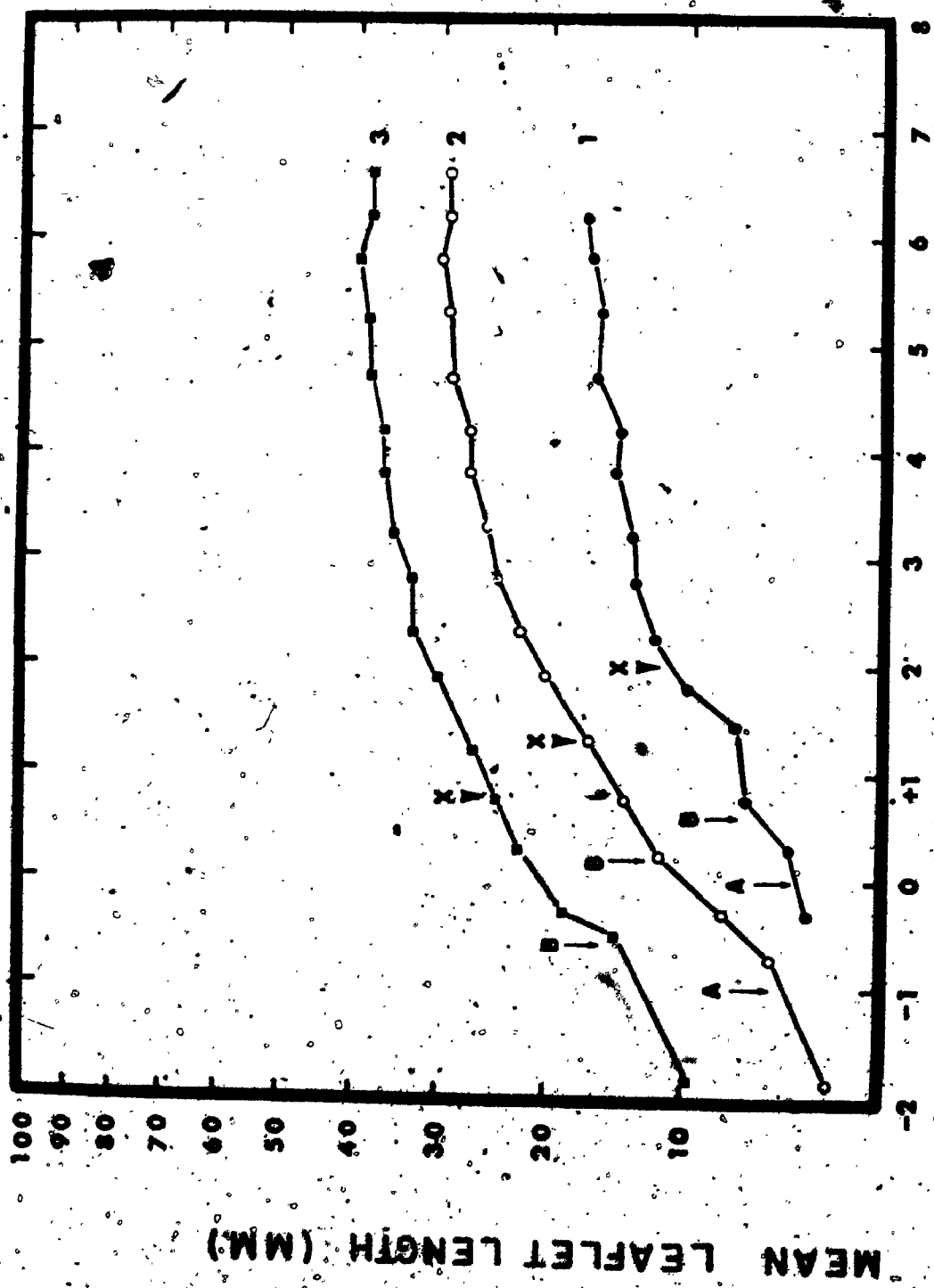

TOTAL CHLOROPHYLL (MG./ LEAF)
1
2
3
4
5
6
-2
0
+2
4
6
8
MEAN LPI3

Figure 3.11. Leaflet growth in the tomato leaf. Closed circles, leaflet 1; open circles, leaflet 2; closed squares, leaflet 3. Points on the curves marked 'A' represent the approximate age when mitotic activity ceased in the apical region of the developing lamina. 'B' represents the similar occurrence in the basal region. 'X' represents the stage of final xylem maturation within the minor vein system of the tomato leaflet.


MEAN LEAFLET LENGTH (MM.)
100
90
80
70
60
50
40
30
20
10
MEAN L.P.I.3
-2
-1
0
+1
2
3
4
5
6
7
8
1
2
3

mesophyll cell areas (cross sectional) and associated blade areas, the basipetal trend in smallness of the individual leaflets appears to be due to fewer cells at maturity and a smaller cell size (Table 3.3).

Regression analysis of the log. width/length values of the individual leaflets indicated that the rate of increase in leaflet width relative to length for the terminal leaflet was consistently greater (at the 5% level) than the lateral leaflets (Table 3.4).

Regression analysis of the log. lengths for the terminal/lateral leaflets indicated that the relative growth rate of leaflet 3 compared to leaflet 1 was significantly greater (at the 5% level) than leaflet 3 to leaflet 2 (Table 3.5). Furthermore, the Y-intercept values suggest that the lateral leaflets are initiated during the first 1000 μ of growth in length of the terminal leaflet.

3.3.2 Ontogenetic Studies

3.3.2.1 The Adult Leaf

a) Leaf shape - The leaves of the tomato plant are arranged spirally in a 2/5 phyllotaxy and develop in a heteroblastic manner (Plate 3.1;1). In 'Farthest North' the first adult leaf form is encountered in leaf number 3. It appears imparipinnate with 5 asymetrically elliptic leaflets. The terminal (3) leaflet is larger than the lateral ones which are arranged in two sub-opposite pairs on the rachis. The

Table 3.3. Cell areas (cross-sectional) and estimated cell number in the palisade mesophyll tissue of mature leaflets.

Leaflet position	LPI_3	Mean cell area (μ^2)*		Estimated cell number ($X10^6$)**	
1. basal lateral	4.45	640		1.8	
	4.64	675		1.9	
	4.88	785	644	3.2	2.7
	5.34	504		4.8	
	5.45	614		2.0	
2. middle lateral	4.45	718		3.8	
	4.64	864		3.5	
	4.88	796	764	3.9	4.0
	5.34	559		5.5	
	5.45	882		3.2	
3. terminal	4.45	859		5.4	
	4.64	861		5.1	
	4.88	876	863	5.5	5.2
	5.34	914		5.1	
	5.45	807		4.7	

* Each value calculated from 10 random cell measurements obtained in the middle lamina region of each fixed leaflet.

** This value is based on the calculation:

$$\text{Est. Cell number} = \frac{\text{area of leaflet } (\mu^2)}{\text{mean area of palisade cell } (\mu^2)}$$

and is uncorrected for intercellular spaces or veins.

Table 3.4. Regression analysis of the log. width/length values for tomato leaflets.

Leaflet	Regression formula	Regression Coefficient
1	log Y = -.0091 + .7388 log X	.7388 ± .0072[a]
2	log Y = -.0250 + .7447 log X	.7447 ± .0177[a]
3	log Y = -.0054 + .7935 log X	.7935 ± .0035[a]

a = 95% confidence intervals

Table 3.5. Regression analysis of the log. terminal leaflet length/lateral leaflet length for tomato.

Leaflet pair	Regression formula	Regression coefficient	Y-intercept
3/1	log Y = .0189 + 1.4748 log X	1.4748 ± .0147[a]	.0189 ± .0219[a]
3/2	log Y = .0265 + 1.1267 log X	1.1267 ± .0097[a]	.0265 ± .0180[a]

a = ± confidence intervals

Plate 3.1. 1. Heteroblastic leaf development in the tomato cultivar 'Farthest North'. Leaves are numbered consecutively from the first true leaf above the cotyledons. Leaflets 1 to 5 of leaf 3 are also numbered. (Approximately 1/3 natural size.)

2. Vein types and distribution in a NaOH cleared, mature, tomato leaflet. Numbers refer to vein orders as described in text. (x 200)


3
2
4
1
5
1
2
3
4
5
1

leaflets are petiolate with a margin possessing a few rounded crenations or small lobes, irregularly spaced.

b) Venation - The venation classification is based upon 2 criteria:

1) size and distribution of veins relative to the primary vein or mid-rib. The terminology of Hickey (1973) for venation classification is quite useful for this purpose.

2) number, type and arrangement of the tracheary elements within the different vein types.

The following venation classification will be used:

i) major venation - Tracheary systems composed of multiple vessels which appear early in the ontogeny of the leaf venation (Plate 3.1;2).

a) primary vein or midrib

b) secondary veins

c) intersecondary veins

d) marginal fimbriate vein - although this vein lacks internal phloem (unlike a, b and c), it is included on the basis of tracheary composition, early ontogenetic appearance and continuity with the primary vein (Plate 3.2;1 and 3.2;2).

ii) minor venation - Tracheary systems composed of tracheids (usually two adjacent strands) which appear relatively late in the ontogeny of the leaf venation (Plate 3.1;2).

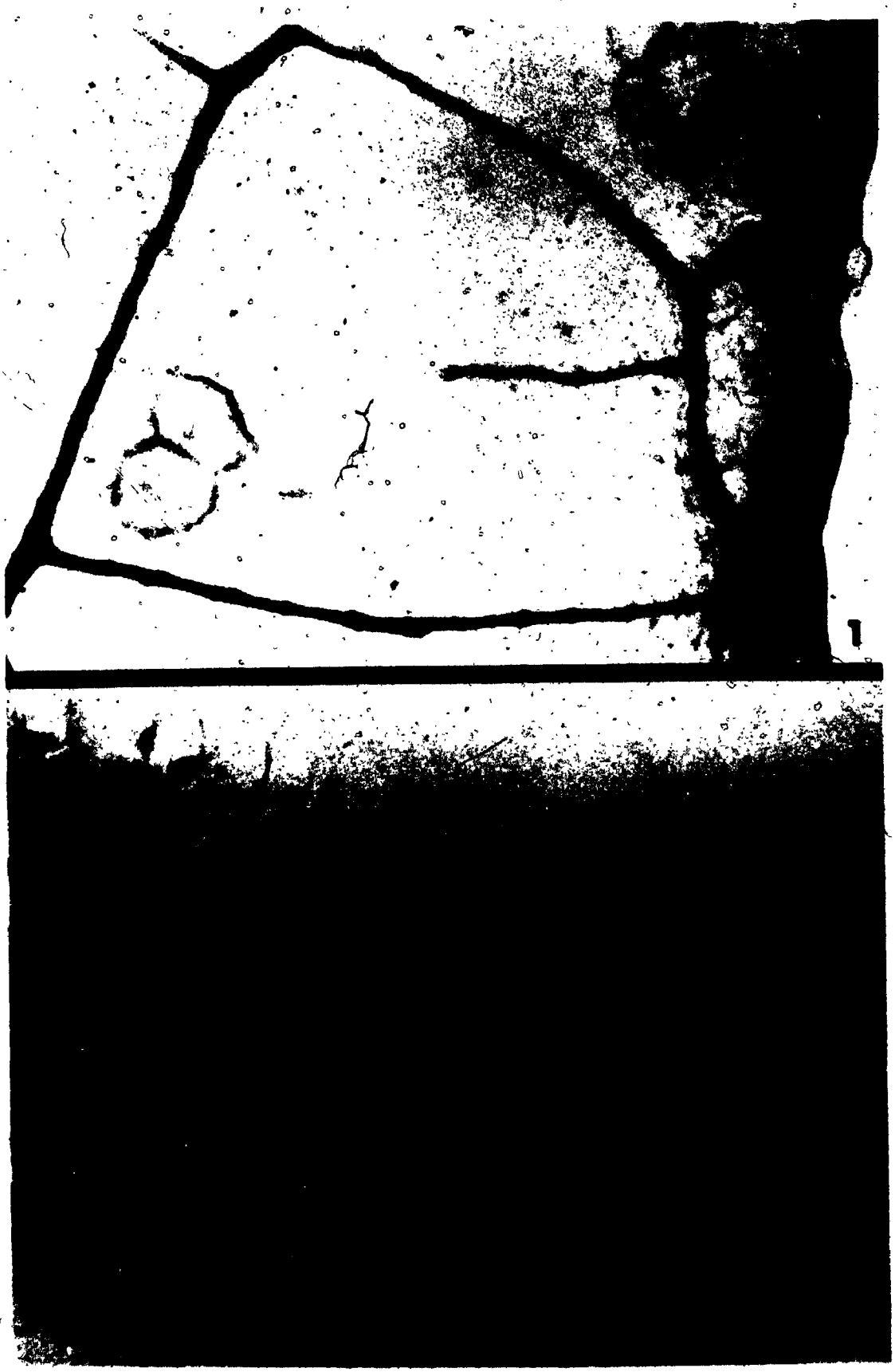
a) tertiary veins

b) quaternary veins

c) vein endings or terminal veinlets

Plate 3.2. 1. Cleared tomato leaflet showing mature vessel elements of the marginal fimbriate vein interconnected with the minor vein system. (x 200)

2. Cross section view of marginal fimbriate vein in a mature leaflet (arrow). (x 1280)



The vascular system is pinnate with the main lateral or secondary veins not terminating at the margins but curving acroscopically into a series of loops with adjacent secondaries or their branches (camptodromous venation; Hickey, 1973). The rounded crenations are supplied with branches of the secondary veins where they join a distinct marginal fimbriate vein.

The secondary veins have a moderately acute (45-65°) angle of divergence from the mid-rib which becomes more obtuse in the distal regions of the leaf. A few simple intersecondary (Hickey, 1973) or intermediate veins (Foster, 1950) are evident in the leaflets.

The tertiary veins exhibit an orthogonal reticulate pattern while the quaternary veins (the highest order venation recognized in this material) exhibit an orthogonal course. Terminal veinlets may be simple, branched or non-existent while the areoles are imperfect, randomly arranged, irregular and small in shape. A survey of 880 areoles revealed that 418 (47.5%) contained vein endings. Chi-Square analysis (Table 3.6) suggests that vein ending formation is probably a random event and ontogenetic evidence to be presented later supports this concept.

c) General Leaf Histology - The mature leaflets possess a thin lamina with a mesophyll consisting of a well defined columnar palisade layer and three layers of spongy mesophyll. Variations in the number of cell layers were not observed in the greenhouse grown material during the course of this

Table 3.6. Chi-Square analysis of vein ending frequency in mature tomato leaves.

Condition	Number observed	Number estimated	Chi-Square Value*
areoles with vein endings	418	440	1.10
areoles without vein endings	462	440	1.10
			X^2 = 2.2 n.s.
Total	880		

*n.s: Not significant at 5% level

investigation. A gradual decrease in thickness of the mature leaflet lamina is apparent from the base to the apex. This thinning effect is due primarily to a progressive failure of the palisade cells to elongate at right angles relative to the epidermal layers and not to the loss of a particular mesophyll layer.

Scattered crystal idioblasts containing calcium oxalate crystals (Solereder, 1908) are observed in the upper mesophyll and rarely in the middle spongy layer. They are also apparent in numerous elongated cortical cells of rachis and petiole. Crystal accumulation within these cells begins immediately after cessation of mitotic activity.

The leaflet, rachis and petiole are covered with three types of glandular hairs (types I, VI and VII of Luckwill, 1943) and three types of non-glandular ones (types II, III and V).

Stomata of the anomocytic type (formerly Ranunculaceous) occur on both of the adaxial and abaxial leaf surfaces (i.e. amphistomatic) although they appear more frequently on the latter. The guard cells protrude slightly above the adjacent epidermal cells. Using the plastic epidermal imprint method of Williams (1973), no stomatal abnormalities such as the occassional "single guard cells" reported to occur in tomato by Ahmad (1964) were observed. Very rarely, stomata were observed adjoining one another at right angles. In view of my ontogenetic study of tomato stomata (see section 3.3.2.3), it would appear that both stoma mother

cell derivatives had pursued an independent, yet similar, developmental course.

The major vascular bundles of the leaf (i.e. the mid-rib and secondary veins) are bicollateral. The xylem possesses tracheary elements with annular, helical or scalariform thickenings (Plate 3.3). While the primary, secondary and marginal fimbriate vein xylem is almost exclusively composed of vessel elements with simple perforation plates, the third, fourth and terminal veinlet xylem consists entirely of helically (with some annularly) thickened tracheids. Contrary to Solereder's (1908) comments that the vessels of the Solanaceae possess simple perforations exclusively, the short vessels of the marginal fimbriate vein at the leaflet tips may possess scalariform perforation plates (Plate 3.15;2).

The internal phloem ventral to the xylem elements consists of sieve tubes, companion cells and phloem parenchyma. Its distribution is limited to the primary and secondary veins. In the former vein type, it fails to develop into the tips of the leaflets and stops at the most distal secondary vein.

The more extensive external phloem consisting of the same cell types occurs as discrete bundles separated by parenchyma. Small phloem strands are observed to occasionally interconnect the external phloem bundles laterally in the primary veins. External phloem is associated with the entire vasculature except the extreme tips of the

leaflets where the large marginal fimbriate vessels are present.

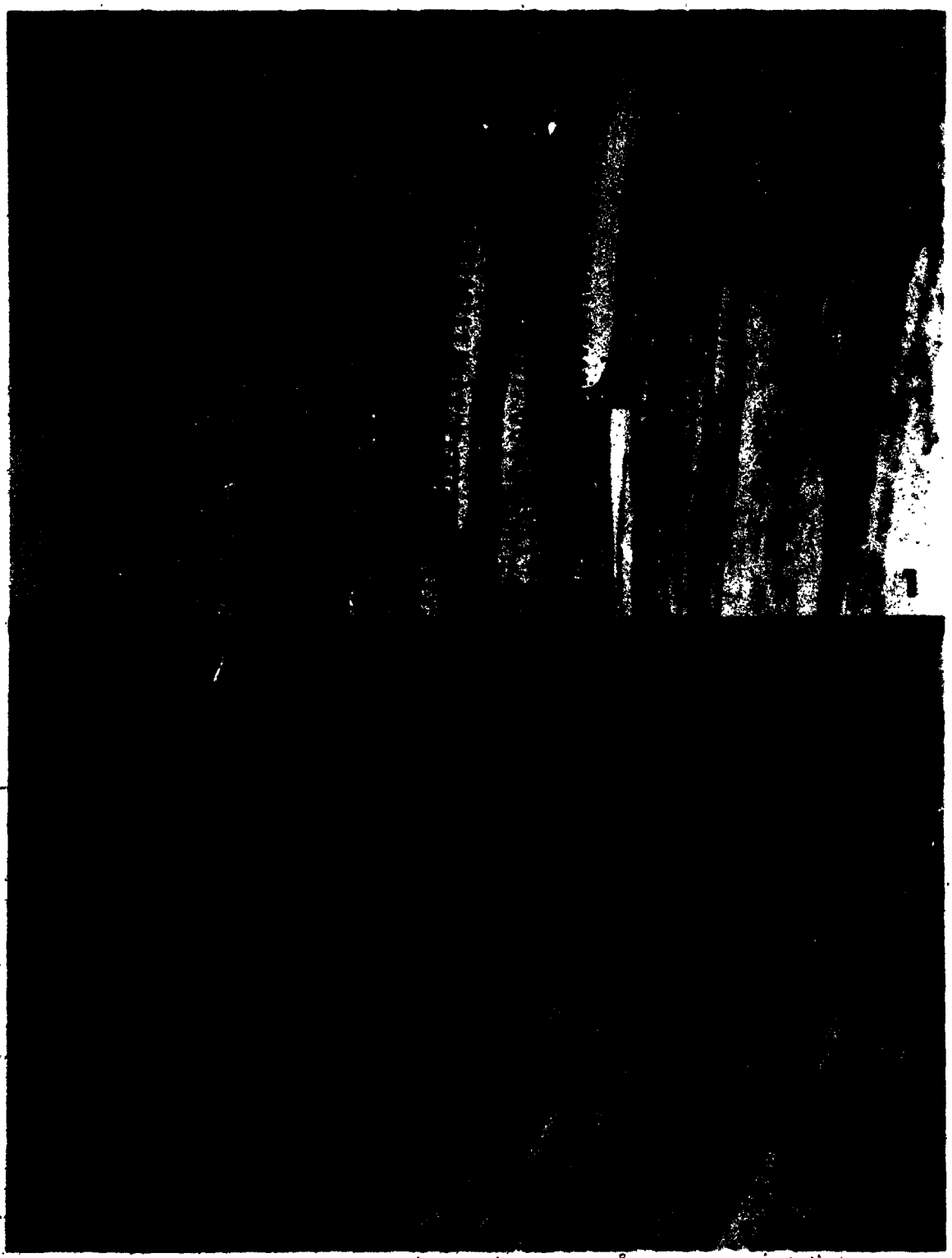
Isolated lignified fibers are occasionally observed in mature petioles within the external and internal phloem parenchyma. No fibers are observed in the mature leaflets or rachis. Bundle sheaths (often containing abundant starch grains within plastids) are evident surrounding the major and minor veins.

Angular collenchyma is common in the adaxial margins of the petioles and rachis, and is present as a 2-3 cell layer underlying a subepidermal chlorenchyma. Small vascular traces containing external phloem and vessels connected with the marginal fimbriate veins of the leaflets are found below the adaxial lateral collenchyma in the rachis and petiole. These traces are interconnected laterally with the main petiolar vasculature by periodic transverse traces (Plate 3.3;1) and subsequently connect with the lateral leaflet traces (Plate 3.3;2).

d) Vein Ending Histology - Virtually all the terminal veinlets of the cultivar 'Farthest North' possess external phloem and tracheids embedded in the upper spongy mesophyll layer. The characterization of vein endings as either principal or secondary types according to Fischer (1895, as cited in Pray, 1954) was not applicable in this study due to the heterogeneous nature of the terminal veinlets. The principal type of vascular tissue arrangement found in the third, fourth, and terminal veins consists of two tracheary

Plate 3.3. 1. Interconnected vascular strands (arrow) in the rachis of a NaOH cleared mature tomato leaf. Single strands within the rachis margins are directly connected to the marginal fimbriate vein system of the leaflets. (x 200)

2. Junction of basal leaflet xylem with the rachis vasculature in a NaOH cleared tomato leaf. Note the short irregular vessel elements at the junction point. (x 200).



strands adaxially located relative to the external phloem strand. In general, the external phloem consists of five nucleated parenchyma cells surrounding two sieve tubes (Plate 3.4;1). One of these parenchyma cells, adjacent to a tracheid, may be considered a xylem parenchyma cell while the others are probably phloem parenchyma (Plate 3.4;2). While the phloem parenchyma of the terminal veinlets forms a tight junction at the vein ending with the adjacent sheath parenchyma cell, the terminal tracheid(s) is often observed to extend slightly further around the margin of the adjacent sheath cell (Plate 3.5;1 and 2). The sieve elements in the terminal veinlets often do not extend to the tip of the vein ending; rather, they appear to stop near the end of the terminal phloem parenchyma cells. The positive identification of the sieve elements is based on: (1) lack of nucleus and other cell contents in cells of the mature leaf; (2) narrow diameter and thick primary cell walls; and (3) continuity with sieve elements in the 4° veins possessing distinct sieve plates in the mature leaf.

A number of aberrations were observed in the phloem and xylem of the minor veins - particularly in relation to the vein endings. These abnormalities included: (1) variation in the number of tracheids (from zero to 3 tracheal strands); and (2) variations in the number of phloem parenchyma. The aberrant xylem development will be discussed further in the section dealing with minor vein ontogeny.

Plate 3.4. 1. Cross section of a mature minor vein showing a common vascular arrangement. T, tracheid; VP, vascular parenchyma; S, sieve tube element; SP, sheath parenchyma. (x 2458)

2. Cross section of a mature minor vein junction. (x 2048)

2

Plate 3.5. 1. Mature minor vein ending in a FPA cleared leaflet focused at the level of the external phloem showing "tight" junctions (arrow) with the adjoining bundle sheath cells. C.I., crystal idioblast cell. (x 800)

2. Similar to 1, except focused at the xylem level. (x 800)

3.3.2.2 Leaf Initiation and Early Primordium Development

The leaf primordium is initiated as a small protruberance from the outermost 2 to 3 cell layers of the shoot apex flank. More specifically, the initiation process involves anticlinal divisions in the outermost layer of the two-layered tunica with periclinal divisions in the inner tunica layer and adjacent corpus cells giving rise to the internal portion of the leaf primordium. Similar features have been observed in the tomato cultivar 'Marglobe' (Bedesem, 1958) and in numerous other studies of dicot leaf initiation (Popham, 1966). Fluctuations in the number of tunica layers during the different stages of leaf initiation and development were not observed in my material.

During the initial growth phase of the leaf primordium, a distinct meristematic cell or group of cells (i.e. an apical meristem) in the epidermal and subepidermal apical region of the leaf primordium was not observed. Rather, growth of the primordium is accompanied by diffuse cell division throughout the developing organ (i.e. an intercalary meristem). (Plate 3.6;1). When the primordium is between 20 to 40 μ high, conspicuous enlargement and vacuolation of the epidermal and cortical cells on the abaxial side become apparent. Large multicellular hairs (type I of Luckwill, 1943) begin to differentiate from the developing epidermis in the leaf tip region at this stage. The remaining types of epidermal hairs subsequently differentiate from the developing epidermis in an

Plate 3.6. 1. Median longitudinal section of a tomato leaf primordium approximately 20 μ high. (x 1056)

2. Median longitudinal section of a leaf primordium approximately 50 μ high. Note the acropetally differentiating procambial strand in the primordium base (arrow). (x 1056)

essentially basipetal manner.

3.3.2.3 Marginal Growth and Lamina Differentiation

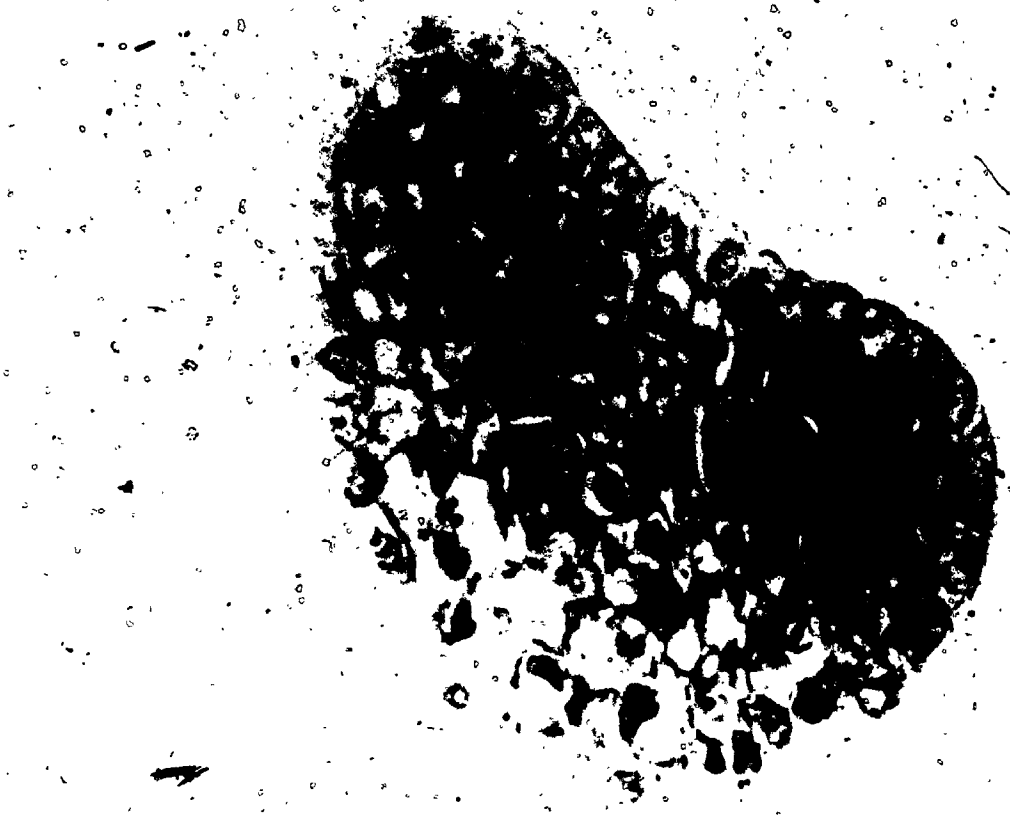
When the peg-like primordium reaches approximately 200 μ in height, a number of diverse events begin to occur. The adaxial meristem becomes apparent in the basal region of the primordium where it increases the radial thickness of the developing petiole by a series of periclinal divisions and the subsequent enlargement of the derivatives. On the abaxial surface, apparently random periclinal divisions in the vacuolating epidermal layer give rise to the different types of glandular and non-glandular epidermal hairs. Simultaneously with these events, marginal growth of the lamina begins.

Initial marginal growth in a tomato leaf primordium can be recognized in transverse sections by the appearance of the marginal and submarginal initials on the adaxial flanks of the young primordium (Plate 3.7;1,2). The initial marginal growth is localized on both sides of the distal leaf region where it will develop into the terminal leaflet (#3) lamina. The most distal lateral leaflet is also initiated at this time from adaxially located marginal and submarginal initials. Subsequent marginal meristem development in the lateral leaflets proceeds in a similar manner and at a comparable phase in their development.

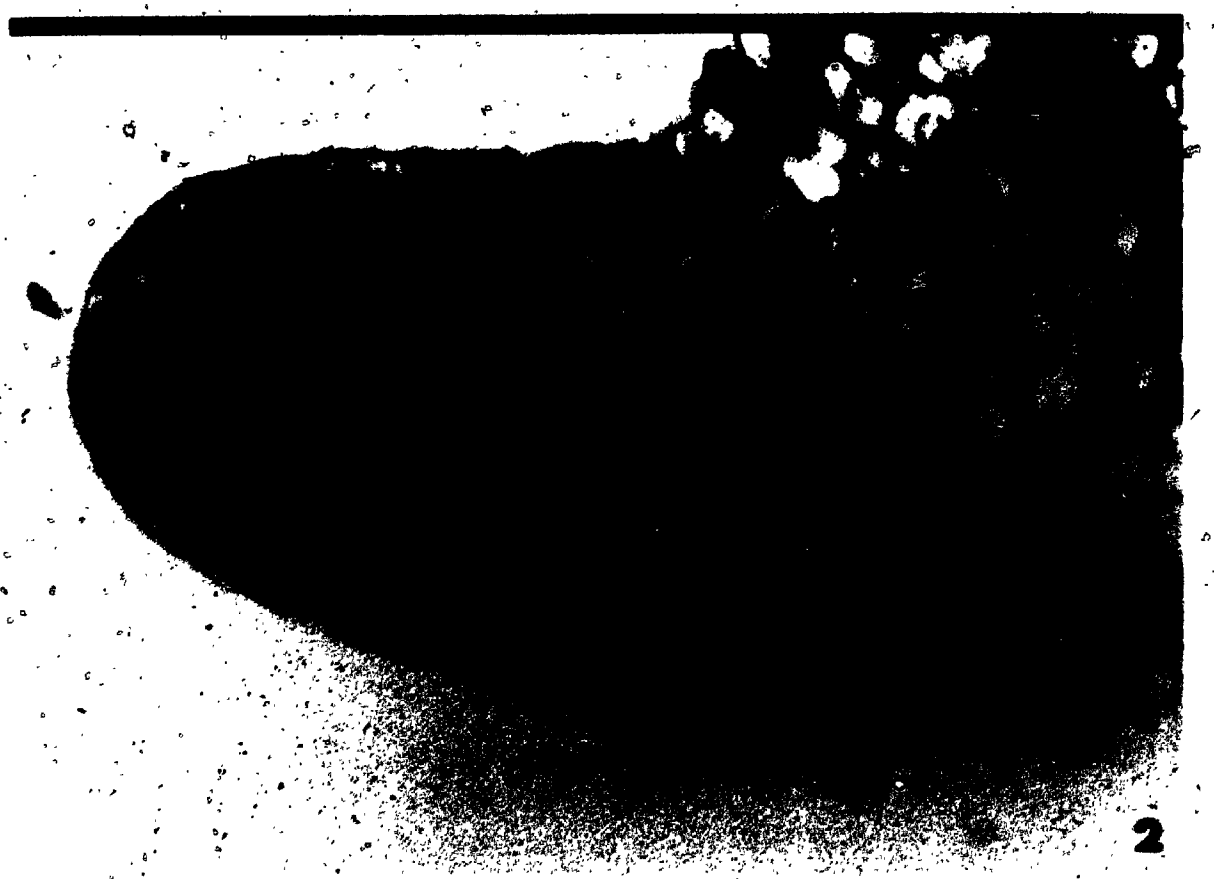
In cross sectional views of the developing leaflets, it is apparent that the marginal initials give rise to the

Plate 3.7. 1. Cross sectional view of a basal lobe of the terminal leaflet showing the early development of the six lamina cell layers (arrows). Leaf 3 is approximately 250 μ high at this stage. (x 800)

2. Slightly later stage of 3.7;1 showing the development of a 2° vein procambium (arrow) from the upper spongy layer. The procambium develops in continuity with the mid rib. (x 1280)



1



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abaxial and adaxial protoderm layers which perpetuate themselves by diffuse anticlinal divisions. In the present study, 4 cell layers can be consistently recognized in the mesophyll with their production following the middle type of marginal growth as described by Hara (1957). Once initiated by the submarginal initials, the four mesophyll cell layers continue to develop by means of anticlinal divisions and cell expansion throughout the lamina (i.e. by means of plate meristem activity). A clear delineation between the marginal and submarginal initials of the marginal meristem and the plate meristem is, at best, indirect when interpreted on the basis of cell wall positions from essentially static cross sections of fixed leaf material. Similar conclusions have already been drawn with regard to the concept of early apical growth of the tomato leaf primordia due to apical and subapical initials (see section 3.3.2.2). Further comments on this dilemma which presently faces the developmental botanist will be found in the discussion.

Marginal growth ceases in the developing leaflets with the formation of the marginal fimbriate vein procambium which develops basipetally and continuously within both margins of the lamina and joins with the derivatives of the secondary vein procambial strands. Further growth in area of the lamina is due to diffuse cell division and enlargement in the plate meristem.

While initial observations of the mitotic activity

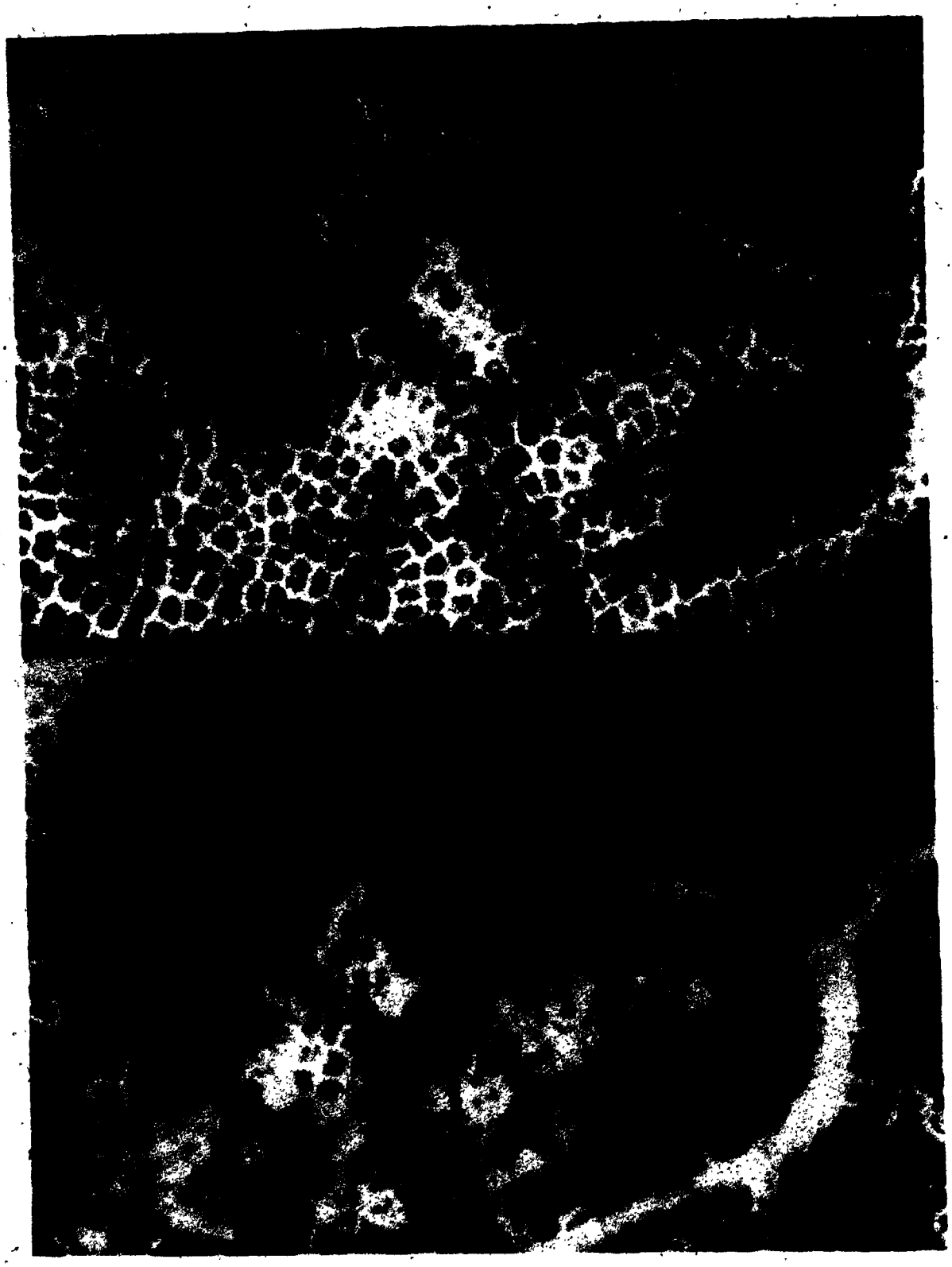
within the developing palisade and upper spongy layers would suggest a diffuse and random pattern, a closer inspection reveals a distinct relationship between the developing procambium reticulum of the minor veins and the planes of division in the previously mentioned two mesophyll layers (Plate 3.8;1,2). As indicated in Plate 3.8, cell division planes within the plate meristem are invariably at right angles or perpendicular to the developing minor veins. Further comments will be made on this observation in the section on minor venation development.

An examination of over one hundred FPA cleared leaves revealed that mitotic activity in the lamina of the most basal leaflet ceases around LPI_3 = +1.0 with the cessation sequence: lower epidermis → lower spongy mesophyll → middle spongy → upper spongy → upper epidermis → palisade mesophyll (Fig. 3.11). However, isolated mitotic divisions are still evident in the lower epidermis (as well as in the upper epidermis although to a lesser extent) after mitotic cessation in the palisade. These late divisions in the epidermal tissue appear to be associated with the last forming stomata. The lower spongy mesophyll has highly vacuolated cells and prominent air spaces at this stage while surrounding epidermal cells are developing irregular borders with mature stomata already present. Furthermore, the last glandular and non-glandular hairs to be formed have nearly completed their development.

As mentioned previously, the tomato leaf is

Plate 3.8. 1. NaOH cleared immature leaflet focused at the level of the developing palisade layer. Note the distinctive cell division patterns (arrows) within the plate meristem. (x 800)

2. Same. Focused at the level of the developing upper spongy layer. Note the relationship between the developing procambial strands (arrows) and the planes of division in the adjacent plate meristem. (x 800)



amphistomatous with stomatal development proceeding more rapidly on the abaxial than on the adaxial surface. Stomatal ontogeny is very similar to that described for tobacco (Esau, 1965a) and proceeds in a basipetal direction within the developing leaf epidermis. Early forming stomatal complexes at the stoma mother cell stage are closely associated with the formation of intercellular spaces within the underlying mesophyll. During leaf development various stomatal stages are often observed in close proximity to one another (Plate 3.9;1). Maturing leaves periodically exhibit guttation droplets associated with the leaflet tips and crenations. Large stomata with underlying cavities adjoining the abundant accumulations of marginal fimbriate vessels are observed in these regions (Plate 3.9;2) and may serve the function of unspecialized hydathodes (Esau, 1965a).

3.3.2.4 Development of the Major Venation

When the leaf primordium attains a height of approximately 50 μ, procambial cells of the incipient primary vein are detected in the base of the primordium (Plate 3.6;2). Differentiation proceeds acropetally and apparently continuously from cambial tissue in the stem and reaches the leaf apex when the primordium is 200 to 300 μ in length (Plate 3.10;1,2).

External phloem differentiates acropetally from the procambial strand and is present in the base of 200 μ high

Plate 3.9. 1. Stomatal cell ontogeny in the lower epidermis of a FPA cleared tomato leaflet. Various ontogenetic stages from the stoma mother cell to a mature stoma are pointed out with arrows. (x 800)

2. Stomatal cavity (arrow) adjacent to marginal xylem vessel elements at the FPA cleared tip of leaflet 3. (x 800)

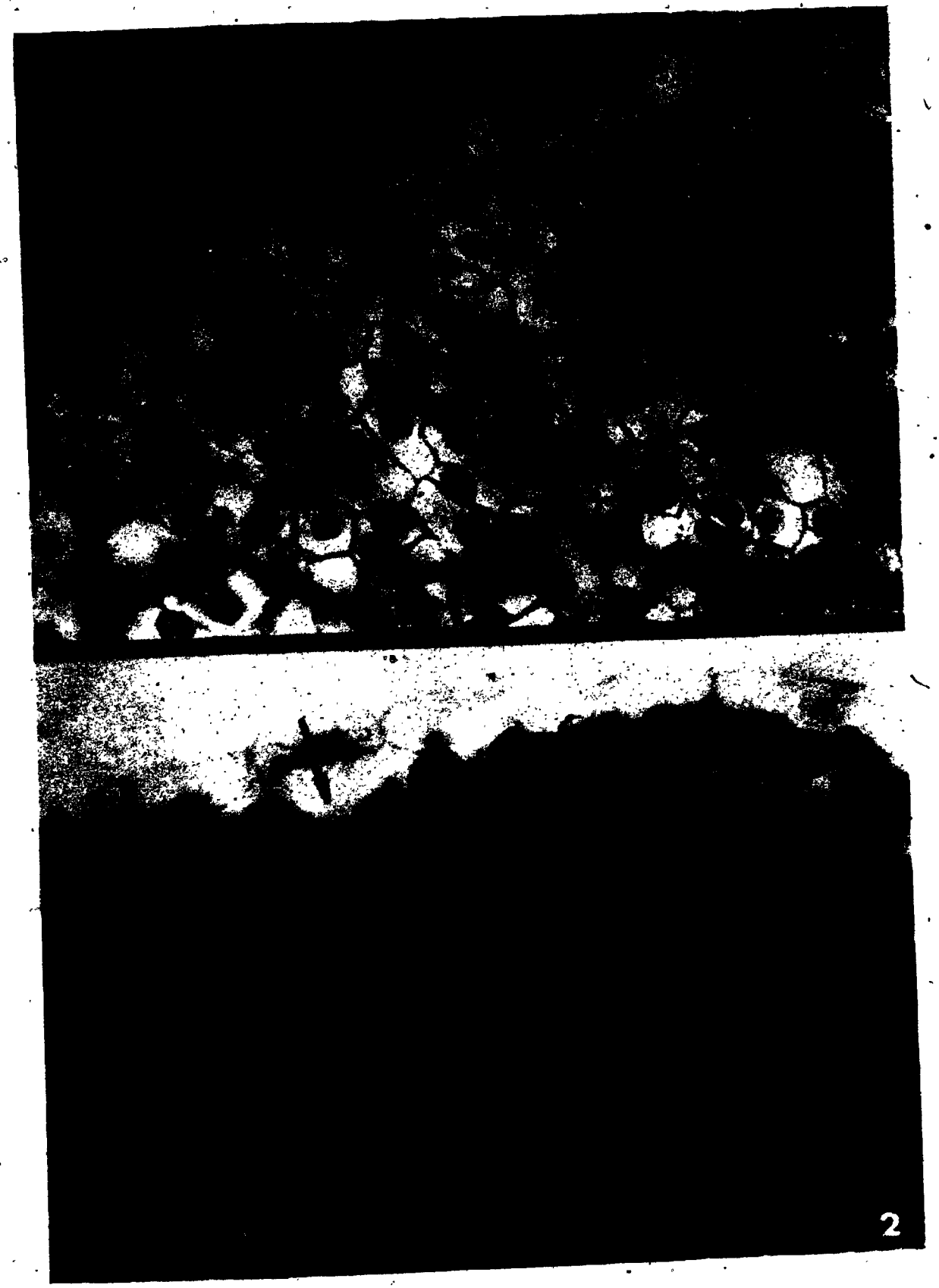

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Plate 3.10, 1. Tip region of leaflet 3 showing the developing procambial strand of the mid rib (arrow). (x 1056)

2. Slightly lower than 1. Note the developing mid rib and secondary vein procambium (arrows). (x 1056)

1

2.

primordia (Plate 3.12;1). The sieve elements, companion cells and associated parenchyma appear to differentiate in a continuous manner towards the leaf apex. The presence of an isolated stem locus of phloem differentiation (cf. Jacobs and Morrow, 1967) cannot be ruled out as a detailed study was not made of this aspect. As will be noted below, however, an isolated xylem locus within the stem is characteristic of this variety. The phloem leaf traces exhibit considerable complexity in the stem when viewed in FPA cleared material. This is particularly true in the hypocotyl regions immediately below the insertion points of the cotyledons where abundant lateral interconnections (literally a "phloem bundle reticulum") are observed among the internal phloem leaf traces (Plate 3.11;1,2).

Xylem vessel elements begin to differentiate in the base of the terminal leaflet when the leaf primordium is approximately 220 μ in height (Plate 3.12;2). Subsequent differentiation takes place basipetally and, to a lesser extent, acropetally. At the 250 μ stage, an isolated stem locus of xylem differentiation can be observed adjacent to the pre-existing leaf traces (Plate 3.13;1,2). Subsequent differentiation at this locus takes place in both directions as well; however, the stem leaf traces are the first to join in the apical region of the shoot when the leaf primordia is 300-340 μ high. Following this junction, xylem elements differentiate basipetally from the stem locus and join with differentiating tracheary elements of an older leaf trace.

Plate 3.11. 1. Cleared region of tomato seedling hypocotyl showing extensive phloem bundle reticulum. Phloem branch point at arrow. (x 200)

2. Close up of the phloem branch point pointed out in 1, showing the nucleated sieve tubes and adjacent phloem parenchyma. (x 960)

Plate 3.12. 1. Base of leaf 3 petiole showing the initial development of the median external phloem strand (arrow) which will develop acropetally into the terminal leaflet. (x 1056)

2. Later stage showing a single median vessel element strand and initiating internal phloem strand (arrow). Note the well developing external phloem strands. Leaf 3 is approximately 1200 μ high. (x 800)

1

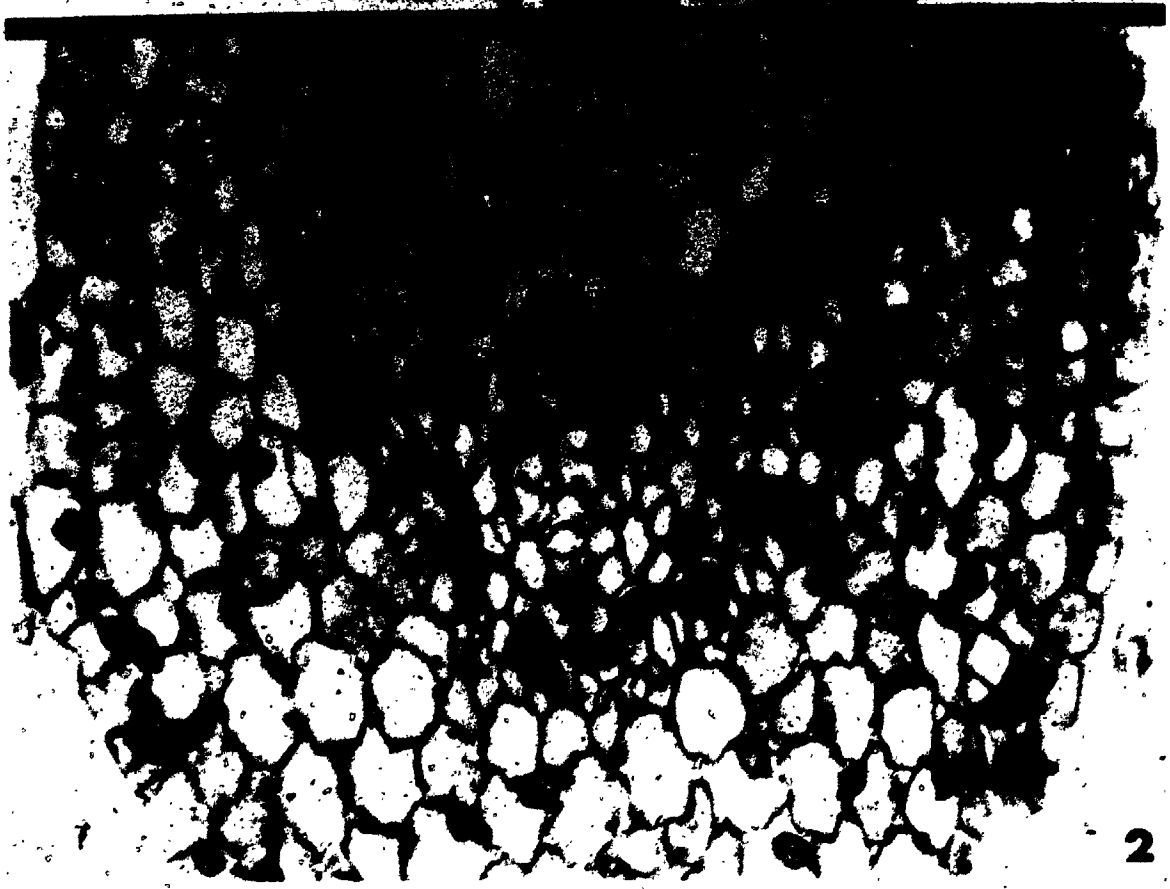

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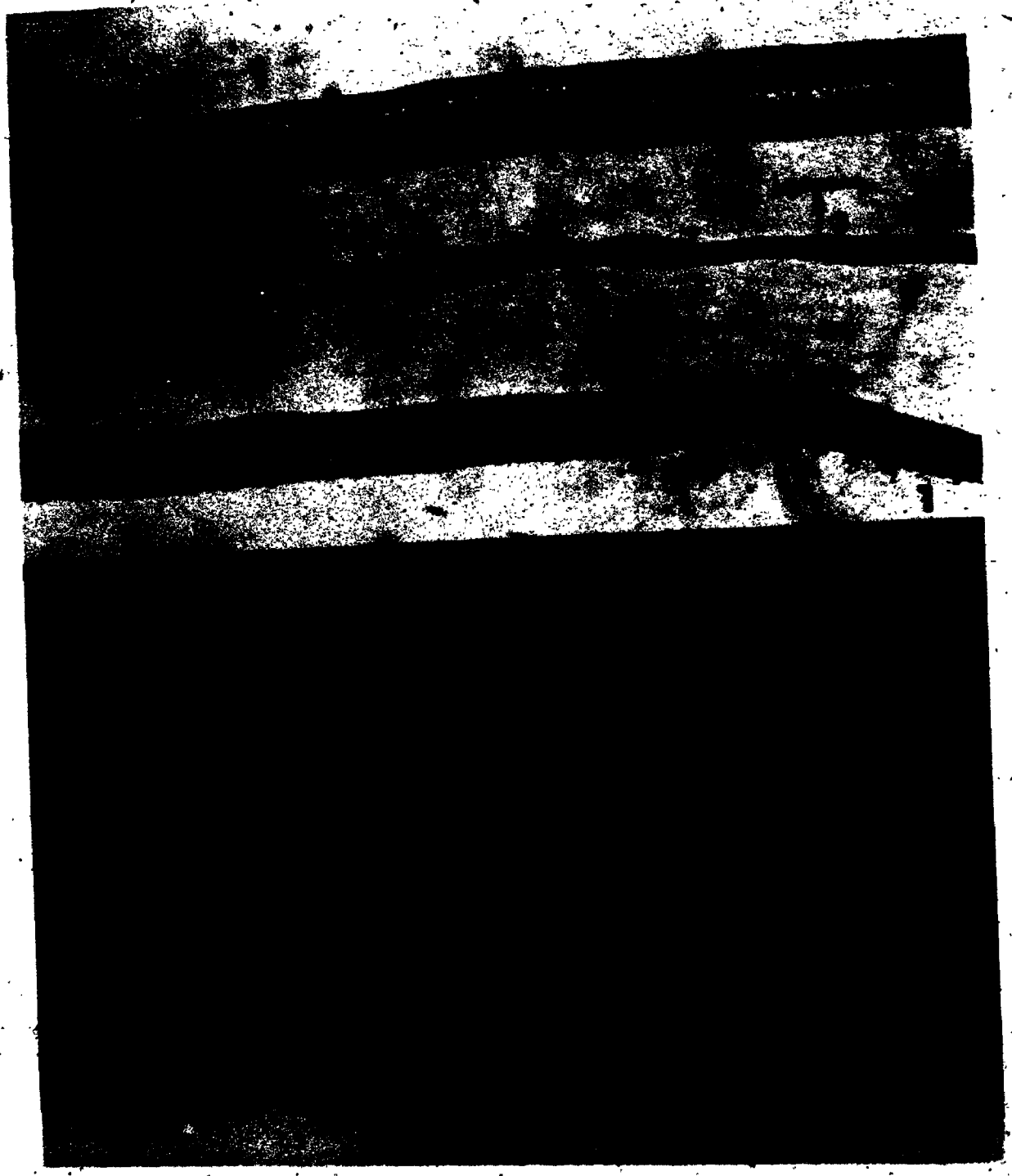
Plate 3.13. 1. Cleared hypocotyl of a tomato seedling showing the isolated stem xylem initiation site of leaf 3 (arrow). Probable initiation site of leaf 2 is also marked. (x 200)

2. Close up of the xylem initiation site for leaf 3 showing the typical vessel member morphology marking the acropetal end of an incomplete vessel. (x 800)



The differentiating tracheary elements of the older traces within the seedling hypocotyl are themselves discontinuous in their development towards acropetally differentiating xylem from the root. While the leaf/stem locii situation is evident in leaves 2 to 6, leaf 1 may exhibit a variation - the first isolated xylem locus is often within the stem adjacent to one of the developing cotyledonary traces. Subsequent development is acropetal and continuous into the leaf. Simultaneously, the xylem develops basipetally and joins one of the cotyledonary traces. Continuous acropetal development of the mid-rib xylem into the tip of the terminal leaflet is achieved by the time the primordium is 360-400 μ in height.

The lateral leaflets subsequently initiate xylem in the basal mid-rib region which then joins the developing tracheary elements within the rachis. However, subsequent acropetal maturation in the leaflet is discontinuous, unlike that found in the terminal leaflet. The mid-rib xylem strands from the two sub-opposite leaflets (#2 and 4) subtending the terminal leaflet develop basipetally where they join with acropetally developing stem loci. The stem loci in turn join with the older, yet still developing leaf traces. However, in leaf 1, the lateral leaf traces are derived from the major secondaries which develop into the basal lobes of the lamina. The presence of the three major xylem traces gives the tomato leaf node its typical trilacunar appearance. Xylem vessel development at the base of the

petiole is positively correlated (r = 90.7; significant at the 5% level) with the developmental status of the leaf (Fig. 3.12).

Secondary vein procambium in the terminal leaflet is apparent in 250 μ long leaf primordia and develops in a continuously acropetal manner from the mid-rib procambium. All the secondary veins are outlined by procambial strands before the leaf is 400 μ in length (Plate 3.14;1). The procambium of the secondary veins originates from the developing upper spongy layer with the middle spongy layer contributing cells to the abaxial surface of the bundle sheath (Plate 3.7;2).

Simultaneously with secondary vein procambium development, external phloem progresses acropetally into the distal region of the leaf where it will develop into the secondary veins. This process of phloem differentiation proceeds in successive secondaries in a basipetal sequence and precedes secondary vein xylem development. The various external phloem cells develop in a continuous sequence with no apparent disjunct differentiaion.

Internal phloem develops in an apparently continuous manner from the internal phloem strands of the older leaf traces and is present in leaf primordia 550-700 μ long (Plate 3.12;2). It appears to terminate its acropetal differentiation at the junction of the most distal secondary veins. Internal phloem proceeds to develop acropetally in successive secondaries in a basipetal sequence.

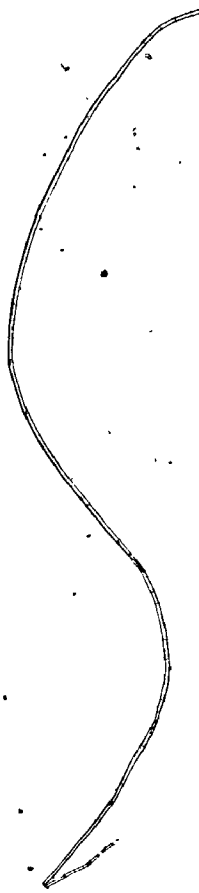


Figure 3.12. Number of xylem vessel elements within the tomato petiole as a function of LPI_3.

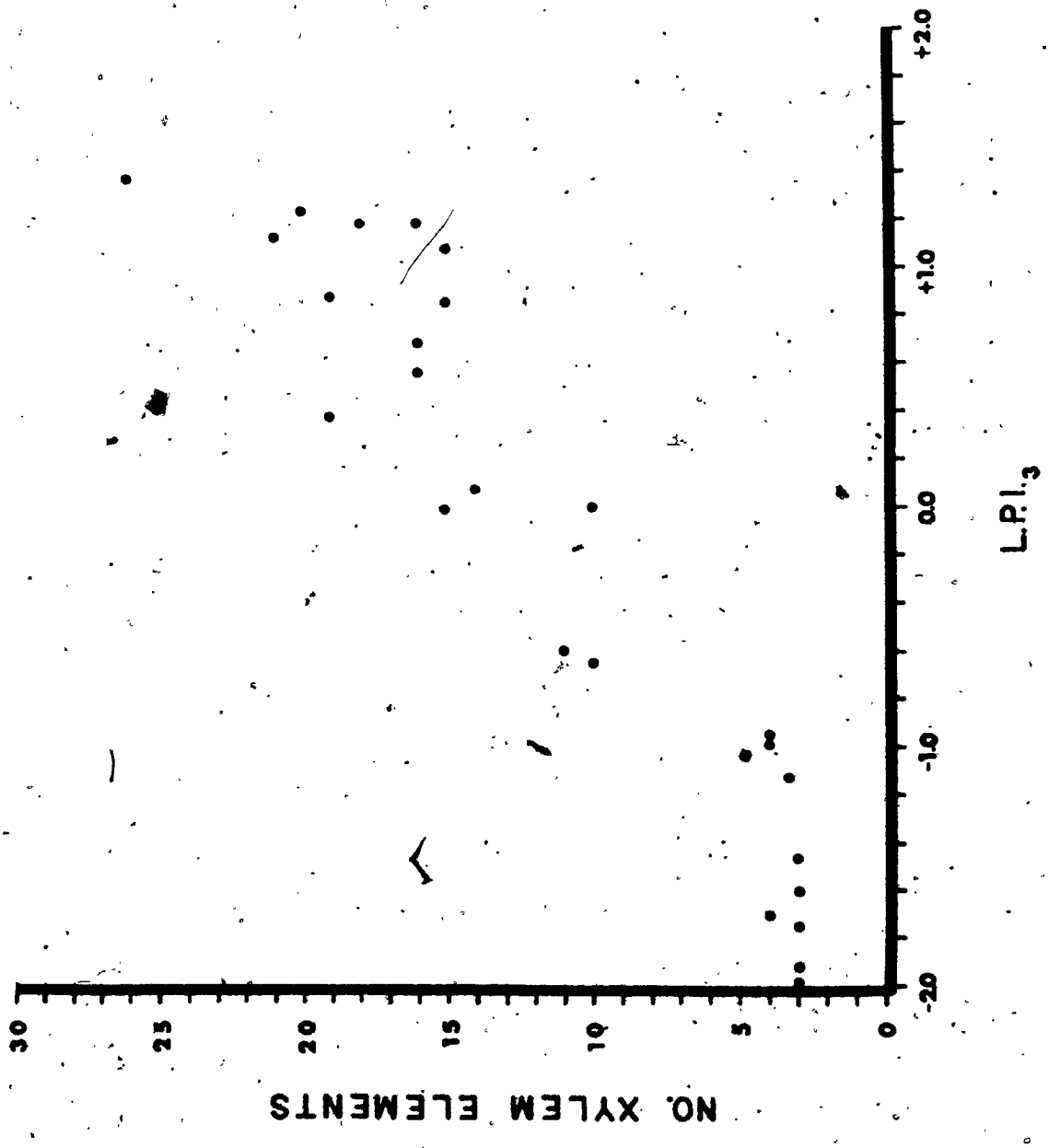
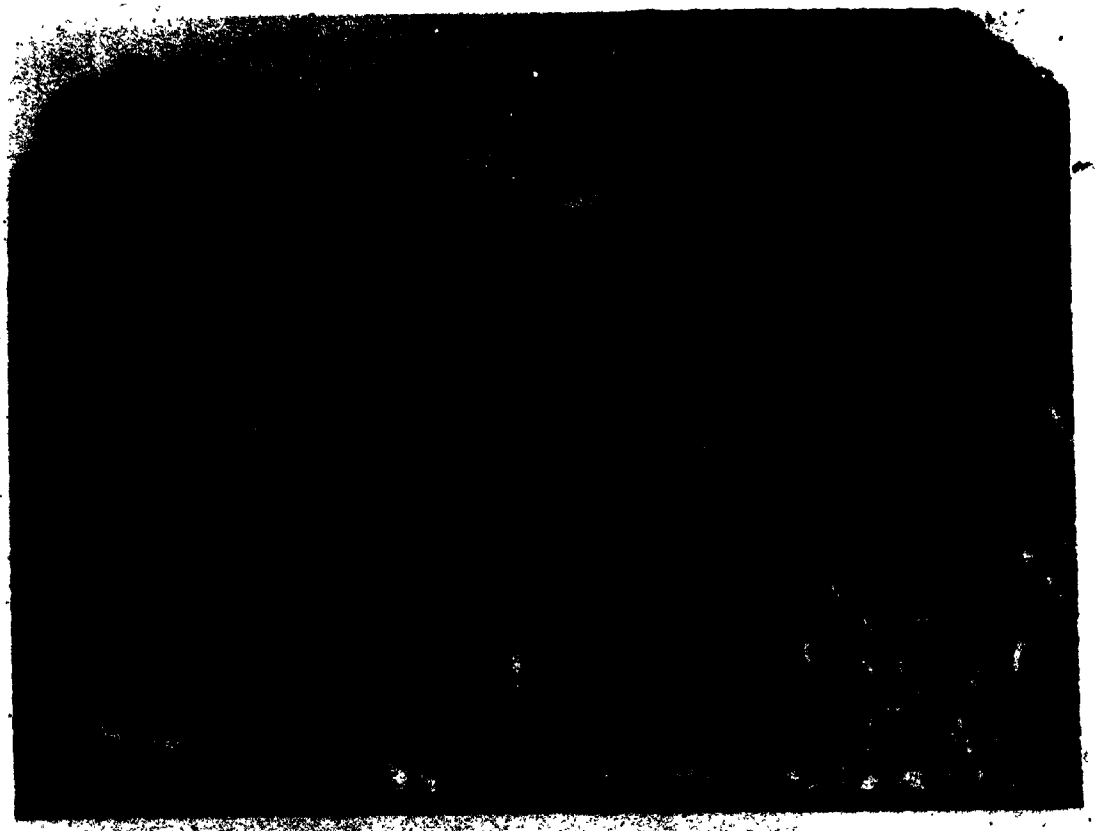

+2.0
+1.0
0.0
-1.0
-2.0
L.P.I.3
30
25
20
15
10
5
0
NO. XYLEM ELEMENTS

Plate 3.14. 1. Progressive and continuous development of the procambial cells of the 2° veins and their derivatives (arrows) from the upper spongy mesophyll layer. (x 800)

2. Later stage than 1 showing the isolated vessel element development of the marginal fimbriate vein (arrow). Examples of discontinuous tracheid maturation in the minor veins are also evident. (x 200)



The first mature xylem elements appear in the distal secondaries of the terminal leaflets when the entire leaf is approximately 450-550 μ long (Plate 3.15;1,2). Irregular maturation of the tracheary elements is consistently observed in the secondary veins (Plate 3.16;1,2). The basipetally differentiating marginal fimbriate vein xylem is also discontinuous (Plates 3.14;2 and 3.16;1). It often develops in an isolated manner at the junctions of 2° vein derivatives within the crenations. Subsequent xylem differentiation in the marginal fimbriate veins is essentially basipetal (Plates 3.16;1 and 3.17;1,2) and its development is in close continuity with the minor vein xylem.

3.3.2.5 Development of the Minor Venation

Minor vein procambium differentiates progressively from the plate meristem and is restricted in origin to the upper mesophyll layer. Initially the earliest occurring procambial strands are formed through a series of periclinal and anticlinal divisions in the distal part of the terminal leaflet, and later, in successive intercostal areas in a basipetal sequence (Plate 3.18;1,2). Similar basipetal sequences are found in the lateral leaflets during later, yet comparable, stages of development.

During the leaf's early increase in area, the minor vein reticulum expands by elongation and is accompanied by occasional transverse divisions of the procambial cells. Some discontinuities were observed in the progression of the

Plate 3.15. 1. Initial stages in the development of the marginal fimbriate vein xylem at the tip of the terminal leaflet. (x 200)

2. Close up of the vessel elements at the leaflet tip. Note the apparent scalariform perforation plate at arrow. (x 800)

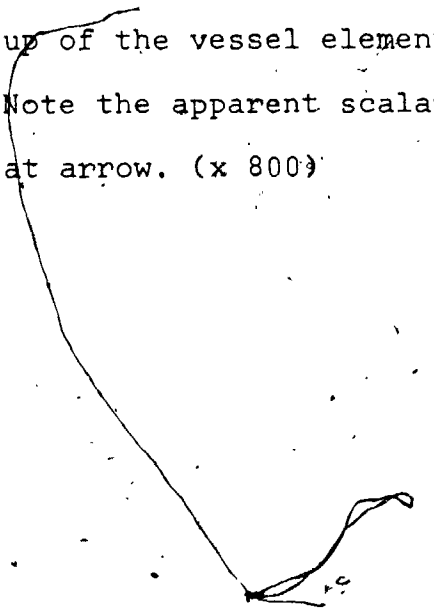


Plate 3.16. 1. NaOH cleared preparation of an immature basal leaflet showing basipetal maturation of the major and minor xylem systems. Discontinuities in the marginal fimbriate vein xylem are noted by arrows. (x 50)

2. Basipetal and discontinuous maturation of the mid rib and 2° vein xylem in an immature terminal leaflet. (x 240)

Plate 3.17. 1. Immature tip of leaflet 3 (NaOH cleared) showing irregular maturation of the minor vein xylem and its relationship with the marginal fimbriate xylem. (x 200)

2. Immature margin near the tip of leaflet 3 (FPA cleared) showing the nucleated vessel element with perforation plate (arrow at left). Note the failure of the external phloem strand (arrow) to develop into the extreme leaflet tip (towards the bottom of the plate). (x 800)

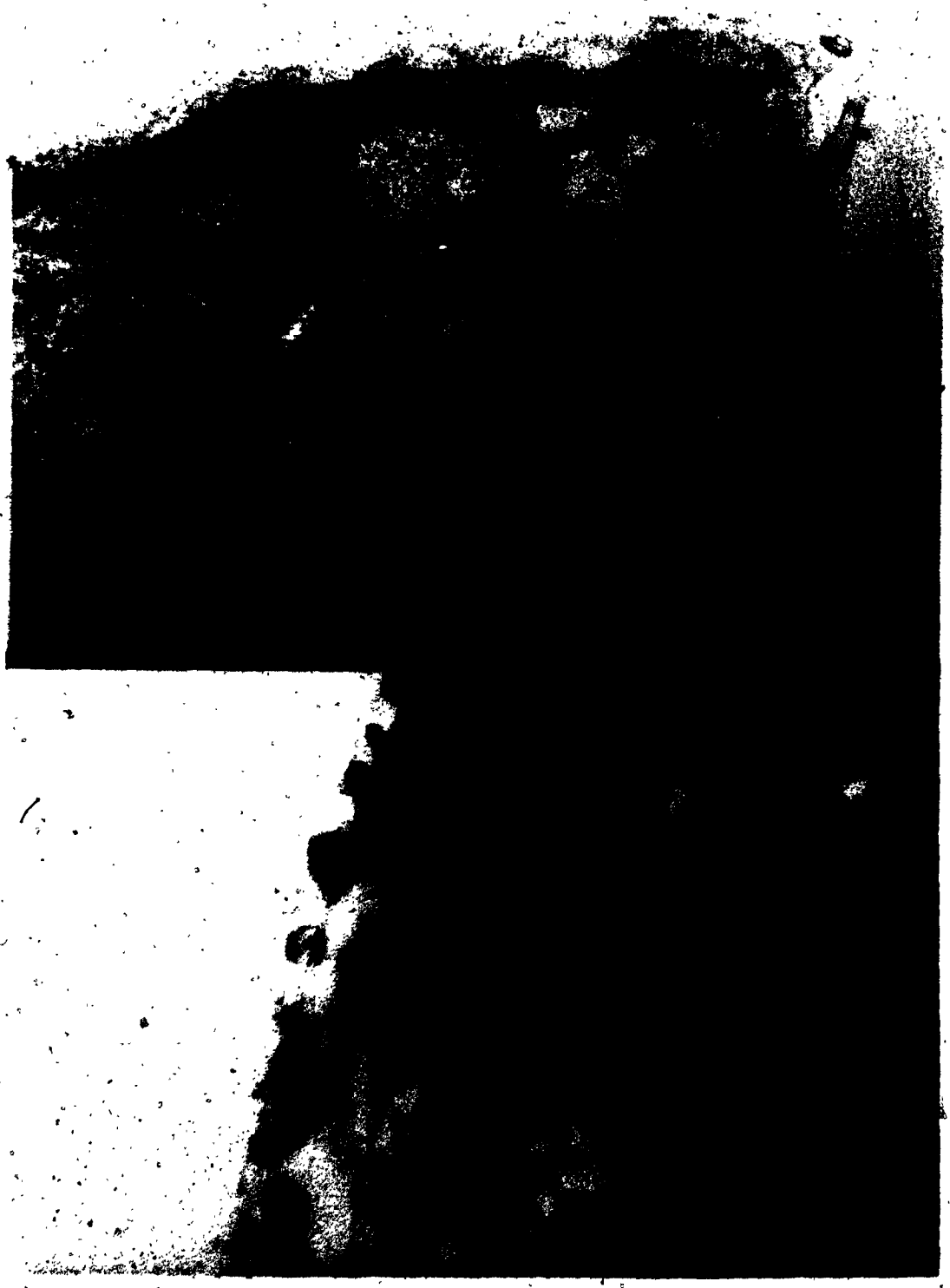


Plate 3.18. 1. Early stages in minor vein development from the upper spongy mesophyll layer as viewed in cross-section. The numbers on the plates refer to the ontogenetic stages defined by Figure 3.17. (x 1280)

2. Progressive development of a vein ending as viewed in a FPA cleared leaf preparation. Arrow marks the end cell which is undergoing a paradermal division into stage 5 or 6. (x 1056)


2

procambial tissue through its developmental sequences. Generally, these discontinuities involved apparently precocious, yet essentially normal, mitotic activity at the intersections of minor veins (Plates 3.19;1,2 and 3.20;1,2). Vein ending formation is essentially unpredictable due to its random nature. In general, however, most if not all developing procambium tissue within clearly defined areoles will become terminal veinlets i.e. late forming procambial strands developing progressively towards a relatively mature minor vein. A few of the variations in development of the procambial network as viewed in paradermal sections are outlined in Figures 3.13 to 3.16. Elongated plate meristem cells in continuity with developing procambial strands are outlined by stippling. These cells are interpreted as potential procambial cells or "extension cells" sensu Hara (1962).

As the figures indicate, minor vein development is progressive with areole formation following 2 distinct patterns:

a) areole formation due to progressive development of a procambial strand (at right angles to the existing strand) and across an earlier formed areole (or "areole precursor") (Figures 3.13A, 3.13B, 3.14A, 3.14B and 3.15A).

b) Areole formation due to multiple procambial strand linkage within an earlier formed areole (Figures 3.15B, 3.16A and 3.16B). This type of areole formation follows from the near simultaneous development of a number of procambial strands into an areole precursor.

External phloem initiation in the minor vein reticulum

Plate 3.19. 1. Stages 6 and 7 in minor vein development as viewed in FPA cleared leaf material. (x 1056)

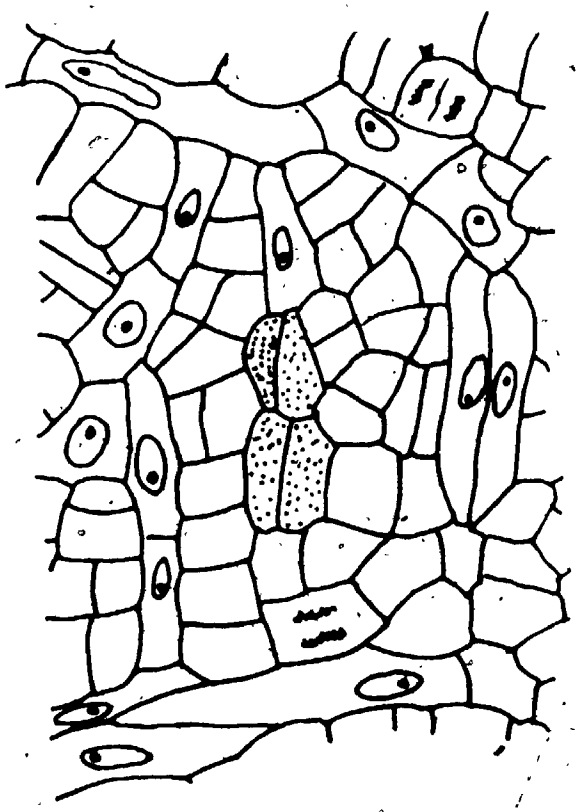
2. Similar to 1. Note the pair of transverse divisions in the minor vein procambium strand giving rise to stage 7. (x 1056)

Plate 3.20. 1. Stages 10 and 11 in minor vein development as viewed in FPA cleared leaf material. (x 1056).

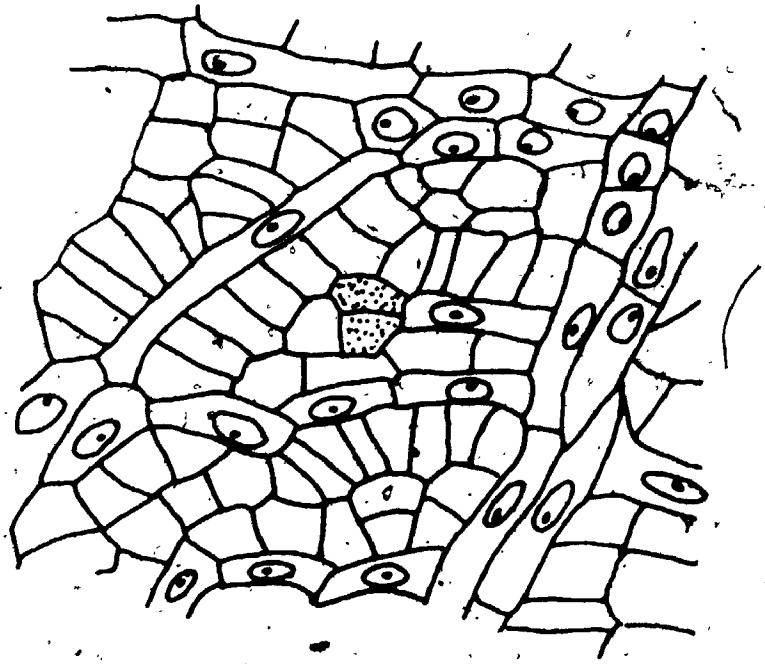
2. Similar to 1. Note the formation of intercellular air spaces within the adjacent spongy mesophyll. (x 1056)


11
11

Figure 3.13;A and B. Progressive formation of minor veins within the developing leaf lamina and viewed in paradermal section. These figures are derived from tracings of photographic prints. Potential procambial cells or "extension cells" are outlined by stippling while procambial cells are nucleated. Note the presence of only one or two plate meristem cells between the developing procambial strands as well as the typical biseriate origins of the minor vein procambium. (Fig. 3:13;A, x 1056; Fig. 3.13;B, x 960).

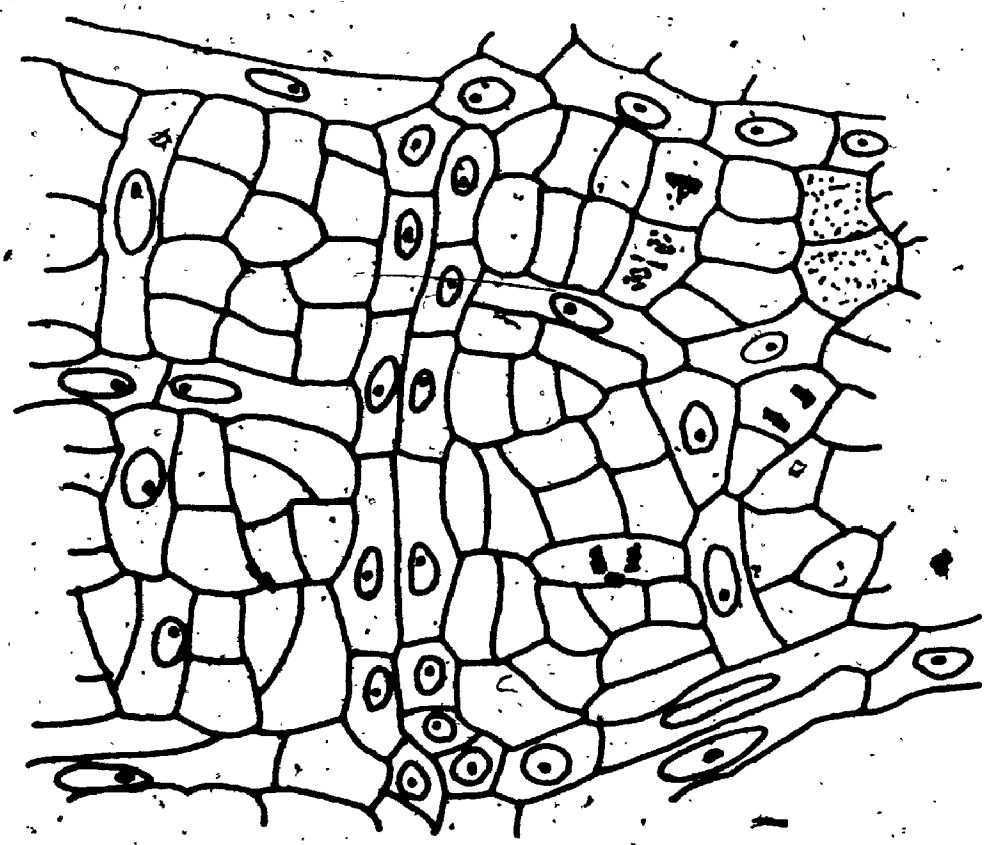


A

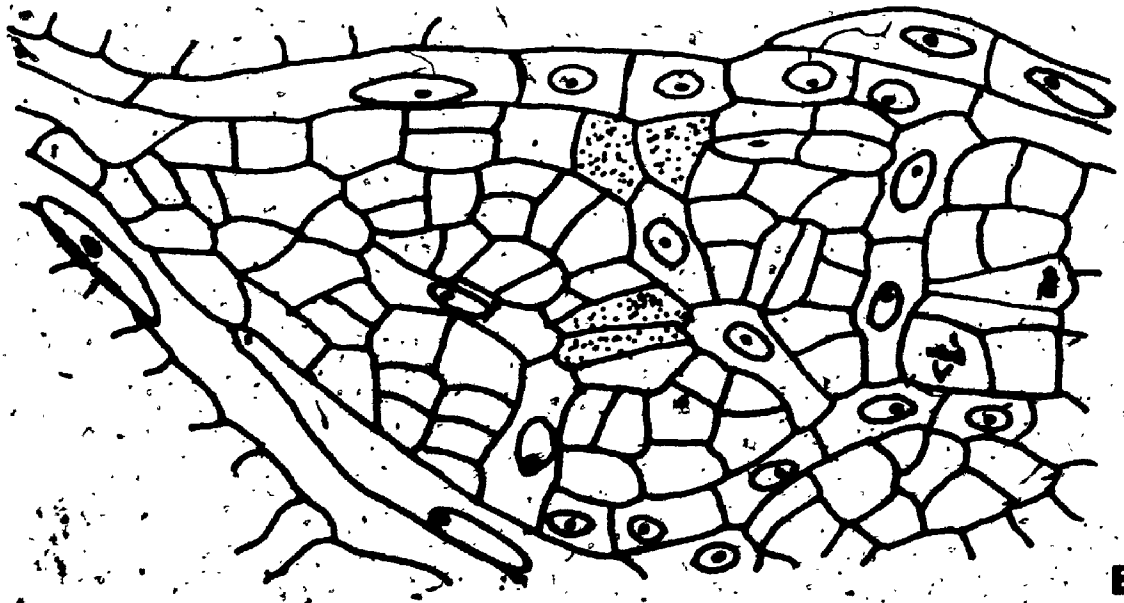


B

Figure 3.14;A and B. Further examples of the progressive formation of minor veins within the developing tomato leaf lamina. Note the apparent extension cell interconnections between developing procambial strands in Figure 3.14;B (x 1056).



A



B

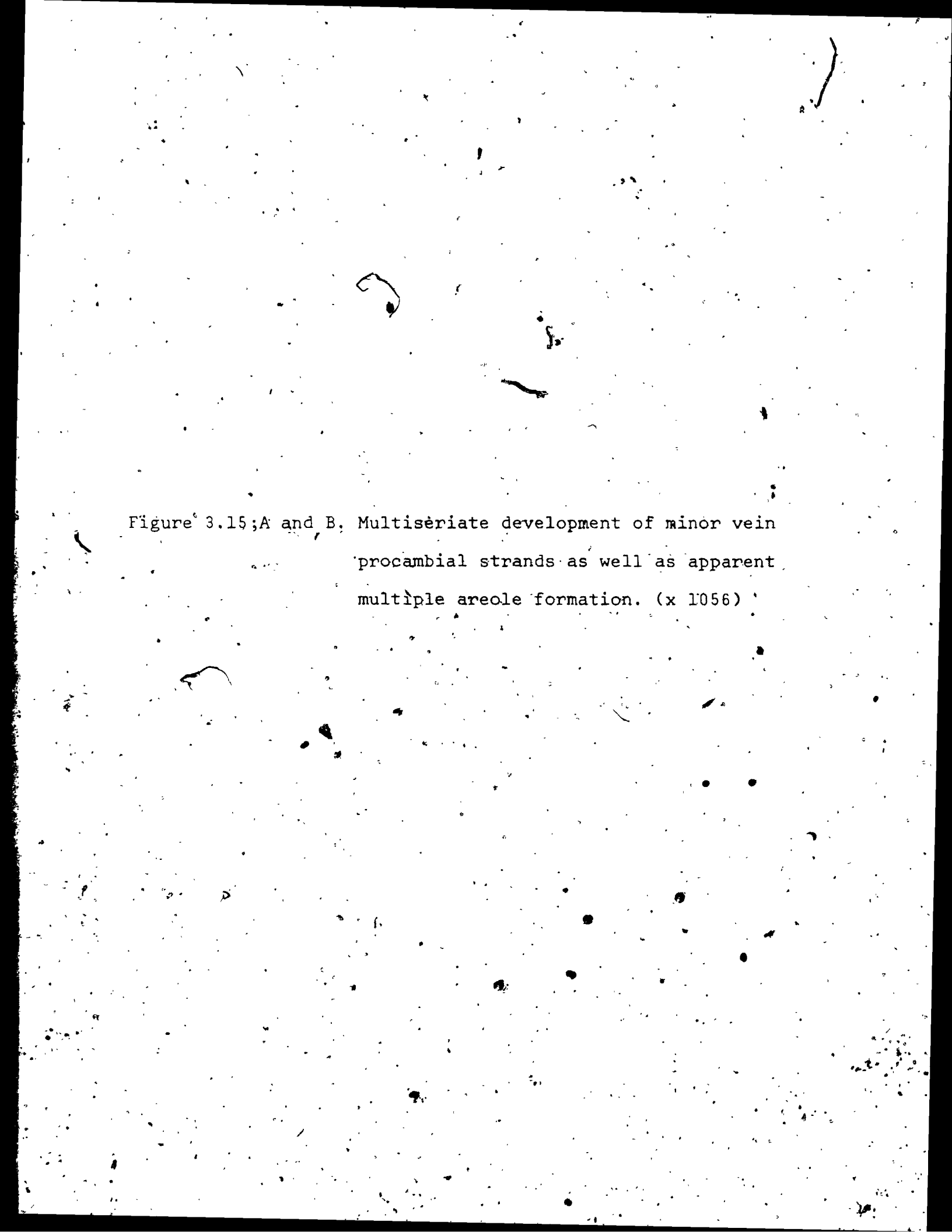
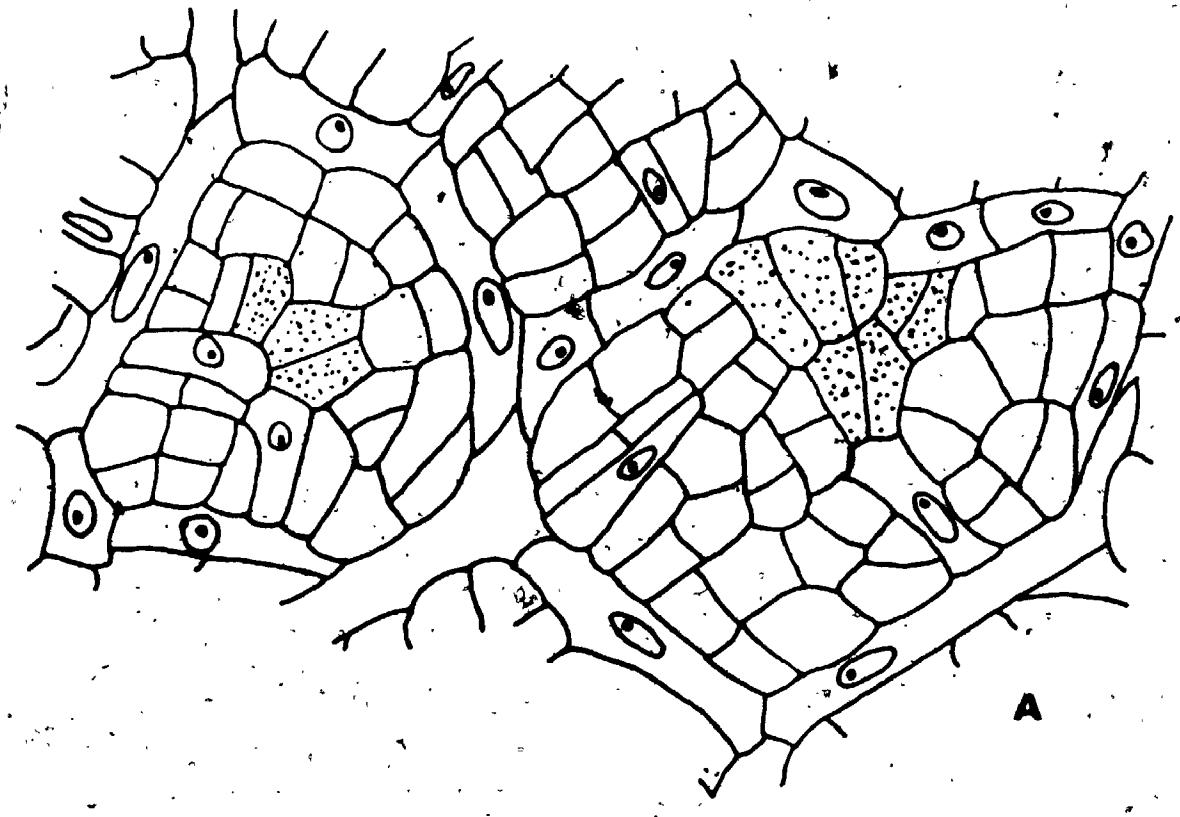
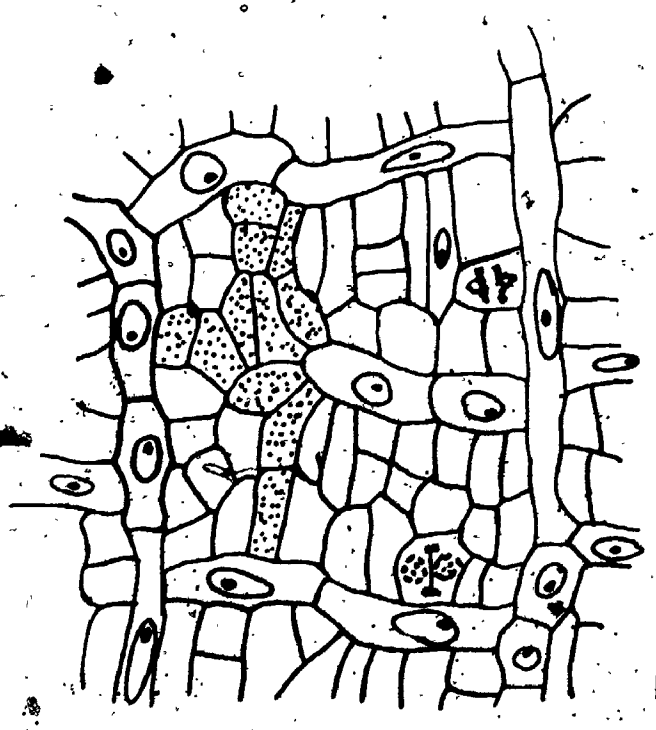


Figure 3.15;A and B. Multiseriate development of minor vein procambial strands as well as apparent multiple areole formation. (x 1056)



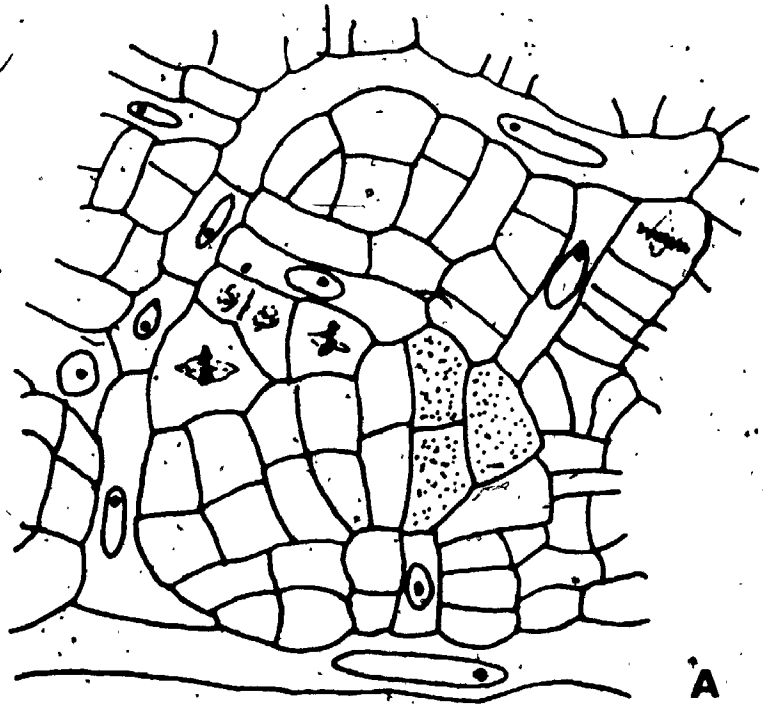
A

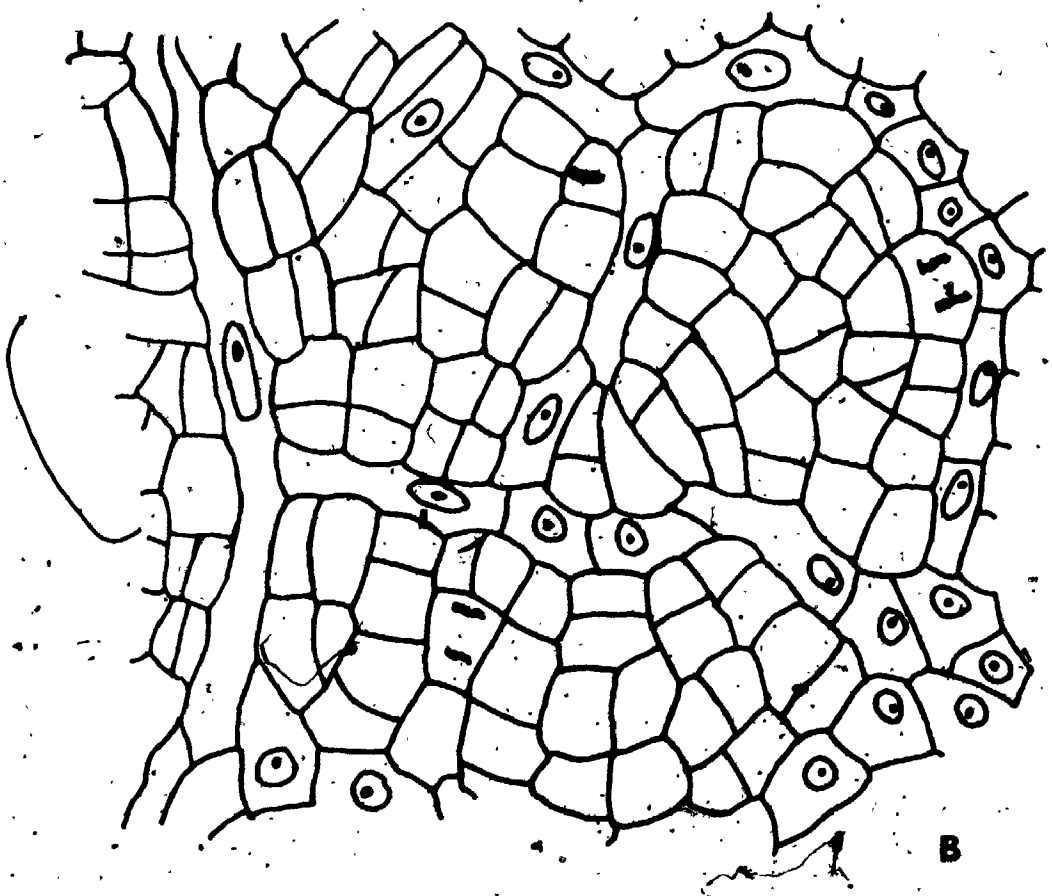


B

Figure 3.16;A. Areole formation due to multiple procambial strand linkage within an earlier formed areole. (x 1056)

B. Later stage than A. Note the planes of the cell wall formation within the plate meristem cells surrounding the developing procambial strands of the minor veins. (x 1056)


A


B

is basipetal and essentially continuous with existing phloem elements. Although tracheid precursors are laid down prior to the phloem within the developing procambial reticulum, maturation of the sieve tubes is completed prior to the differentiation of adjacent tracheids. The tracheids mature in a typically discontinuous yet basipetal manner with complete development within the basal leaflet lamina occurring during the exponential phase of growth (i.e. at approximately $LPI_3 = +2$) when the surrounding mesophyll cells are non-mitotic, highly vacuolated and undergoing cell separation (Plate 3.14;2 and Plate 3.21;1,2). A generalized summary of minor vein ontogeny is presented in Figure 3.17.

In mature leaves (i.e. $LPI_3 > 4.0$), anomalies in xylem development are evident in the minor veins and particularly in association with terminal veinlets. The most common aberrations include lack of any intervening mature tracheids between a seemingly isolated, yet mature, vein-ending and the occurrence of only one tracheid within the vascular strand. No discontinuities were observed in the external phloem strands at these aberrant xylem sites.

Variation in xylem development could be traced to early differences in the direction and/or number of mitotic events around stage 5 (Fig. 3.17). These inferred anomalies in xylem ontogeny include:

a) transverse divisions of cell 3 followed by normal phloem development from derivatives of cells 4 and 5

Plate 3.21; 1 and 2. Examples of incomplete tracheid maturation in the minor vein system. These "precocious" tracheids often develop at the point of vein intersections. (Plate 3.21;1, x 800 and Plate 3.21;2, x 960)

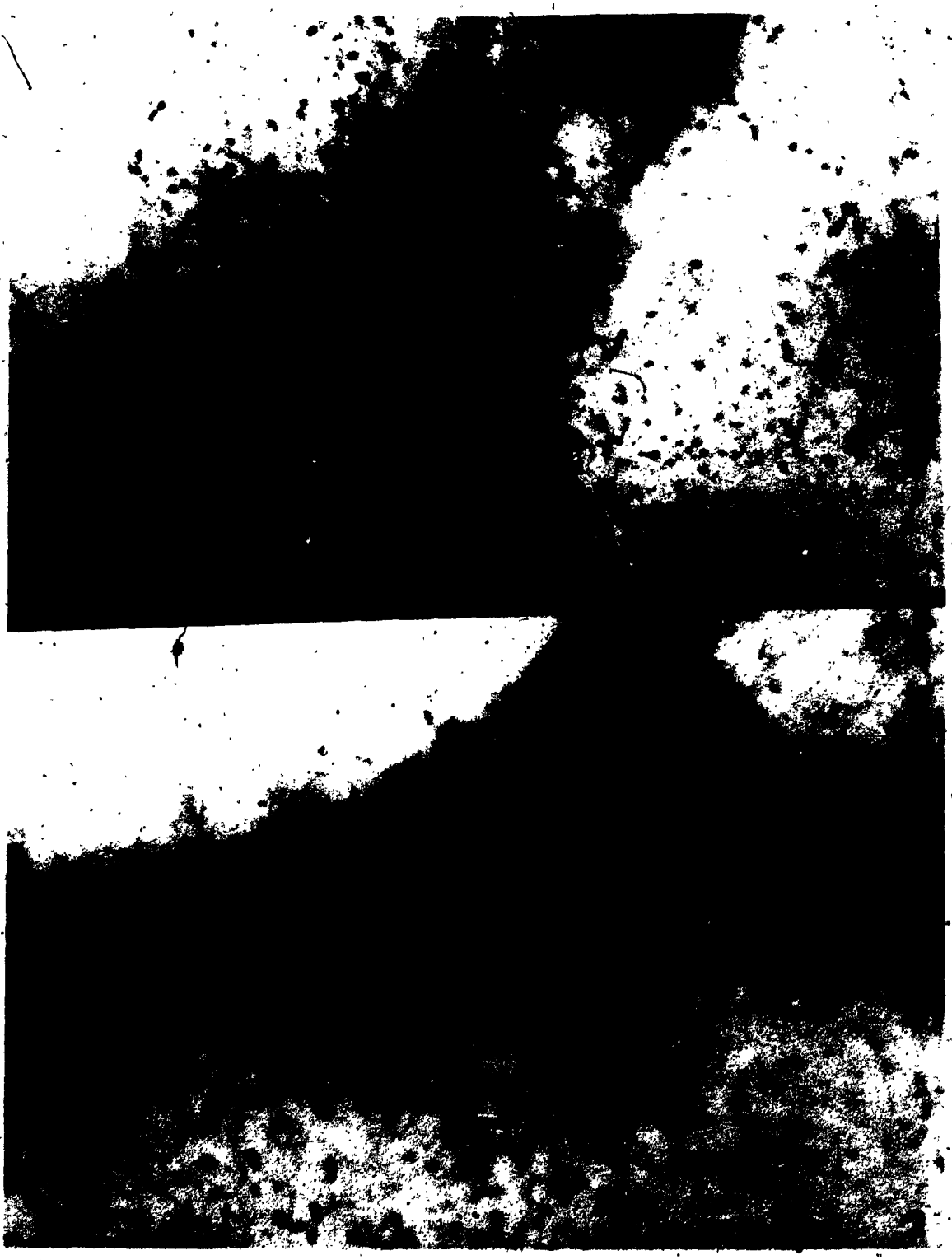
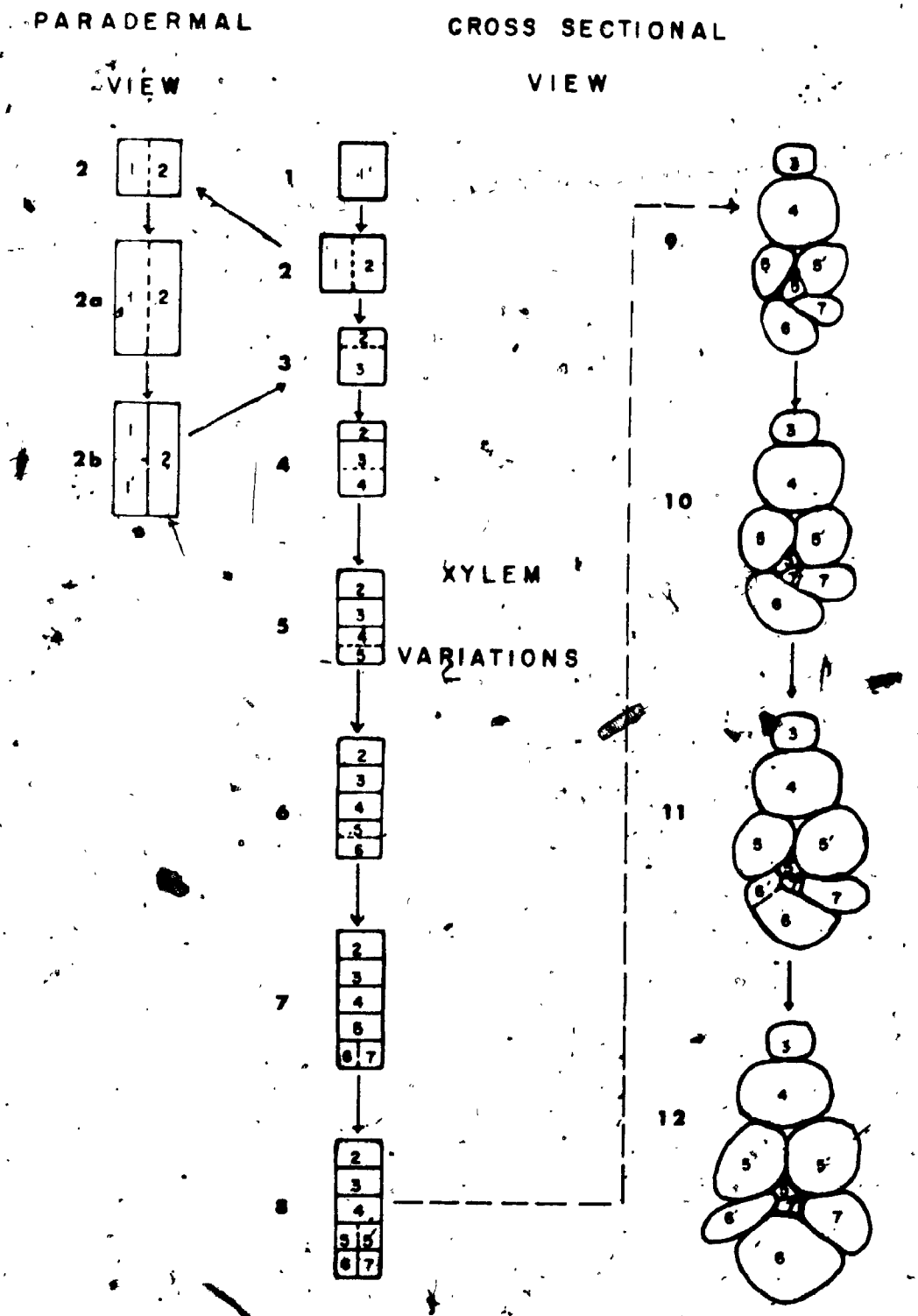


Figure 3.17: Generalized summary of minor vein ontogeny in the developing tomato lamina. Cell types at the mature stage (12): 3 and 4, tracheids; 5, 5', 6, 6' and 7, vascular parenchyma; 5" and 7', sieve tubes.


PARADERMAL
VIEW
CROSS SECTIONAL
VIEW
XYLEM
VARIATIONS
1
2
2a
2b
3
4
5
6
7
8
9
10
11
12

b) cell 4 develops as xylem parenchyma; normal phloem development from derivatives of cell 5;

c) cell 3 develops as xylem parenchyma; normal phloem development from derivatives of cells 4 and 5;

d) cell 3 develops as xylem parenchyma; no tracheids form; normal phloem development from derivatives of cells 4 and 5;

e) cell 4 divides paradermally; the upper derivative becomes a tracheid; the lower derivative becomes a xylem parenchyma. This appears to be the most common aberrant form of the five observed.

Xylem strand separations (Plate 3.22,1,2) were occasionally observed between minor veins and veins of increased complexity (e.g. between 2° and 3° veins). These separations resemble Slade's example (1959; Plate 5, Fig. 3 of her article) of a small veinlet becoming disconnected from a main lateral vein. A close examination of these sites in NaOH cleared mature tomato leaflets ($LPI_3 > 4.0$) revealed, however, that the "separations" were often restricted to the xylem elements and not to the associated phloem and parenchyma. Slade's example appears quite similar. Whether these xylem separations were due to lamina expansion or histological processing is unknown. However, no evidence was found of vein ending formation by a rupturing mechanism at any stage of the leaf's development as postulated by Slade.

Plate 3.22. 1. Incomplete xylem development at the junction of a 2° vein with two 3° veins (arrows) in a NaOH cleared leaflet. Phloem development is complete at these sites. (x 200)

2. Incomplete xylem development in a minor vein. Complete phloem strands are evident (arrows). (x 800)

3.4 Discussion

The growth and biochemical studies outlined above demonstrate that the PI and LPI are applicable, within limits, for describing the morphological status of the vegetative tomato shoot. Furthermore, this application is valid either to individual plants sampled over a specific time interval or to a large number of plants sampled simultaneously at different times of the year.

The non-linearity in the PI/time curve (Fig. 3.3) is due to flower bud production after 9-10 leaves are produced. Although this index is, as a consequence, limited in accuracy to the early vegetative stages, it is apparent that the PI and LPI are quite adequate for studying developmental features of the early formed leaves (i.e. leaves 1 to 7).

True leaf number 3 goes through four distinct growth stages which can be characterized in terms of the LPI_3:

i) $LPI_3 > -2.0$. This period includes all features of leaf development until the leaf is 2-6 mm long e.g. initiation of the terminal and lateral leaflets in a basipetal manner and associated anatomical features (summarized in Table 3.7).

ii) $LPI_3 = +1.0$. Cessation of mitotic activity in the lamina of the most basal leaflets. Subsequent lamina growth by cell expansion and cell separation only. A summary of lamina development is presented in Figure 3.18.

iii) $LPI_3 = +2.0$. Completion of minor vein maturation

3 6

OF/DE

Table 3.7. A summary of important early ontogenetic events during tomato leaf growth and development.

Approximate leaf length	
20- 40 μ	vacuolation of epidermal and cortical cells on the abaxial surface; differentiating epidermal hairs evident
50 μ	midrib procambium in leaf base
200- 300 μ	midrib procambium in leaf apex
200 μ	marginal growth initiated; external phloem in leaf base; adaxial meristem in leaf base
220 μ	isolated xylem leaf locus
250 μ	isolated xylem stem locus
250- 400 μ	secondary vein procambium apparent
270- 300 μ	leaflets 2 and 4 present as small bulges on the differentiating rachis
300- 340 μ	complete xylem connection between stem and leaf
360- 400 μ	mature vessel elements at leaflet 3 apex
400- 450 μ	formation of apical fimbrial vessels
550- 600 μ	first mature secondary vein xylem connection with basipetally differentiating marginal fimbrial vein xylem
550- 700 μ	internal phloem strands differentiating acropetally towards leaf apex
1000-1100 μ	leaflet 3 approximately 800 μ long; leaflets 2 and 4 approx. 200 μ; leaflets 1 and 5 approx. 50 μ; fimbrial vein procambium halfway down leaflet 3 margin
1-2 mm	cessation of mitotic activity in apical lamina region of leaflet 3
5 mm	mature marginal fimbriate vein (LPI$_3$ ≈ -2.0 approximately) xylem at the tips of leaflets 2 and 4

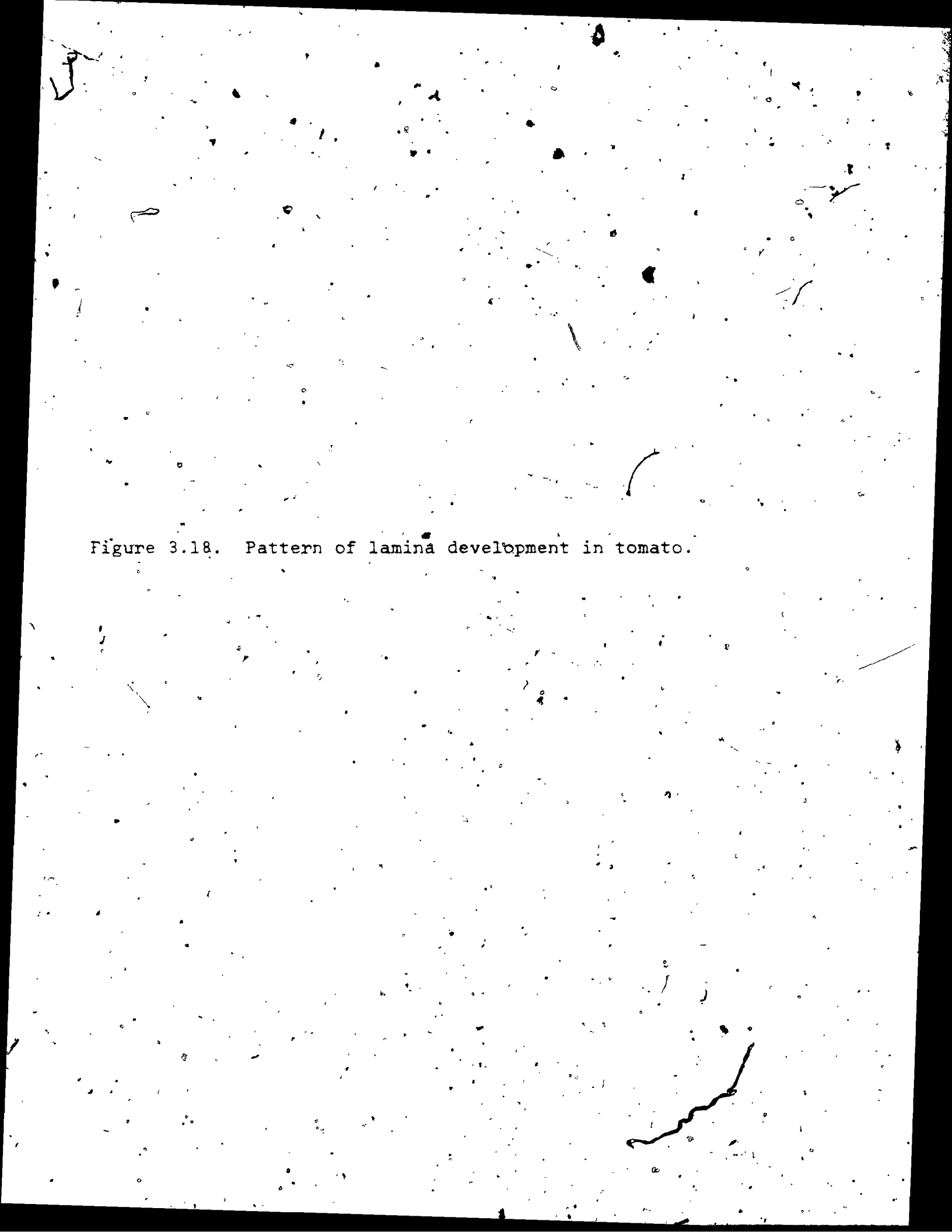
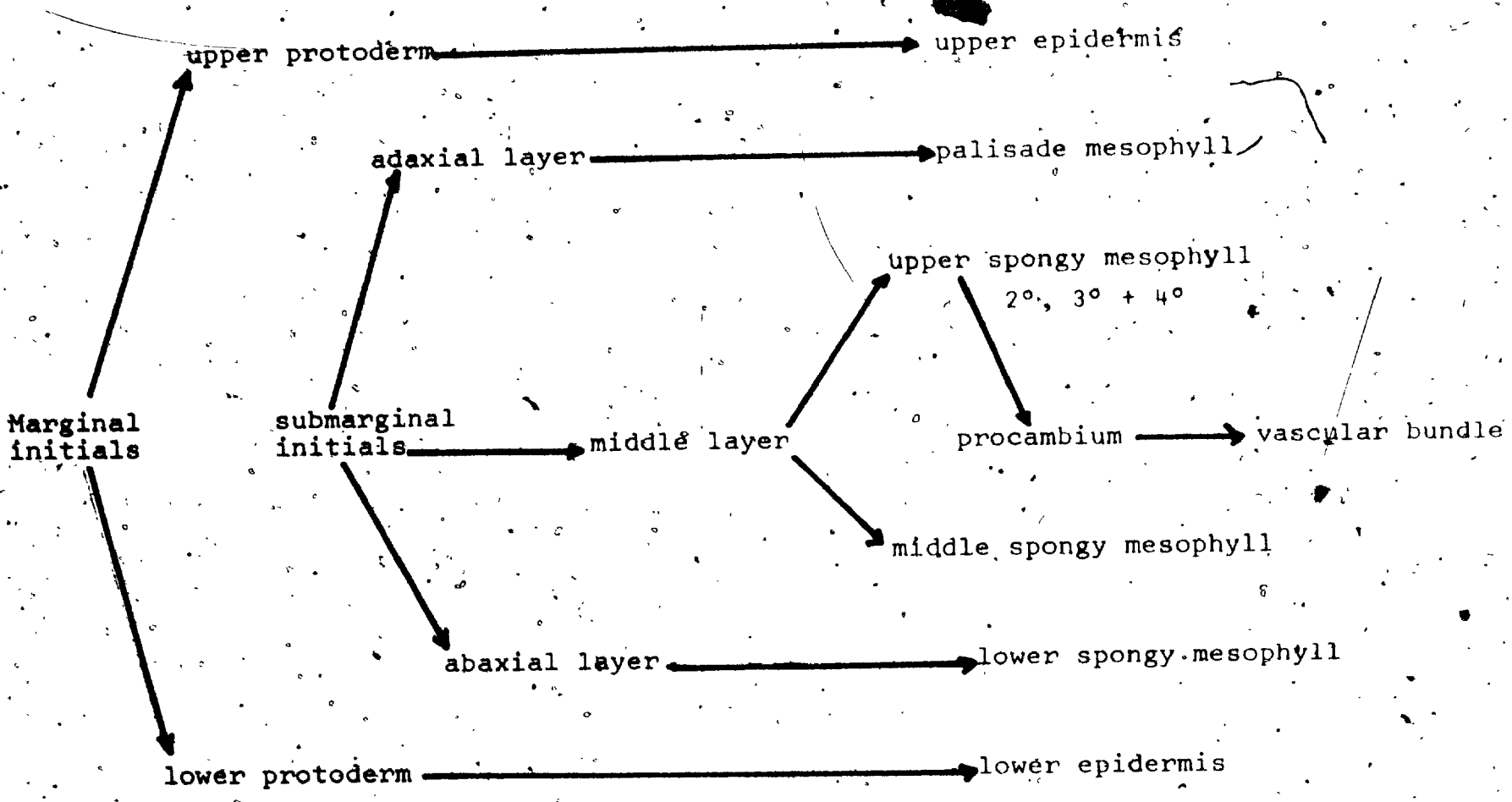


Figure 3.18. Pattern of lamina development in tomato.


upper protoderm
upper epidermis
adaxial layer
palisade mesophyll
upper spongy mesophyll
2°, 3° + 4°
Marginal initials
submarginal initials
middle layer
procambium
vascular bundle
middle spongy mesophyll
abaxial layer
lower spongy mesophyll
lower protoderm
lower epidermis

(i.e. vein ending tracheid maturation) which immediately preceedes initiation of a rapid decline in total soluble carbohydrates within the developing leaf. Approximate point of maximum growth rate in leaf length, fresh weight. Cell enlargement and separation is taking place throughout the lamina of the individual leaflets.

iv) LPI_3 = +4.0. Cessation of exponential phase of leaf 3 growth (i.e. phase 1) in terms of leaf length, area, fresh/dry weight, and total chlorophyll level. Minimum level of total soluble carbohydrates is evident at this point. Leaf is anatomically and physiologically mature.

During the initiation process, the amount of internal leaf tissue derived from the outermost cells of the corpus region within the shoot apex is difficult to determine. However, it would appear that the majority of internal leaf tissues are derived from the T_2 layer through a series of periclinal and anticlinal divisions with minor contributions to the leaf primordium base by derivatives of the corpus. The role played by the different regions of the tomato shoot apex in the organogenic process is unknown. The results of Lange (1927) and Hussey (Hussey and Turner, 1969 ; Hussey, 1971) suggest that the initial organogenic event (i.e. formation of a "growth center" of increased mitotic activity) is relatively deep seated within the shoot apex and is probably within the outer periphery of the corpus. This is contrary to Schüepp's theory of leaf primordium formation (Schüepp, 1914, as cited in Foster, 1936) and also

contrary to Lyndon's recent evidence for leaf initiation in pea (Lyndon, 1971). However, Hussey (1972) has re-evaluated Lyndon's work and added his own analysis based on colchicine-blocked metaphase distribution and carbon particle marker movement on the surface of the pea and tomato apices. The data substantiates the concept of a "growth center" (i.e. region of increased mitotic activity) within the corpus for both species and is contrary to Lyndon's original conclusions.

The present histological evidence substantiates the allometric analysis of terminal leaflet/lateral leaflet growth i.e. the lateral leaflets are initiated during the first 1000 μ of terminal leaflet growth. It is also apparent from histological evidence that the leaflets are initiated in a basipetal sequence. This initiation of leaflets in a basipetal manner is contrary to the relatively few studies in other compound leaves such as Carya (Foster, 1935), Clematis (Tepfer, 1960), Acacia (Rickson, 1969) and Doxanthus (Sistrunk and Tucker, 1974) which develop their lateral appendages in acropetal sequence. As a consequence, direct ontogenetic comparison with these leaves is limited. Nevertheless, it is evident that the basic developmental sequences (e.g. procambial strand differentiation in the midrib and subsequent lamina development) observed in the compound leaves are probably quite similar.

Leaflet growth in width relative to length is consistently greater for the terminal leaflet than the lateral ones. The earlier development and terminal position are

probably important factors in allowing successful competition for nutrients from the rest of the plant and possibly no restriction on lateral growth due to limited spacing as found with lateral leaflets in the leaf primordium stage. An examination of palisade mesophyll cell areas (cross sectional) and associated blade areas in mature ($LPI_3 > 4.0$) leaflets (Table 3.4) suggests that the basipetal trend in smallness of the individual leaflets is due to both fewer cells at maturity and a smaller average cell size. The decreased cell numbers may be due to the progressively later initiation of the leaflets in a basipetal direction with the consequent production of fewer cell generations.

Certain differences were noted between this study of tomato leaf development and the one carried out by Bedesem (1958). Contrary to Bedesem, but in agreement with Lange (1927), no discrete apical meristem was observed in the emerging leaf primordium. Rather, the early growth in length of the leaf primordium appears to be due to scattered cell division and enlargement throughout the developing organ (i.e. due to a diffuse or non-localized intercalary meristem). While the presence of an apparently mitotically active epidermal and sub-epidermal group of cells is evident in the young primordia, it is questionable whether the distinction between an apical meristem and an intercalary meristem can be made on the basis of histological examination alone. Following their extensive labelling studies of DNA

synthesis during lamina development in Xanthium, Maksymowych and Wochok (1969) expressed similar doubts in any purely histological attempts at clearly delineating the marginal meristem from the plate meristem. It is apparent that the classical concepts of the various leaf meristems desperately need a reevaluation in terms of timing, duration and relative intensity of the mitotic cycle with emphasis placed upon the characterization of discrete cell cycle populations within the developing primordia.

The size of the tomato leaf primordium (cv. 'Farthest North') at anatomical stages comparable to Bedesem's leaf study of the cv. 'Marglobe' (Bedesem, 1958) is approximately one half. In view of the considerably larger final size attained by leaves of 'Marglobe' over the 'Farthest North' cultivar and associated studies in other dicot species (e.g. tobacco; Avery, 1933; clover; Lersten, 1965), it would appear that the increased duration of various early developmental stages in the young primordia may be correlated with increased final size in the mature form.

The development of an apparently optional fourth spongy mesophyll layer in the 'Marglobe' lamina was not observed in 'Farthest North'. Whether the formation of this additional spongy layer is a function of leaf position, varietal differences and/or environmental modification is unknown.

Bedesem's observation that "the ends of the veinlets on the mature blade margin have clusters of somewhat

disoriented spiral tracheids" (p. 452) is not supported. Rather, his failure to recognize a discrete marginal fimbriate vein composed predominately of large vessel elements with spirally thickened walls suggests that the "disoriented spiral tracheids" may indeed be the marginal vein. Sections of mature lamina tissue cut transversely through a small lobe or crenation would give the appearance of disoriented tracheary tissue along the blade margin. An examination of the mature leaf vasculature of six other cultivars (i.e. 'Alsia Craig', 'Bonny Best', 'Bush Beefsteak', 'Golden Queen', 'Rutgers' and 'Tiny Tim') has revealed similar xylem patterns, suggesting that this feature is probably ubiquitous to most, if not all, commercial tomato cultivars.

A locus of xylem initiation within the tomato stem was not observed by Bedesem. In the present study, it is apparent that the differentiation of xylem vessels at isolated leaf and stem locii occurs quite early in the development of leaf number three. While the initiation of xylem at the leaf locus appears to slightly precede the stem locus, examples were found of a reverse order of initiation. The latter initiation sequence is in agreement with Jacob's work in Coleus (Jacobs and Morrow, 1957). A second variation in the locus of xylem initiation is observed in the vascularization of leaf number one and involves the acropetal differentiation of xylem which is initially connected to an older trace. A similar developmental feature has been reported for Coleus and Anagallis by De Sloover (1958). These variable

occurrences in timing and location of xylem formation are probably due to locally altered cellular environments caused by minor fluctuations in the levels of various endogenous factors (both nutritional and hormonal).

While the minor venation of the tomato leaflets may show considerable localized variation in terms of number and arrangement of vascular tissue, a generalized scheme of minor vein ontogeny can be presented (Fig. 3.17).

My views on terminal veinlet formation coincide with Lersten's (1965) in the sense that "...the procambial strands are quite complex even at an early stage and probably very quickly attain a level of structural complexity and a degree of differentiation that can be assumed to prevent the formation of junctures with newly forming procambial strands" (p. 772).

The apparent control of planes of cell division in the upper spongy layer by the early stages of the developing procambial reticulum may have an important bearing on this problem (Plate 3,8;1,2). This observation is contrary to Schüepps' view (1926, as cited in Avery, 1933) that all plate meristem cells show a complete lack of arrangement. Certainly, the right-angled development of successive members of the minor vein procambial reticulum appears to reflect the prior orientation of the plate meristem cells in the upper spongy mesophyll layer which, in turn, was initially caused by earlier developing procambial strands (e.g. 2° and 3° veins). This cyclic 'cause and effect'

system would lead inevitably to: a) an orthogonal reticulate pattern of minor venation; and b) a threshold areole size above which further subdivision by newly initiated procambial strands would be expected.

From the physiological point of view, one could hypothesize vein ending formation caused by the alteration of "hormonal/nutritional" fields emanating from the earlier formed procambial stages of the surrounding reticulum and prior to the completion of the later forming procambial strand within the areole. However, Hara's (1962) suggestion that tensions within the leaf during its rapid expansion could be removed by differentiation of potential procambial cells into mesophyll cells is equally valid in view of the total lack of experimental evidence.

While no disjunct minor veins were observed in tomato as found in Euphorbia (Herbst, 1972) it is interesting to note that Bedesem (1958) was able to induce gaps in the developing minor veins as well as the formation of apparent disjunct minor veins by TIBA application to young tomato leaf primordia. Whether these disrupted minor veins were formed by breakage or by subsequent differentiation of procambial cell precursors into mesophyll cells (similar to Hara's extension cells) was not established. It is apparent, however, that an experimental approach to the problem of disjunct vein formation could be initiated through the use of an ontogenetic system precisely defined in terms of a

developmental index and coupled with highly localized TIBA applications on the developing lamina of tomato leaflets.

An important feature of the present observations on tomato minor vein ontogeny is the apparent "multiple choice" allowed the procambial strand for its further development from the 'extension cells' (see fig. 3.13 to 3.16). While it is conceivable that all mesophyll cell precursors have the potential for procambial cell development, the final selection must rest on position relative to earlier formed, adjacent procambial strands. The signal for either vein completion or non-completion (i.e. vein ending formation) probably comes from the procambial strand towards which the younger strand is developing. The critical procambial stage, after which no connection with a younger stage is possible, is unknown. However, a thorough examination of the sectioned and cleared material would appear to suggest that once stages 4 or 5 (Fig. 3.17) had been reached, no further connection with a developing procambial strand is possible.

In view of the precision with which the developmental status of the tomato leaf can be described both qualitatively and quantitatively (at the organ, tissue, physiological and biochemical levels), it is apparent that the LPI should prove to be an adequate index for studying the regenerative capacity of the tomato leaf.

CHAPTER 4

ROOT REGENERATION FROM LEAF CUTTINGS

4.1 Introduction

The pre-eminence of the leaf as a source of hormonal and nutritional substances necessary for root formation in cuttings has been recognized for almost 100 years (e.g. see the early review of Went and Thimann, 1937; and individual works such as Cooper, 1935; Went, 1938; Van Overbeek and Gregory, 1945; Van Overbeek, Gordon and Gregory, 1946; Adams, 1967).

Due to its sensitivity and simplicity, the tomato leaf cutting has been a favourite system for studying the effects of natural and synthetic auxins on induced adventitious root formation (Hitchcock and Zimmerman, 1937; 1938). However, no recent studies on the effects of the other major groups of plant growth substances appear to have been pursued in this regenerating system. With regard to the gibberellins, only one published report has appeared which deals with the effects of GA_3 on root regeneration in tomato. Using shoot cuttings, Jansen (1967) confirmed some of the earlier results of Brian et al. (1960) who worked with pea cuttings:

i). GA_3 inhibits root regeneration over a wide concentration range (i.e. from 10^{-3} mg/l to 1000 mg/l);

ii) GA_3 exerts its effect during root primordia induction with no apparent effects on root development; and

iii) GA_3 is unable to inhibit rooting completely.

Unlike Brian and his co-workers, however, Jansen did not rule out the possibility that the GA_3 effect was not localized at the site of primordia formation. Instead, he postulated that the GA_3 response would be indirect due to its effects in the shoot tip (e.g. growth stimulation) which would, in turn, give rise to such correlative events as inhibition of root formation in the base of the shoot. Interestingly, Jansen found that IAA was inhibitory to the rooting of tomato shoot cuttings. He suggested that the regenerating shoot cuttings were probably saturated with endogenous IAA, and hence, exhibited a toxic response to exogenous IAA applications. This appears to be a plausible explanation in view of the high levels of IAA found in tomato shoots (Schneider, Wightman and Gibson, 1972). However, Jansen's observations with tomato shoot cuttings did not significantly extend the original observations of Brian et al. (1960) nor did they explore possible links between altered metabolic patterns and GA_3 inhibition of root regeneration. As a tool for studying regeneration, the use of a complex and developmentally undefined shoot system with its attendant failure to respond to exogenous auxins, make an evaluation of GA_3-auxin interactions

impossible. It should be pointed out that modifications of the GA_3 inhibition of root regeneration by other growth substances or nutrient substrates have generally been considered in terms of concentration effect with little regard to the timing and sequence of application. Finally, it should be noted that Jansen's use of root dry weight as a measure of root formation would not allow a complete separation of root initiation from root growth - two developmental events which show considerable divergence in their responses to growth substances.

As indicated in Chapter 2, the hypotheses for GA_3 inhibition of organ regeneration centre on inhibition of primordium initiation, and/or subsequent development. A metabolic link between an altered starch metabolism and GA_3 induced regeneration inhibition has been developed by Thorpe and his co-workers over the past seven years for *in vitro* bud formation from tobacco callus. However, no such links have been established for root regeneration except in a very cursory and qualitative fashion by Münzel (1968) who noted that starch accumulations were less in GA_3 treated petiole segments of detached *Begonia* leaves than in controls. No consideration was given to possible GA_3 induced correlative effects which might have accounted for this observation.

As a consequence, the present study involves four interrelated areas:

i) to relate the developmental state of a tomato leaf (as

defined by LPI to that leaf's ability to regenerate roots;

ii) to evaluate the theory that root formation and growth rate of the regenerating organ are related;

iii) to physiologically define the inhibitory aspects of gibberellins on root regeneration with emphasis placed upon an altered carbohydrate metabolism in the region of the induced primordia; and

iv) to evaluate other plant growth regulators in terms of their ability to modify the gibberellin response.

4.2 Materials and Methods

4.2.1 Preparation of Leaf Cuttings

Cuttings of true leaf number 3 were obtained from tomato plants (cv. 'Farthest North') at the LPI_3 designated in the individual experiments. LPI_3 values were calculated (as outlined in Chapter 3) on the basis of ten to twenty LPI_3 determinations from randomly selected plants. In the plant growth regulator studies, each petiole base was immersed in 5 ml of test solution for up to 48 hours in continuous darkness at 25°C unless otherwise noted. Approximately 20 to 24 leaf cuttings were used for each treatment. The cuttings were subsequently rinsed in tap water and placed in plastic trays (16" x 10" x 4") containing a mixture of standard greenhouse potting soil and vermiculite (1:1 v/v). Approximately 4 litres of rooting mixture were prepared for each plastic tray and 1 litre of

tap water was used to moisten the mixture. The plastic trays were wrapped in clear plastic bags and placed in a growth cabinet at a constant 20°C with 16 hour day length.

Lighting was provided at 800 f.c. by a bank of 4 fluorescent tubes (Sylvania Cool White). Fourteen days after the cuttings were prepared, they were removed from the rooting medium, cleared in 70% ethanol and the number of roots (internal and external) counted.

4.2.2 Histochemistry

4.2.2.1 Starch

Localization of starch grains in fresh sections of petiole material was carried out with I-KI staining (Jensen, 1962). Starch distribution within the tomato leaflets was observed after boiling the leaflets in 95% ethanol for 20 minutes, washing them in hot water, and flooding the bleached tissues with I-KI solution for a few minutes. The iodine solution was then rinsed off and the starch containing areas observed as dark purple colourations (Machlis and Torrey, 1956).

4.2.2.2 Amylase Activity

The enzyme was localized at the cellular level using the substrate film method of Molnar and La Croix (1972b) with slight modification. Instead of filtering the 3% suspension of soluble starch after heating, the suspension

was centrifuged at 6,500 x g for 15 minutes at room temperature prior to substrate film preparation: Control tissue was heat killed at 100°C for 5 minutes.

4.2.2.3. Phosphorylase Activity

This enzyme was localized at the cellular level using the starch synthesis method of Dyar (1950, as cited in Jensen, 1962), with glucose-1-phosphate as the substrate. Controls included i) heat inactivation of the tissue and ii) incubation without glucose-1-phosphate substrate.

4.2.2.4 General Histology

An aqueous solution of .05% Toluidine blue was used to obtain fresh sections in order to evaluate the morphological changes (O'Brien, Feder and McCully, 1964).

4.2.3 Carbohydrate Analysis

Starch and total soluble carbohydrate analysis were carried out as outlined in sections 3.2.1.4 and 3.2.1.6.

4.2.4 Starch Synthesis in Isolated Petiole Segments

The bases of the leaf cuttings were pretreated with distilled water or GA_3 at 1×10^{-4} M for a 48 hour period in continuous darkness. The basal 20 mm of the petioles were subsequently severed and weighed out in groups of 250 mg (fresh weight). They were cut into .5 and 1.0 mm segments and incubated in 0.1 M sodium acetate buffer (pH 6.1) in

the presence or absence of glucose-1-phosphate at a final concentration of 30 mM. Preliminary experiments indicated that lower concentrations of G-1-P were not effective in supporting starch synthesis while higher levels (i.e. 40 and 50 mM) did not substantially increase endogenous starch levels above those induced by 30 mM G-1-P. Incubation conditions included incandescent lighting (1000 f.c. light intensity) for 2 or 3 hours with the temperature maintained at 30°C by means of a water bath. Three samples were used for each treatment. At the end of the incubation period, the petiole segments were extracted with hot 80% ethanol four times and the dried pellet subsequently analysed for starch content according to the method of Dubois et al. (1956) as outlined in section 3.2.1.5. The results are expressed in ug starch per 250 milligrams fresh weight tissue.

4.2.5. Total Amylase Activity

Petiole tissue from GA_3 treated and untreated material were homogenized in the presence of a small quantity of purified sand for 5 minutes at 0-2°C in 0.05 M sodium citrate buffer (pH 5.8) with 0.2% $CaCl_2$ (3 ml buffer solution/gm fresh weight tissue) in a cold mortar and pestle (Baun, Palmiaro, Perez and Juliano, 1970). The homogenate was stirred for 30 minutes in the dark and subsequently centrifuged at 30,000 x g at 2-4°C for 30 minutes. Acid washed Polyclar AT (5% final concentration) was added to the resulting supernatant and stirred for 15 minutes in order to remove

phenolic compounds which might cause enzyme inactivation (Loomis and Battaile, 1966; Anderson, 1968). The suspension was centrifuged at 20,000 x g at 4°C for 15 minutes in a Sorval swinging bucket HB-4 rotor. The supernatant was subsequently assayed for total amylase activity using either the gel diffusion method of Briggs (1962) or the spectrophotometric procedure of Schuster and Gifford (1962). The gel diffusion method was modified slightly. Instead of impregnating 9 mm filter paper discs with the plant extract, 8 mm circles were lightly marked on the surface of the agar/starch plates using a stainless steel cork-borer. The enzyme preparations were subsequently placed in .02, .04 ml or .06 ml amounts within these circles and incubation of the plates carried out at 40°C for 3 days. The plates were stained with I-KI solution for 10 minutes and the average diameters of the clear areas noted. A preliminary experiment with a commercial amylase (Sigma Co., St. Louis, Mo.) indicated a linear relationship between the diameter of the clear zones and the log. concentration of amylase in accordance with the results of Briggs (1962). The spectrophotometric assay of Schuster and Gifford (1962) was modified slightly by the use of 1.5 ml iodine solution and 4.5 instead of 5.0 ml water due to the presence of iodine-reducing substances in the extract (Juliano and Varner, 1969). Duplicate samples were evaluated from each treatment. The assay gave reproducible values which were proportional to enzyme concentration. Specific activity is defined as the change in absorbance at 620 nm

per milligram of protein in 15 minutes at 37°C. Total protein determinations for the enzyme analyses were carried out using the filter paper disc-xylene Brilliant Cyanin G method of Bramhall, Noack, Wu and Loewenberg (1969). Bovine serum albumin (Type IV, Sigma) was used as a reference standard after an appropriate correction for water content (Rutter, 1967).

4.2.6 Determination of Membrane Permeability Changes

Using the method of Lee and Wilkinson (1973), four hundred milligrams (fresh weight) of tomato petiole tissue were cut into 2 mm segments and washed thoroughly in distilled water for 5 minutes. The sections were placed in a 25 ml Erlenmeyer flask containing 3 ml of citrate buffer (5 mM, pH 5.2) and GA_3 at various concentrations. Two sample flasks were prepared for each treatment. The flasks were incubated at room temperature (ca. 25°C) for 1 hour on a rotary shaker (approx. 60 revolutions per minute). The tissue was then removed, thoroughly rinsed in distilled water, blotted on filter paper, and transferred to 5 ml of distilled deionized water for a further 30 minute shaking. The tissue was removed and the amount of intracellular materials released into the water was estimated by measuring the absorbance at 240, 260, and 280 nm. The difference in leakage of cell contents between the treated and control tissues was used as a measure of change in membrane permeability.

4.2.7 Photography

Black and white photography of fresh leaf sections was carried out as described in section 3.2.3.

4.2.8 Statistical Analysis

Standard error, regression and correlation analyses were performed according to Woolf (1968). The count data was transformed by the square root transformation and subsequently analysed by the Student-Newman-Kreul Multiple Range Test (Sokal and Rolf, 1968; Woolf, 1968).

4.3 Results

4.3.1 Leaf Position

An examination of the rooting potential of the different tomato leaves along the shoot axis of differently aged groups of plants revealed a progressive increase in regenerative capacity followed by a decline (Fig. 4.1). This data suggested that the capacity for root regeneration varied (a) with the position of the leaf and (b) with the age of the shoot from which the leaf was taken.

4.3.2 LPI

When the regeneration responses of the various leaves were plotted in terms of the developmental index, LPI, a consistent trend was observed (Fig. 4.2). A similar response curve was obtained for leaf number 3 (Fig. 4.3). In all of

Figure 4.1. Effect of shoot age and leaf position on root regeneration capacity from tomato leaf cuttings. Black bars, leaves from 4 week old tomato shoots. Hatched bars, leaves from 6 week old tomato shoots.

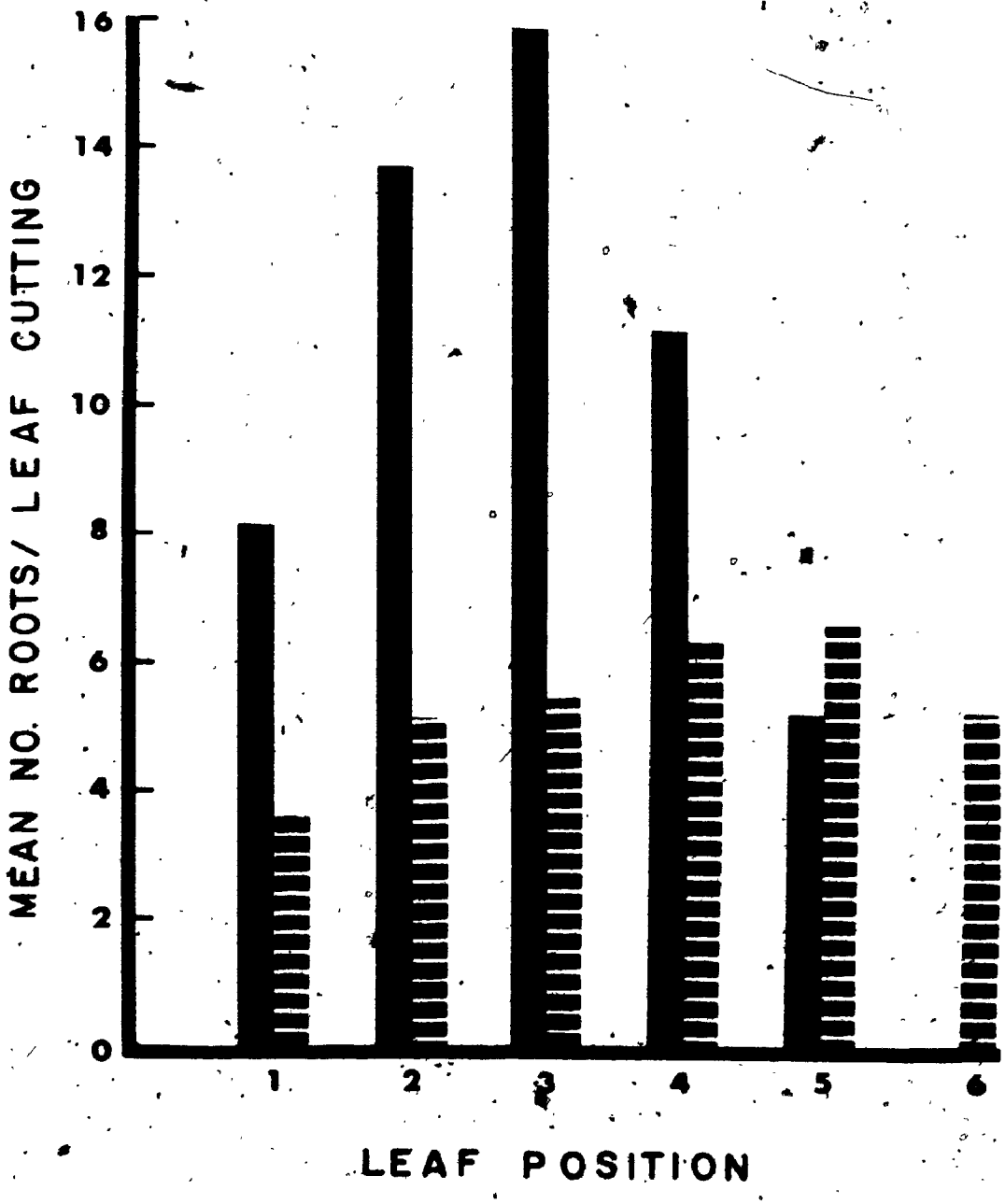

MEAN NO. ROOTS/ LEAF CUTTING
0
2
4
6
8
10
12
14
16
1
2
3
4
5
6
LEAF POSITION

Figure 4.2. Root regeneration of different tomato leaves from 4 week old plants as a function of their developmental age. The dashed line is drawn to emphasize the general trend only. Leaf 1, circle; leaf 2, triangle; leaf 3, inverted triangle; leaf 4, open square; leaf 5, closed square.

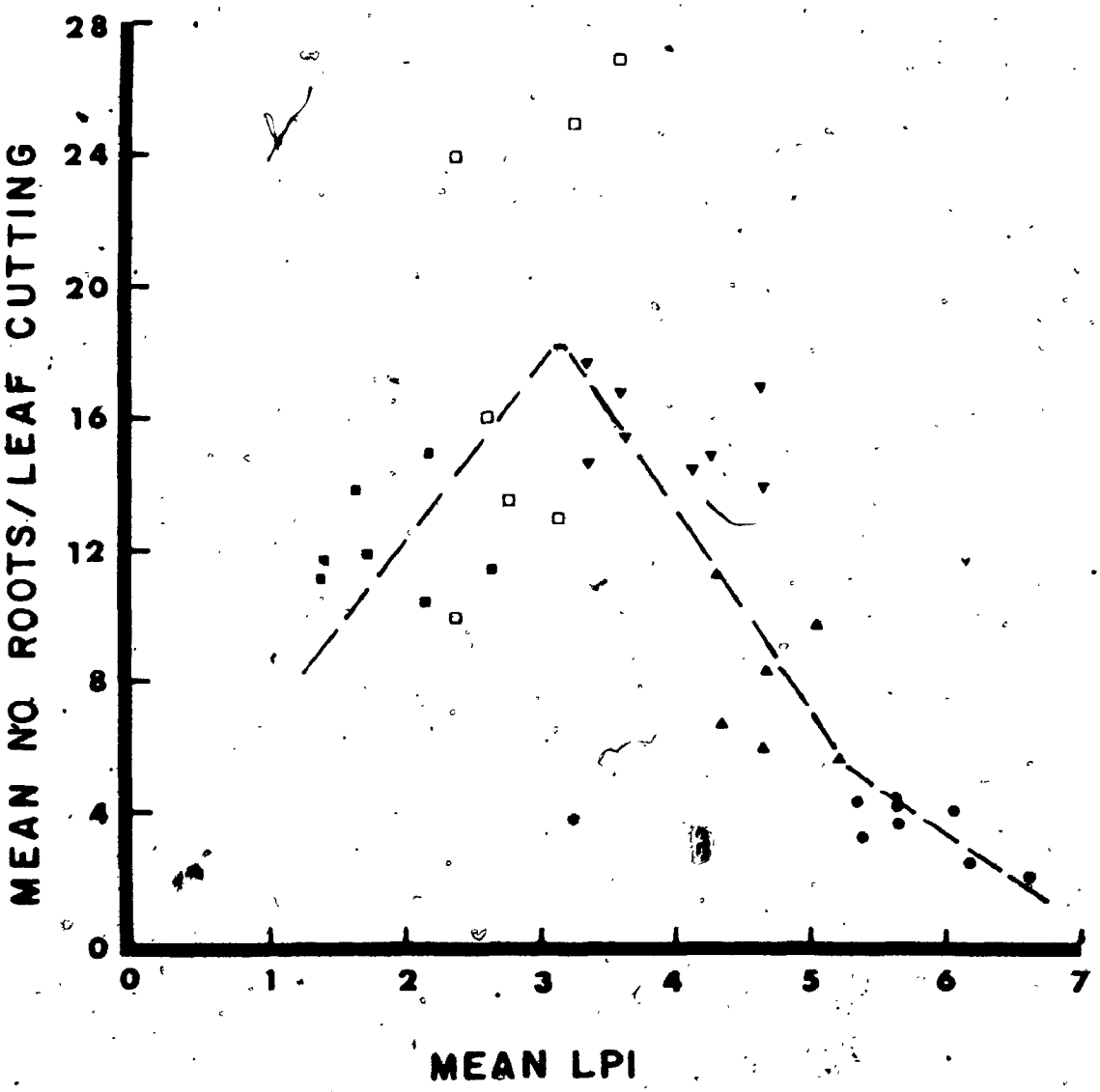
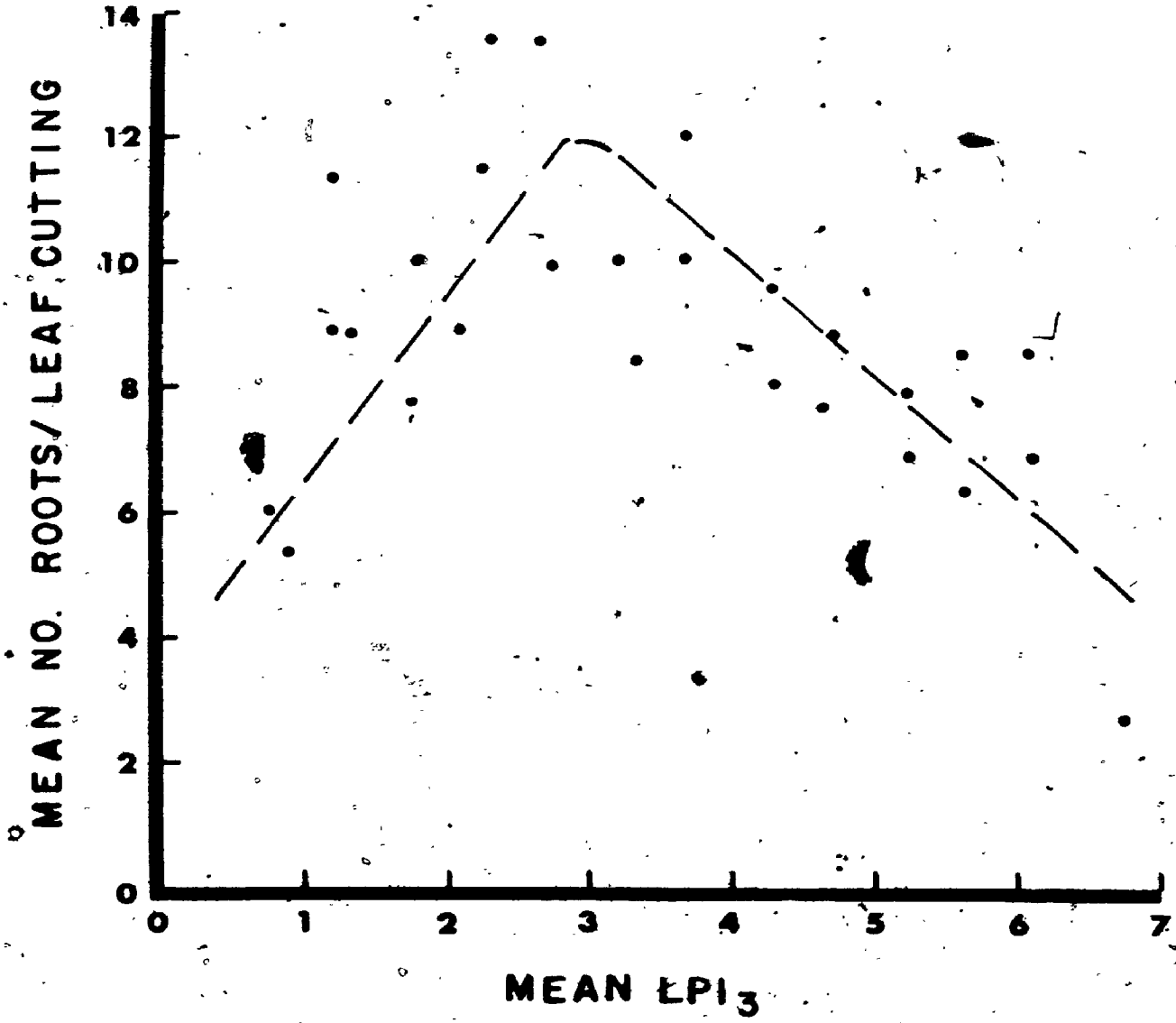

MEAN NO ROOTS/LEAF CUTTING
28
24
20
16
12
8
4
0
0
1
2
3
4
5
6
7
MEAN LPI

Figure 4.3. Root regeneration of tomato leaf no. 3 as a function of its developmental age. The dashed line is drawn to emphasize the general trend only.


MEAN NO. ROOTS/LEAF CUTTING
0
2
4
6
8
10
12
14
0
1
2
3
4
5
6
7
MEAN LPI3

the above experiments, the cuttings were rooted directly in the vermiculite/potting soil mixture for the 14 day period.

4.3.3 Auxins

4.3.3.1 Different Auxins: A Comparison

When different auxins were evaluated for their ability to stimulate regeneration in mature tomato leaves, a rooting response (in declining order of effectiveness) was observed as follows: IBA > NAA > IAA > distilled water (Table 4.1). 2,4-D proved toxic at the concentration tested (i.e. 1×10^{-4}). No significant stimulation of rooting was observed with this growth regulator over the concentration range of 10^{-9} M to 10^{-5} M (Table 4.2).

4.3.3.2 IAA

In terms of root regeneration, tomato leaf cuttings were sensitive to IAA concentrations above 1×10^{-5} M (Table 4.3). Plotting the LPI_3 against rooting for IAA treated and untreated leaf cuttings revealed typical response curves (Fig. 4.4). However, optimal regeneration capacity was shifted from approximately LPI_3 = 2.5 - 3.0 to LPI_3 = 3.0 - 4.0 in the presence of high exogenous IAA levels. Petiole and rachis plus terminal leaflet growth were significantly correlated with regeneration response. IAA did not alter this situation (Table 4.4).

Table 4.1. Effects of different auxins on root regeneration from mature leaves.

Treatment*	Mean number roots per cutting	
Distilled water control	6.8	a
IAA	14.8	b
NAA	22.7	c
IBA	28.9	d
2,4-D	--	-

*Auxins supplied to petioles at concentrations of 1×10^{-4} M. 2,4-D treated petioles were necrotic.

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 4.8

Table 4.2. 2,4-D concentration effects on root regeneration.

Treatment	Mean number roots per cutting	
Distilled water control	11.3	a
2,4-D (1×10^{-9} M)	11.3	a
" (1×10^{-8} M)	9.7	a
" (1×10^{-7} M)	11.9	a
" (1×10^{-6} M)	12.0	a
" (1×10^{-5} M)	12.2	a

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.1

Table 4.3. IAA concentration effects on root regeneration.

Treatment	Mean number roots per cutting	
Distilled water control	9.5	a,b
IAA (1×10^{-8} M)	10.4	a
" (1×10^{-7} M)	6.6	b
" (1×10^{-6} M)	6.4	b
" (1×10^{-5} M)	6.9	b
" (1×10^{-4} M)	35.8	c

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 4.4

Figure 4.4. Effect of IAA on root regeneration of tomato leaf no. 3 as a function of its developmental age. The dashed lines are drawn to emphasize general trends only. Distilled water control, closed circles; 5×10^{-5} M IAA, open circles.

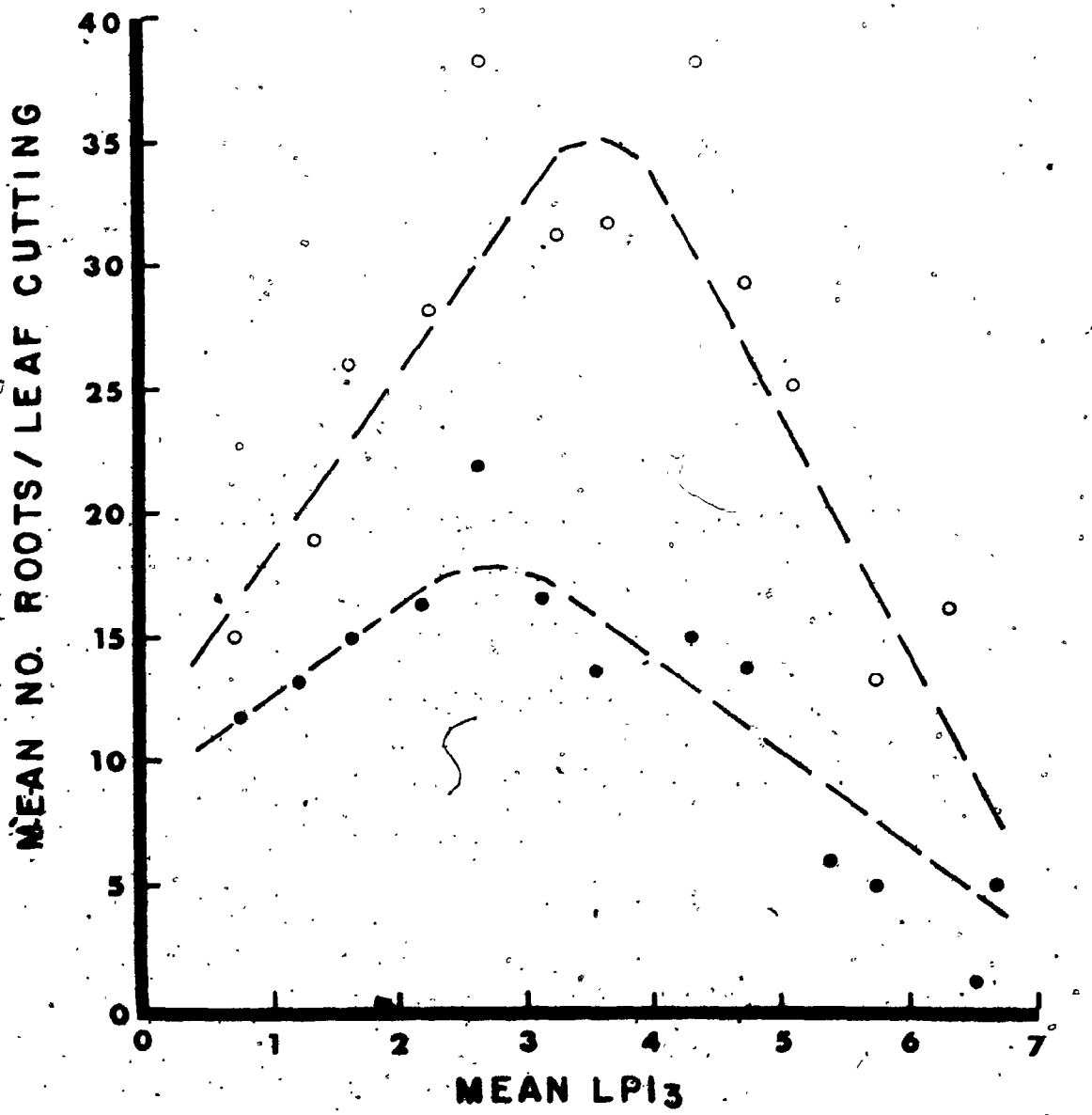

MEAN NO. ROOTS / LEAF CUTTING
40
35
30
25
20
15
10
5
0
0
1
2
3
4
5
6
7
MEAN LPI3

Table 4.4. Effects of IAA on the growth/regeneration correlation coefficient.

Comparison	Treatment	Correlation coefficient(r)
Petiole growth vs Rooting response	distilled water	.34**
	IAA (1×10^{-4} M)	.42***
Rachis/terminal leaflet growth vs Rooting response	distilled water	.52***
	IAA (1×10^{-4} M)	.38**

** significant at 1% level

*** significant at the .1% level

4.3.3.3 IBA

Regeneration was significantly stimulated by IBA concentration above 1 x 10^{-6} M (Table 4.5). Similar rooting-LPI_3 response curves were obtained with IBA as were found with IAA (Fig. 4.5).

4.3.4 Abscisic Acid

ABA had no significant effect on root regeneration over the concentration range of 10^{-8} M to 10^{-4} M (Table 4.6). Slight variations were observed between regenerating leaves treated with ABA (1 x 10^{-4} M) over the LPI_3 range of -0.5 to +7.5 and distilled water controls (Fig. 4.6). In the presence of IAA or IBA, ABA had no significant effect on regeneration (Table 4.7).

4.3.5 Kinetin

Petiolar application of kinetin solutions for a 48 hour period caused a progressive and significant decrease in regeneration over the concentration range of approximately 10^{-6} M to 10^{-4} M. Lower concentrations of kinetin (i.e. 10^{-8} to 10^{-7} M) had no significant effect (Table 4.8). IAA or IBA were unable to completely reverse the kinetin inhibition (Table 4.9).

Table 4.5. IBA concentration effects on root regeneration.

Treatment	Mean number roots per cutting	
Distilled water control	16.0	a
IBA, 1×10^{-8} M	19.0	a,b
IBA, 1×10^{-7} M	21.2	a,b
IBA, 1×10^{-6} M	21.5	b
IBA, 1×10^{-5} M	44.8	c
IBA, 1×10^{-4} M	82.7	c

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.7

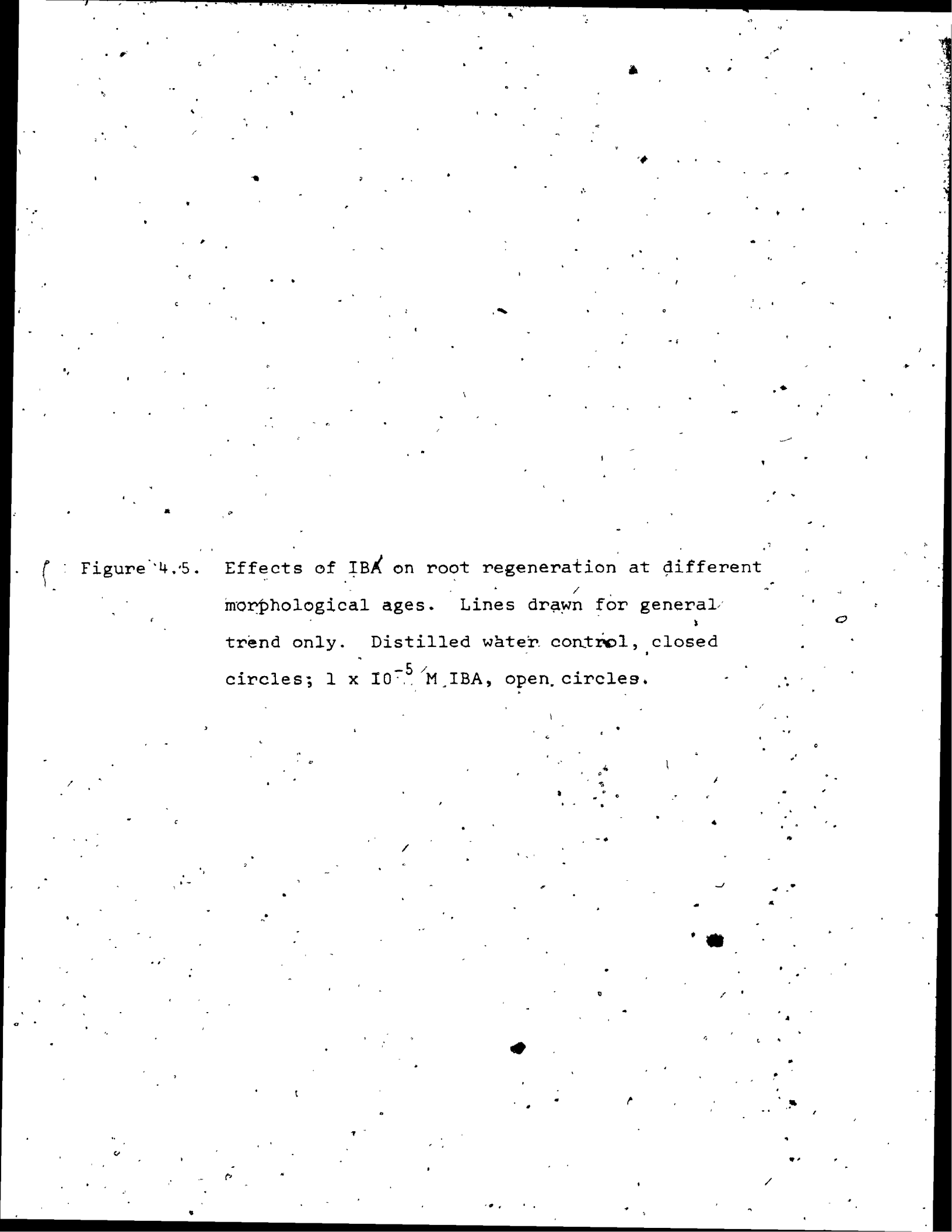


Figure 4.5. Effects of IBA on root regeneration at different morphological ages. Lines drawn for general trend only. Distilled water control, closed circles; 1 x 10^{-5} M IBA, open circles.

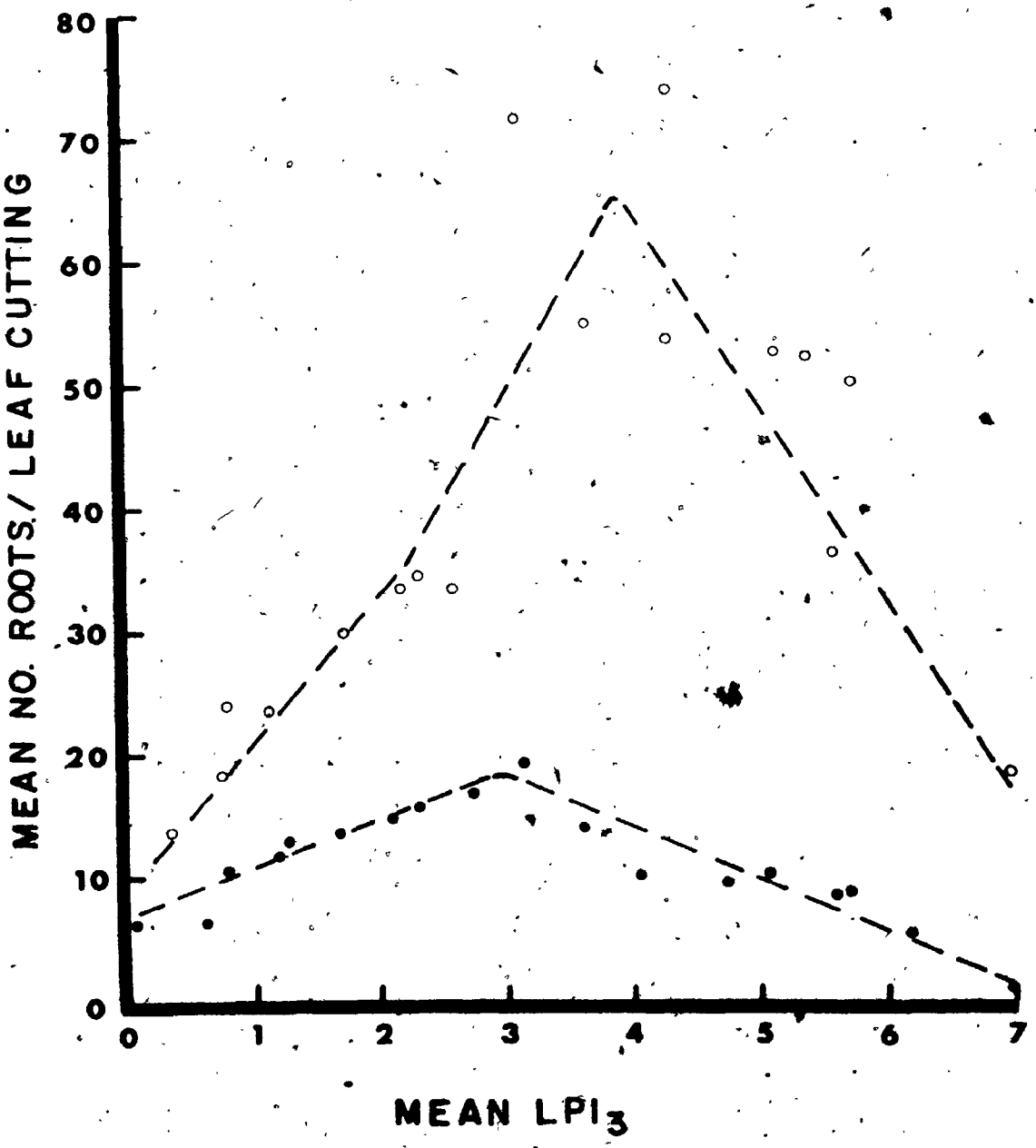

MEAN NO. ROOTS/ LEAF CUTTING
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
MEAN LPI3

Table 4.6. ABA concentration effects on root regeneration.

Treatment	Mean number roots per cutting	
Distilled water control	12.7	a
ABA (1×10^{-8} M)	12.3	a
" (1×10^{-6} M)	11.5	a
" (1×10^{-5} M)	12.5	a
" (1×10^{-4} M)	10.6	a

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.4

Figure 4.6. Effects of ABA on root regeneration at different morphological ages. Lines drawn for general trends only. Distilled water control, closed circles; 1 x 10^{-4} M ABA, open circles.

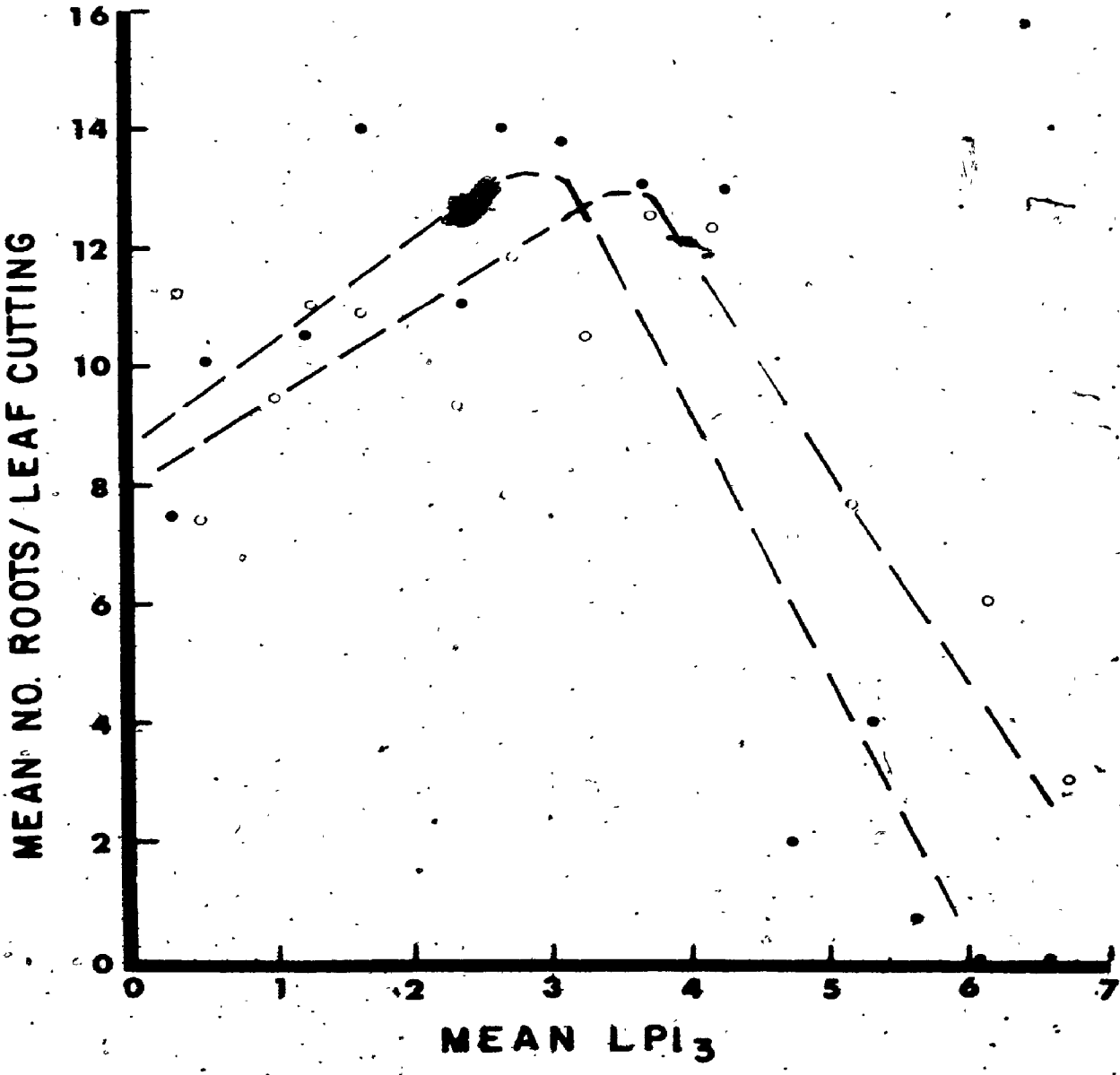

MEAN NO. ROOTS/LEAF CUTTING
0
2
4
6
8
10
12
14
16
0
1
2
3
4
5
6
7
MEAN LPI3

Table 4.7. ABA effects on auxin induced rooting.

Treatment	Mean number roots per cutting	
Distilled water control	16.3	a
IAA (5×10^{-5} M)	23.3	b
IBA (5×10^{-6} M)	23.0	b
ABA (5×10^{-6} M)	17.3	a
ABA + IAA	18.4	a,b
ABA + IBA	25.7	b

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.1

Table 4.8. Kinetin concentration effects on root regeneration.

Treatment	Mean number roots per cutting	
Distilled water control	12.5	a,b
Kn (1×10^{-8} M)	14.0	a
Kn (1×10^{-7} M)	10.7	b
Kn (1×10^{-6} M)	8.1	c
Kn (1×10^{-5} M)	2.0	d
Kn (1×10^{-4} M)	0.0	e

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.0

Table 4.9. Kinetin inhibition of auxin-induced rooting.

Treatment	Mean number roots per cutting	
Distilled water control	11.2	a
Kn (1×10^{-5} M)	3.9	b
IAA (5×10^{-5} M)	21.8	c
IBA (5×10^{-5} M)	36.8	d
Kn + IAA	7.8	b
Kn + IBA	11.9	a

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.9

4.3.6 Gibberellins

4.3.6.1 Inhibition of Regeneration and its Reversal

GA_3 inhibited root regeneration over the concentration range of 10^{-8} M to 10^{-4} M (Table 4.10). However, complete inhibition of root regeneration was not possible regardless of the LPI_3 (Fig. 4.7). While blade growth rate was unaffected by GA_3 treatment, petiole growth was significantly altered (Table 4.11). As indicated by the regression coefficients, significantly more petiole growth occurred in the GA_3 treated tissues for each new root formed than in the control material. Very little petiole growth took place prior to and during root initiation and GA_3 treatment did not significantly change this situation (Table 4.12).

Treatment with GA_3 invariably (a) delayed the formation of the root primordia and (b) reduced the total number of roots formed after 14 days (Table 4.13). However, the tendency for root formation to occur at the base of the cuttings (i.e. within the basal 10 mm) was not altered. Treatments of GA_3 (at 1×10^{-4} M concentration) as short as 5 minutes duration were effective in significantly inhibiting regeneration (Table 4.14). Thirty minute pulses of GA_3 at different time periods during the regeneration experiment revealed that the greatest sensitivity to GA_3 was during the initial 96 hour period immediately following leaf excision, i.e. prior to and during the primordia formation phase (Table 4.15).

Table 4.10. GA_3 concentration effects on root regeneration.

Treatment	Mean number roots per cutting	
Distilled water control	6.6	a
GA_3 (1×10^{-8} M)	0.6	b
GA_3 (1×10^{-6} M)	1.2	b
GA_3 (1×10^{-5} M)	1.4	b
GA_3 (1×10^{-4} M)	1.0	b

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 4.3

Figure 4.7. Effects of GA_3 on root regeneration at different morphological ages. Lines drawn for general trends only. Distilled water control, closed squares; 1×10^{-4} M GA_3, open squares.

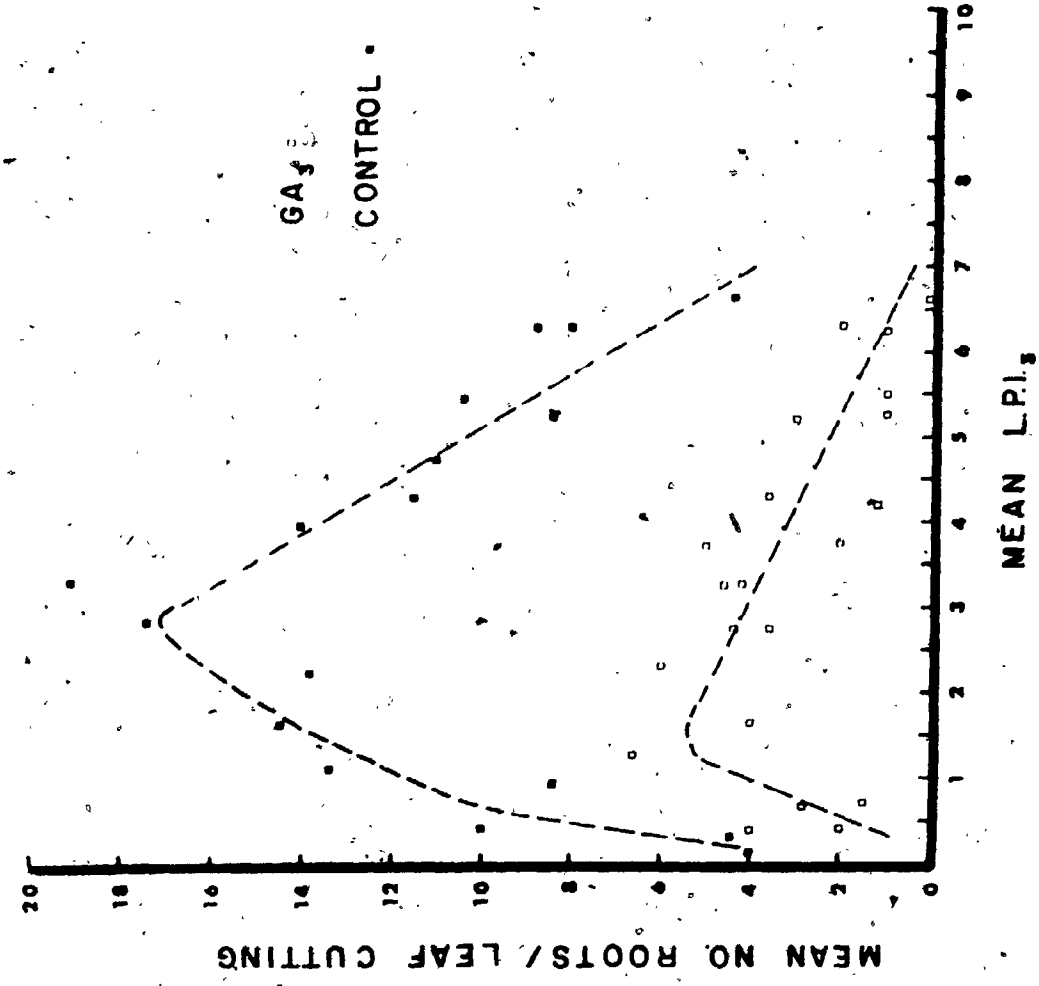

GA3
CONTROL
MEAN L.P.I.3
MEAN NO. ROOTS/LEAF CUTTING
0
2
4
6
8
10
12
14
16
18
20

Table 4.11. Correlation and regression analysis of GA_3 effects on rooting response/organ growth.

Variable	Treatment	Correlation coefficient	Regression formula	Regression coefficient	Y-intercept
petiole growth	H_2O	.49***	Y = -.10 + 1.648X	1.648 ± .245[a]	-.10 ± 1.24[a]
	GA_3	.33*	Y = .563 + 2.280X	2.280 ± .378[a]	.563 ± 1.024[a]
rachis/terminal leaflet growth	H_2O	.42***	Y = -.07 + 4.401X	4.401 ± .692[a]	-.07 ± 3.531[a]
	GA_3	.64***	Y = .174 + 4.570X	4.570 ± .67[a]	.174 ± 1.85[a]

* Significant at 5% level

***Significant at .1% level

[a] ±95% confidence intervals.

Table 4.12. Effect of GA_3 on petiole length prior to and during root regeneration.

Time (days)	Mean petiole length (mm) ± 95% confidence intervals	
	$-GA_3$	$+GA_3$
0	25.0	--
2	27.5	28.4
4	27.2	28.7
6	27.7 ± .7	28.8 ± .7

Mean LPI_3 = 3.6

Table 4.13. Time course of GA_3 (1×10^{-4} M) inhibition of rooting in tomato leaf cuttings.

Day	Mean number of roots per cutting	
	$-GA_3$	$+GA_3$
4	0.0	0.0
6	.4	0.0
8	3.5	.1
10	8.3	.7
12	12.2	1.8
14	16.5	4.4

Mean LPI_3 = 3.6

Table 4.14. Duration of GA_3 treatment on root regeneration.

Treatment duration	Mean number of roots per cutting	
Distilled water control	15.4	a
5 minutes, GA_3, 1 x 10^{-4}M	3.1	b
30 minutes	2.3	b
1 hour	3.0	b
24 hours	2.3	b
48 hours	1.7	b

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 2.9

Table 4.15. Timing of GA_3 (1×10^{-4} M) treatment (30 min. pulse) on subsequent rooting in tomato leaf cuttings.

Time of 30 minute GA_3 pulse	Mean number of roots per cutting	
Control	11.7	a
0 hr.	1.8	c
1 hr.	1.7	c
24 hr.	2.1	c
48 hr.	4.3	b
72 hr.	5.8	b
96 hr.	5.9	b
144 hr.	10.0	a
192 hr.	12.3	a

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.5

Attempts were made to alter the endogenous gibberellin status during root regeneration through the use of inhibitors of the gibberellin biosynthetic pathway. Phosphon D progressively inhibited regeneration over the concentration range of 10^{-8} M to 10^{-5} M (Table 4.16). This inhibition was not auxin reversible (Table 4.17). Both CCC and AMO-1618 at high concentrations caused slight stimulation of root regeneration (Tables 4.18 and 4.19). This effect was not altered by the age of the leaf material.

Both IAA and IBA were capable of reversing GA_3 inhibition of root formation regardless of whether the auxin application was prior to, during or after GA_3 treatment (Table 4.20, 4.21 and 4.22).

ABA was also capable of reversing GA_3 inhibition provided that ABA was applied at a high concentration prior to or during the GA_3 treatment (Tables 4.23 and 4.24). No reversal was possible when ABA treatment followed GA_3 application. GA_7 inhibition of regeneration was also reversed by simultaneous ABA treatment (Table 4.25).

Kinetin at 1×10^{-8} M did not reverse GA_3 inhibition regardless of time of application (Table 4.26) and did not significantly affect the ABA reversal (Table 4.27).

GA_3 inhibition was not significantly affected by exposing the leaf cuttings to continuous low intensity fluorescent lighting (250 f.c.) during the 48 hour treatment period. However, leaf cuttings failed to regenerate if left in total darkness for the 14 day period. If high

Table 4.16. Phosphon D concentration effects on root regeneration in tomato leaf cuttings.

Treatment	Mean number roots per cutting	
Distilled water control	20.4	a
Phosphon D (1×10^{-6} M)	14.0	b
Phosphon D (5×10^{-6} M)	3.1	c
Phosphon D (1×10^{-5} M)	0.9	d

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.3

Table 4.17. Non-reversibility of phosphon D inhibition by auxins.

Treatment	Mean number roots per cutting	
Distilled water control	13.4	c
Phosphon (5×10^{-6} M)	2.9	a
IAA (5×10^{-5} M)	19.4	d
IBA (1×10^{-5} M)	25.3	e
Phosphon + IAA	3.2	a
Phosphon + IBA	4.1	b

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.3

Table 4.18. CCC concentration effects on root regeneration in tomato leaf cuttings.

Treatment	Mean number roots per cutting	
Distilled water control	18.3	a
CCC (5×10^{-5} M)	17.6	a
" (1×10^{-4} M)	20.2	a,b
" (5×10^{-4} M)	25.7	c
" (1×10^{-3} M)	23.5	b,c

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.3

Table 4.19. AMO-1618 concentration effects on root regeneration in tomato leaf cuttings.

Treatment	Mean number roots per cutting	
Distilled water control	13.3	b
AMO (5×10^{-6} M)	15.3	b,c
" (1×10^{-5} M)	17.0	b,c
" (5×10^{-5} M)	16.5	b,c
" (1×10^{-4} M)	17.6	c
" (5×10^{-4} M)	9.3	a

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 2.9

Table 4.20. Reversal of GA_3 inhibition of root regeneration in tomato leaf cuttings by simultaneous auxin treatment.

Treatment	Mean number roots per cutting	
Distilled water control	6.0	a
GA_3 (1×10^{-5} M)	0.2	b
IAA (1×10^{-4} M)	27.0	e
IBA (1×10^{-5} M)	22.5	d,e
GA_3 + IAA	16.7	c
GA_3 + IBA	19.1	c,d

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.9

Table 4.21. Timing of IAA treatment on GA_3 inhibition of root regeneration in tomato leaf cuttings.

Treatment		Mean number roots per cutting	
0 - 24 hr.	24 - 48 hr.		
H_2O	H_2O	10.9	c
GA_3 (1×10^{-6} M)	H_2O	2.3	a
IAA (5×10^{-5} M)	H_2O	22.1	d
H_2O	IAA	14.5	c
GA_3	IAA	7.9	b
IAA	GA_3	13.9	c

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.5

Table 4.22. Timing of IBA treatment on GA_3 inhibition of root regeneration in tomato leaf cuttings.

Treatment		Mean number roots per cutting	
0 - 24 hr.	24 - 48 hr.		
H_2O	H_2O	13.2	a
GA_3 (1×10^{-6} M)	H_2O	4.5	b
IBA (5×10^{-5} M)	H_2O	23.0	c
H_2O	IBA	24.3	d
GA_3	IBA	17.3	a
IBA	GA_3	17.2	a

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 2.7

Table 4.23. Reversal of GA_3 inhibition by simultaneous ABA treatment.

Treatment	Mean number roots per cutting	
H_2O	12.8	a
GA_3 (1×10^{-6} M)	1.5	b
ABA (1×10^{-8} M)	10.8	a
ABA (1×10^{-4} M)	11.3	a
GA_3 + ABA (1×10^{-8} M)	4.3	c
GA_3 + ABA (1×10^{-4} M)	12.6	a

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.1

Table 4.24. Timing of ABA treatment on GA_3 inhibition of root regeneration in tomato leaf cuttings.

Treatment		Mean number of roots per cutting	
0 - 24 hr.	24 - 48 hr.		
H_2O	H_2O	14.0	a
GA_3 (1×10^{-6} M)	H_2O	5.2	c
ABA (1×10^{-4} M)	H_2O	12.3	a,b
H_2O	ABA	15.5	a
GA_3	ABA	3.6	c
ABA	GA_3	9.4	b

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.5

Table 4.25. Reversal of GA_7 inhibition of root regeneration in tomato leaf cuttings by simultaneous ABA treatment.

Treatment	Mean number roots per cutting	
H_2O	9.1	a
GA_3 (1×10^{-6} M)	0.5	b
GA_7 (1×10^{-6} M)	1.5	b
GA_7 + ABA (1×10^{-4} M)	8.6	a
ABA (1×10^{-4} M)	9.3	a

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.8

Table 4.26. Timing of kinetin treatment on GA_3 inhibition of root regeneration in tomato leaf cuttings.

Treatment		Mean number roots per cutting	
0 - 24 hr.	24 - 48 hr.		
H_2O	H_2O	12.8	a
GA_3 (1×10^{-6} M)	H_2O	6.0	b
Kn (1×10^{-8} M)	H_2O	14.6	a
H_2O	Kn	15.0	a
GA_3	Kn	4.2	c
Kn	GA_3	8.7	d

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 2.7

Table 4.27. Effect of kinetin/ABA treatment on GA_3 inhibition of root regeneration.

Treatment		Mean number roots per cutting	
0 - 24 hr.	24 - 48 hr.		
H_2O	H_2O	13.4	a,b
H_2O	GA_3 (1×10^{-6} M)	6.3	c
ABA (1×10^{-4} M)	GA_3	11.5	b
Kn (1×10^{-8} M)	GA_3	7.6	c
ABA + Kn	GA_3	11.0	b
ABA + Kn	H_2O	15.8	a

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.9

concentrations (i.e. 4% to 8%) of sucrose were applied to the petioles for the initial 48 hours, root regeneration took place at low but consistent levels. This stimulation of rooting in the dark by exogenous sucrose application was inhibited by simultaneous GA_3 application (Table 4.28).

4.3.6.2 Biochemical and Histochemical Changes Associated with GA_3 Inhibition

Having established a definite inhibition of root regeneration by GA_3, I decided to explore the possibility that this inhibition might be related to an altered carbohydrate metabolism (see section 2.4 for the reasons behind this decision). An examination of total soluble sugar content within the GA_3 treated and untreated leaf cuttings during regeneration revealed no significant changes (Fig. 4.8A). However, starch levels failed to increase appreciably in the GA_3 treated material when the cuttings were placed in a 16 hour photoperiod of high light intensity (Fig. 4.8B). A closer examination of the starch levels revealed that the inability to accumulate starch was observed initially in the petiole during the 48-96 hour period (Fig. 4.9B). Later, (i.e. during day 6 to 10), decreased starch levels were also observed in the GA_3 treated leaf blades (Fig. 4.9A).

Although total soluble sugars displayed no pronounced differences between the treated and untreated leaf cuttings, a closer examination of their distribution demonstrated

Table 4.28. Effect of GA_3 on sucrose stimulation of root regeneration in tomato leaf cuttings maintained in continuous darkness.

Treatment	Mean number roots per cutting	
4% sucrose	3.5	a
4% sucrose + GA_3 (1×10^{-4} M)	0.1	b
6% sucrose	2.9	a
6% sucrose + GA_3	0.4	b
8% sucrose	3.6	a
8% sucrose + GA_3	0.4	b

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 2.7

Figure 4.8;A. Total soluble carbohydrate changes in tomato leaf cuttings during root regeneration in the presence or absence of GA_3. After a 48 hour treatment period in continuous darkness, the cuttings were transferred to a 16 hour photoperiod (arrow). Vertical lines ± S.E.

B. Starch changes in tomato leaf cuttings during root regeneration in the presence or absence of GA_3.

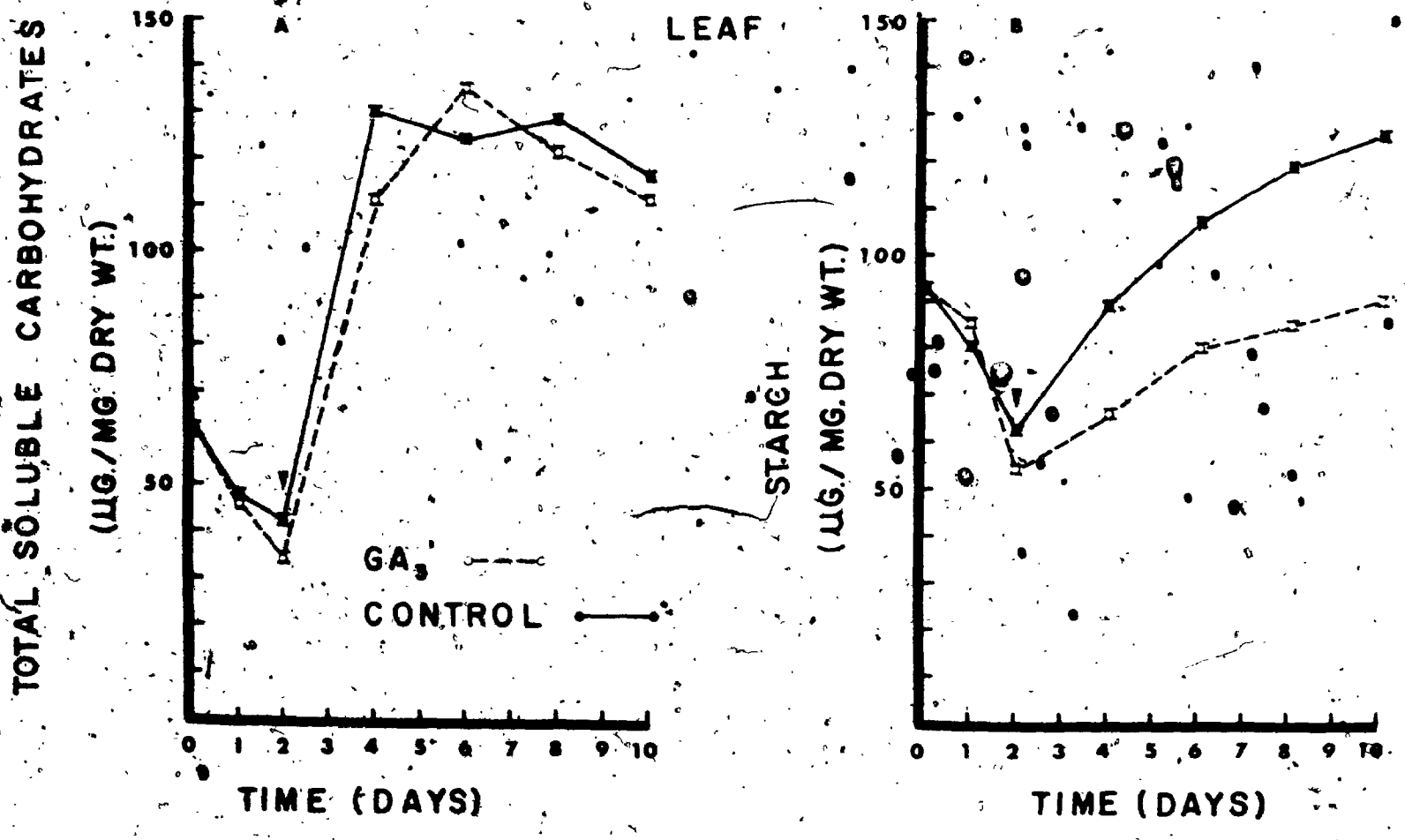
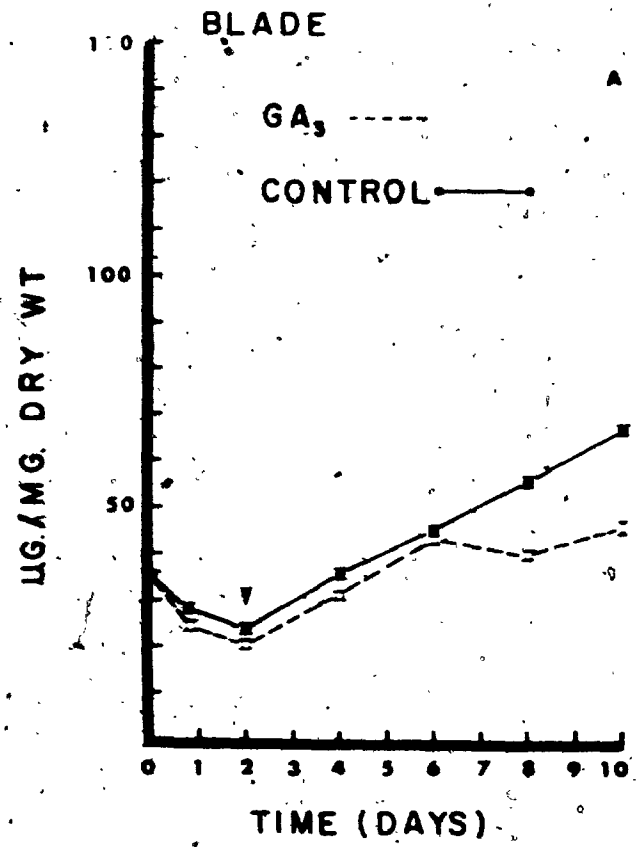
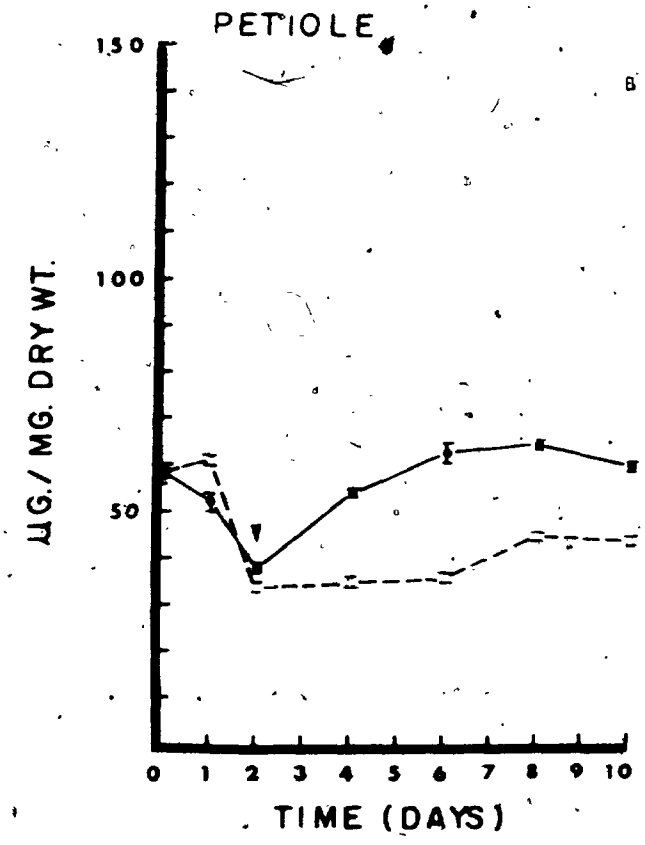

LEAF
A
B
TOTAL SOLUBLE CARBOHYDRATES
(µG./MG. DRY WT.)
STARCH
(µG./MG. DRY WT.)
150
100
50
0 1 2 3 4 5 6 7 8 9 10
TIME (DAYS)
GA3
CONTROL

Figure 4.9;A. Starch changes in tomato leaf blades during root regeneration in the presence or absence of GA_3. Vertical lines ± S.E.

B. Starch changes in tomato leaf petioles during root regeneration in the presence or absence of GA_3.

STARCH


BLADE
A
GA3 ----
CONTROL
µG./MG. DRY WT
100
50
0 1 2 3 4 5 6 7 8 9 10
TIME (DAYS)


PETIOLE
B
150
100
50
µG./MG. DRY WT.
0 1 2 3 4 5 6 7 8 9 10
TIME (DAYS)

consistent variations in the petiole and blade levels (Fig. 4.10B and 4.10A) after 2 days and 4 days respectively.

A histochemical examination of the petiole bases substantiated the quantitative observations of a failure to accumulate starch by the GA_3 treated material in the chloroplasts of vascular parenchyma and adjoining cortical tissue prior to and during primordia formation. However, vascular cambial activity and phloem fiber ("pericyclic fibers") development were apparent by day six in both the treated and untreated petioles (Plate 4.1;1 to 6). Starch distribution within the blade tissue of the treated and untreated cuttings was similar; most of the starch was accumulated in the cortical parenchyma of the basal regions of the mid-rib and secondary veins.

When the leaf cuttings were treated for 48 hours in the dark with distilled water or GA_3, followed by an additional 96 hours in continuous darkness, the petiolar starch content in the treated and untreated material showed similar starch breakdown patterns (Table 4.29).

In view of the polar nature of the regeneration response, an analysis of starch content distribution was carried out using successive 5 mm petiole segments from GA_3 treated and untreated leaf cuttings. The results (Fig. 4.11A and B; 4.12A and B) indicated that GA_3 treatment markedly affected starch distribution within the basal regions of the regenerating petiole tissue. In the basal 20 mm region, where the roots will develop, the data

Figure 4.10;A Total soluble carbohydrate changes in tomato leaf blades during root regeneration in the presence or absence of GA_3. Vertical lines ± S.E.

B. Total soluble carbohydrate changes in tomato leaf petioles during root regeneration in the presence or absence of GA_3.

TOTAL SOLUBLE CARBOHYDRATES

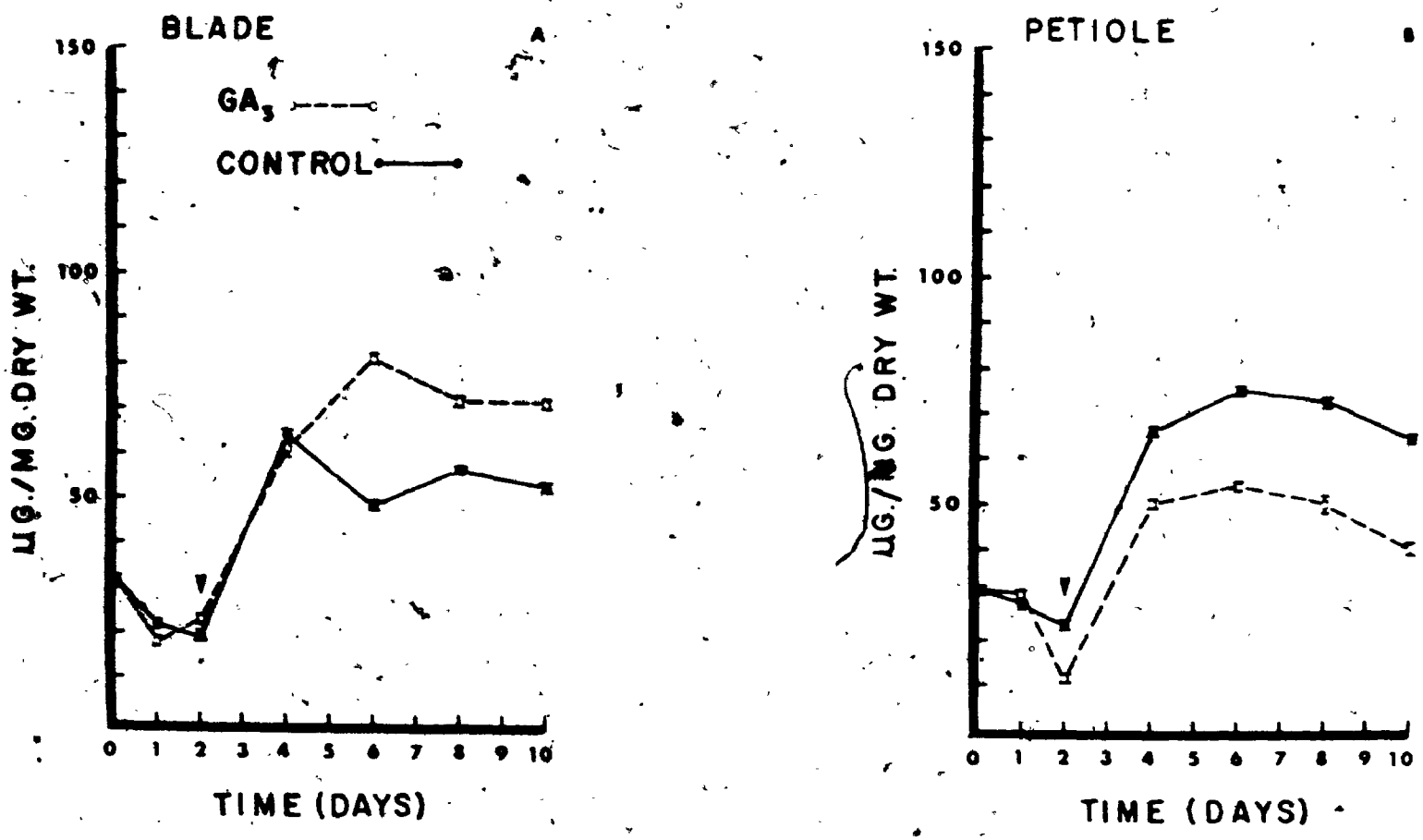


Plate 4.1. 1. Cross sectional view of a mature tomato petiole showing a portion of the vascular strand. V, vessel element; EP, external phloem; CP, cortical parenchyma. (x 400)

2. IKI staining of starch grains within plastids occurring in the starch sheath cells. (x 480)

3. Cross sectional view of a basal petiole region treated for two days in distilled water and subsequently stained for starch with IKI. Note the numerous large starch grains within the starch sheath as well as the smaller grains forming within the vascular and cortical parenchyma. (x 100)

4. Similar to 3, except petioles were treated for 2 days with GA_3 and subsequently stained for starch with IKI. Note the paucity of starch grains within the starch sheath. (x 130)

5. Day 6. Control petiole tissue stained for starch. Prolific starch production and an active vascular cambium are evident. (x 100)

6. Day 6. GA_3 treated petiole tissue stained for starch. An active vascular cambium and lack of starch are evident. (x 100).

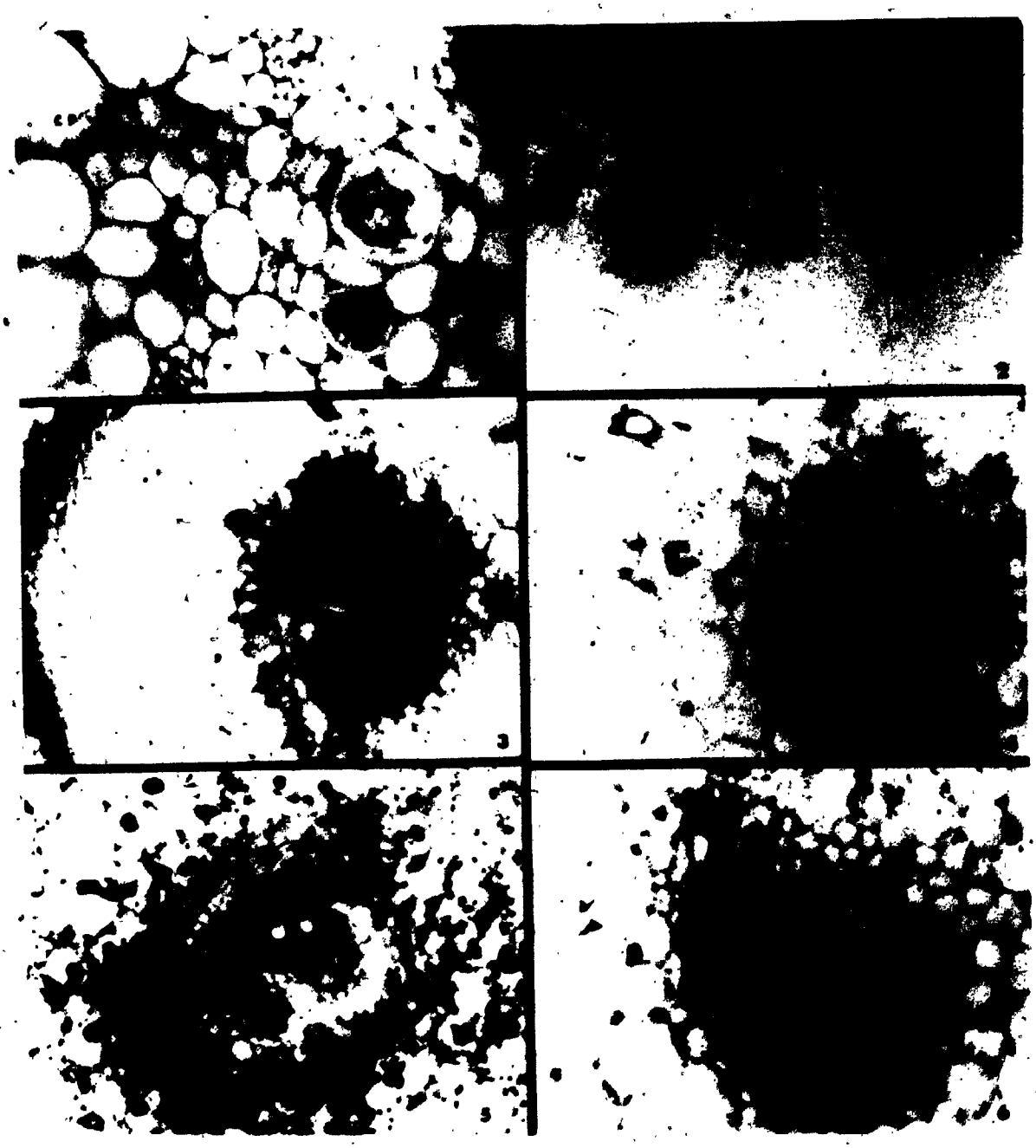
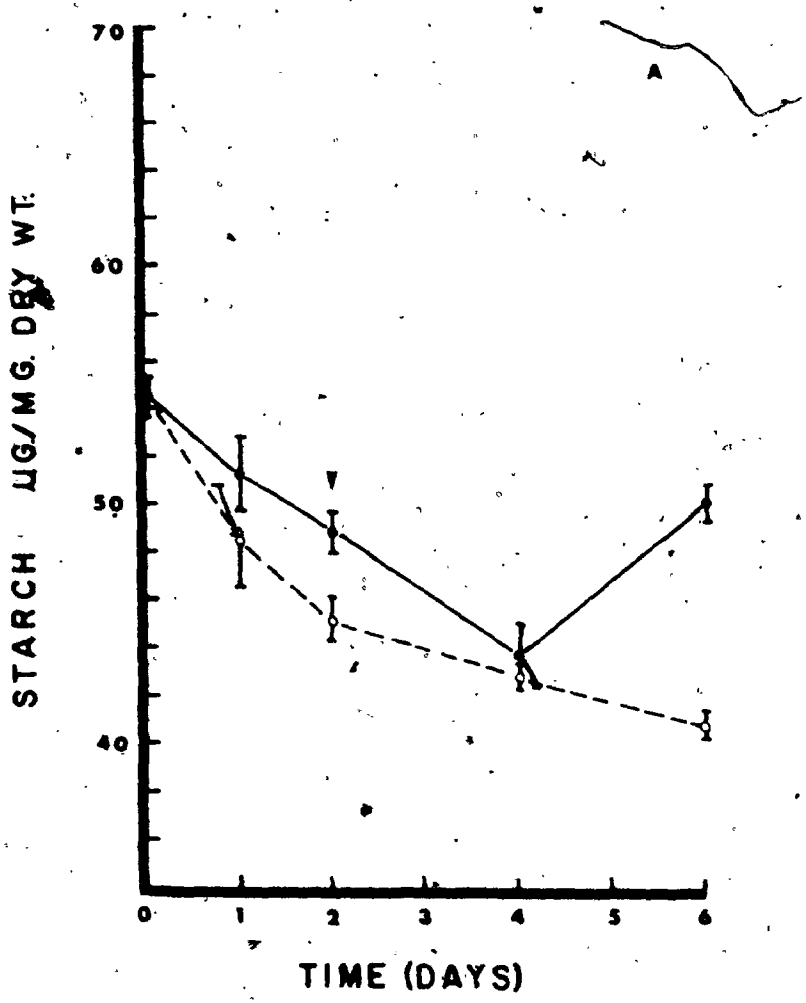


Table 4.29. Starch levels in GA_3 treated and untreated petioles after 6 days in continuous darkness.

Time (days)	Starch content (µg starch/mg dry wt tissue) ± S.E.	
	$-GA_3$	$+GA_3$ (1×10^{-4} M)
0	43.4 ± 1.0	-----
6	33.9 ± 1.4	33.6 ± .7

Figure 4.11; A. Starch changes in petiole segments (0 - 5 mm region measured from the basal petiole end) treated with 1×10^{-4} M GA_3 (o - - - o) or distilled water (● — ●). After a 48 hour treatment period in continuous darkness, the cuttings were transferred to a 16 hour photoperiod (arrow). Vertical lines ± S.E.

B. Starch changes in basal petiole segments (5 - 10 mm region).


STARCH µG/MG. DRY WT.
70
60
50
40
0
1
2
3
4
5
6
TIME (DAYS)

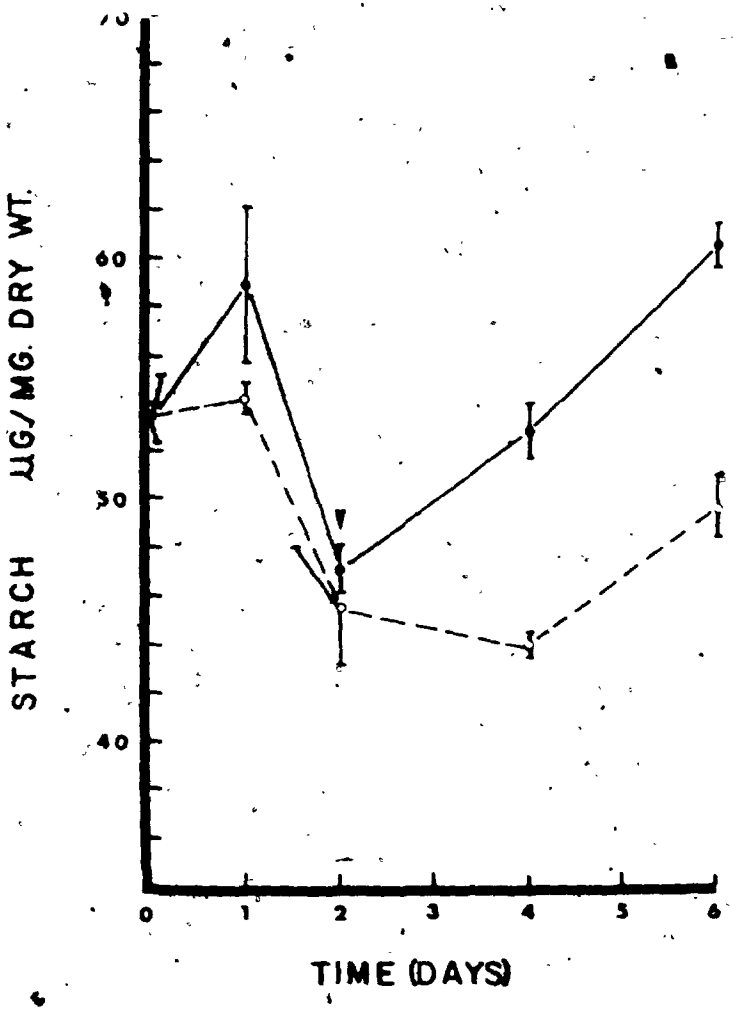
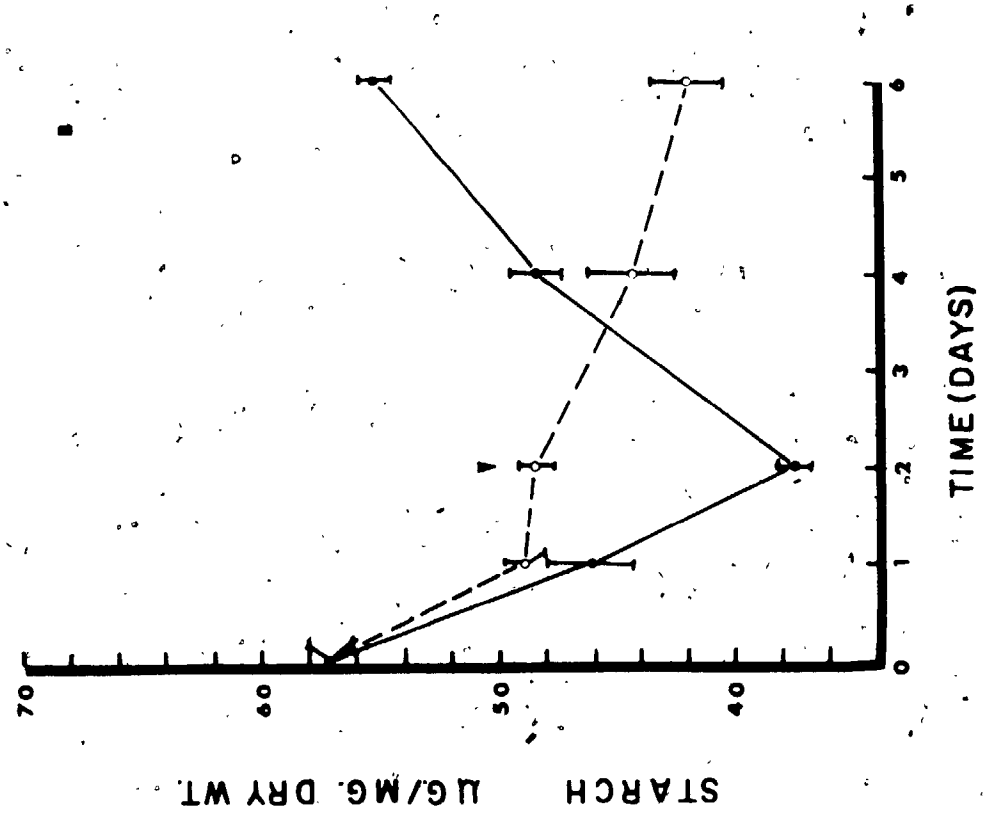
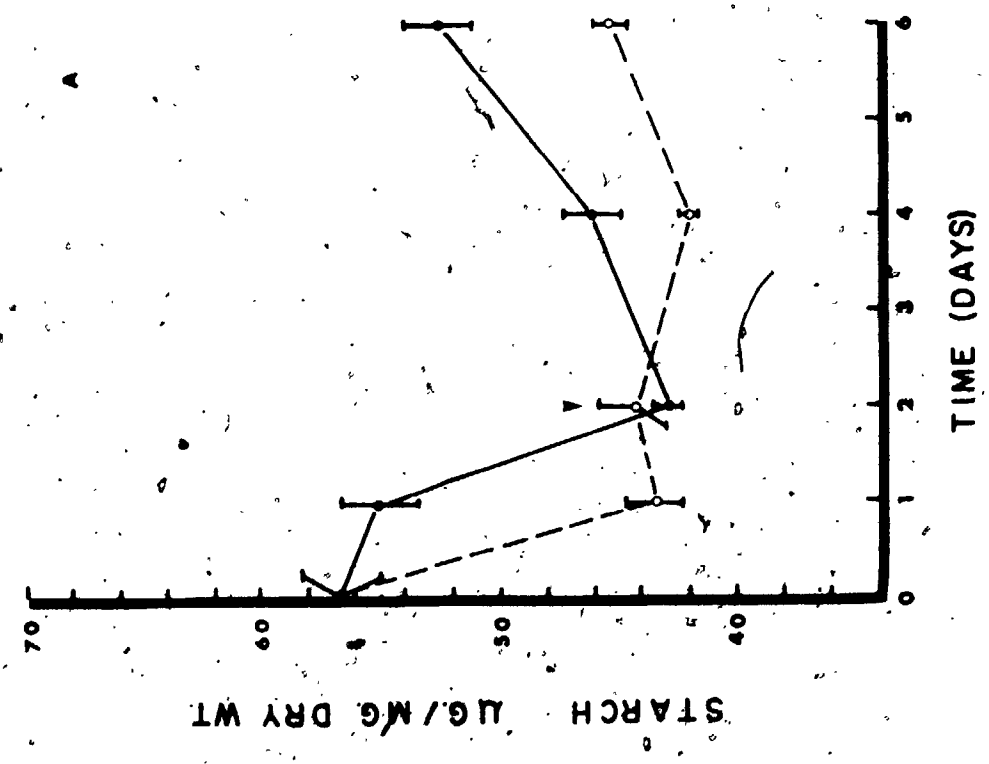

STARCH µG/MG. DRY WT.
60
50
40
0
1
2
3
4
5
6
TIME (DAYS)

Figure 4.12;A. Starch changes in petiole segments (10 - 15 mm region measured from the basal petiole end) treated with 1×10^{-4} M GA_3 (o --- o) or distilled water (● —— ●).

B. Starch changes in petiole segments (15 - 20 mm region). Vertical lines ± S.E.


TIME (DAYS)
0
1
2
3
4
5
6
70
60
50
40
STARCH μG/MG. DRY WT


TIME (DAYS)
0
1
2
3
4
5
6
70
60
50
40
STARCH μG/MG DRY WT

suggested that GA_3 primarily inhibits the localized accumulation (synthesis?) of starch when the cuttings are placed in a photosynthetic environment (i.e. from day 2 to day 6) rather than the degradation of existing starch reserves during the dark treatment period (i.e. from 0 to day 2). Coincident with this view was the observation that the specific amylase activity in both the treated and untreated petiole material increased during the first four days with no treatment effect evident. (Table 4.30). No activity could be detected using Brigg's (1962) agar diffusion method. Most of the amylolytic activity was confined to the vascular parenchyma associated with the three major traces (Plate 4.2; 1,2) during the initial four days of the experiement.

Net starch synthesis of isolated petiole segments in the presence of the starch precursor, glucose-1-phosphate, was slight for the distilled water treated material during the 2 hour incubation period and was not confined to a particular tissue region although it was localized within the plastids. However, petiolar material pre-treated for 48 hours with GA_3 at a concentration of 1 x 1 failed to accumulate starch. Rather, the treated tissues continued to lose starch regardless of the presence or absence of G-1-P (Table 4.31) GA_3 had no apparent effect on net starch synthesis if it was added to the incubation medium (Table 4.32). This feature suggested that GA_3 action on starch accumulation was not a rapid event involving, e.g., a cellular membrane change. In line with

Table 4.30. Changes in the specific activity of total amylase in GA_3 treated and untreated petioles.

Time (days)	Specific activity ($\times 10^{-2}$) ($\pm$ S.E.)	
	$-GA_3$	$+GA_3$ (1×10^{-4} M)
0	47.6 $\pm$ 0.0	---
2	63.3 $\pm$ 13.8	68.2 $\pm$ 1.0
4	69.8 $\pm$ 4.4	66.7 $\pm$ 0.0

Plate 4.2;1 and 2. Amylase activity localized by the starch substrate film method within the vascular strands of basally located petiole tissue treated with GA_3 (1) for 2 days or with distilled water (2). Clear areas are regions of hydrolyzed starch and correspond to vascular strand regions within the petiole. (x 280)

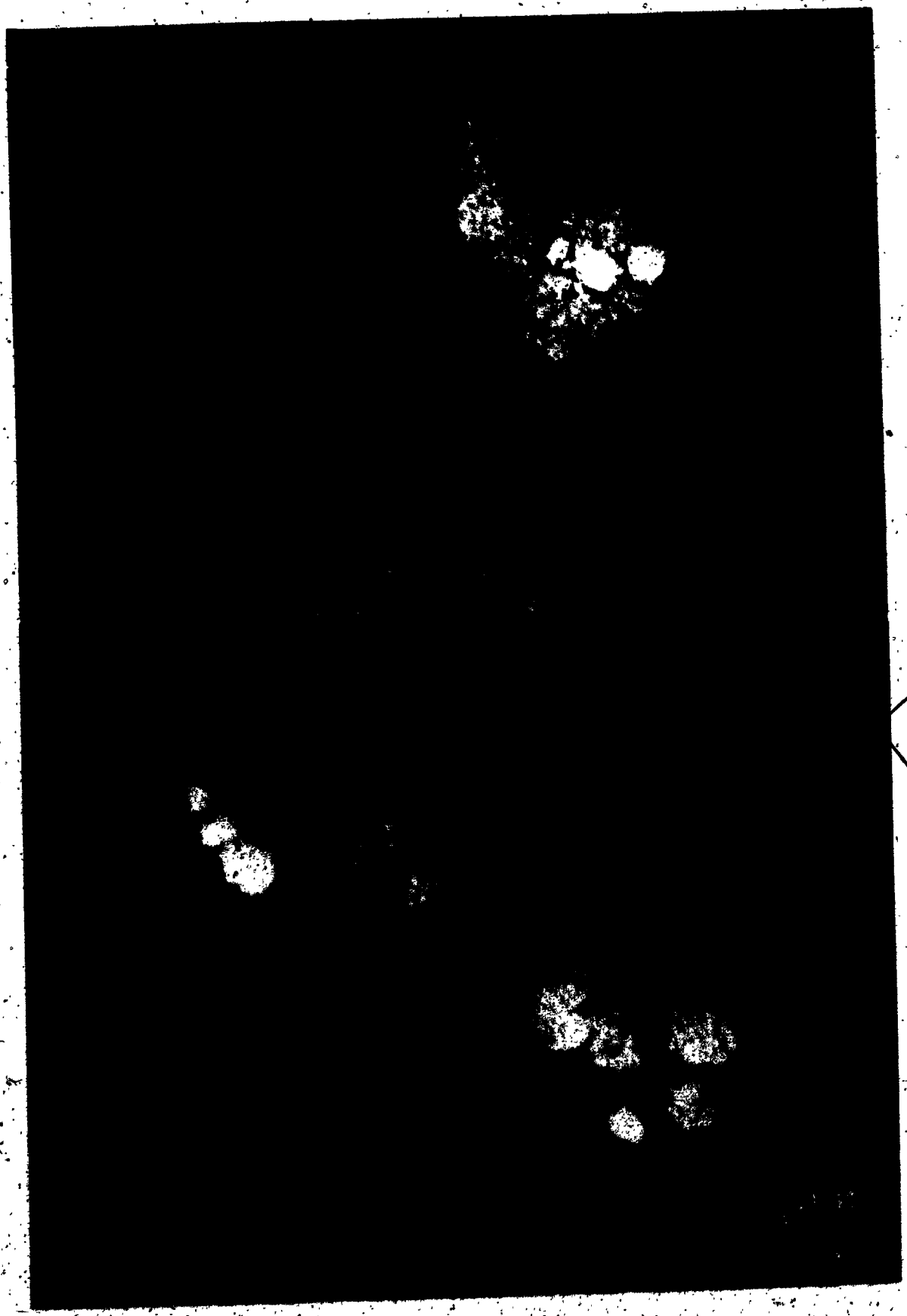


Table 4.31. Effect of GA_3 pretreatment on subsequent starch levels in isolated petiole segments exposed to 30 mM G-1-P for 2 hours.

48 hr. pretreatment	Incubation medium	Mean starch content (± S.E.) (ug/250 mg fresh weight)
H_2O	----	321.8 ± 2.3
	buffer only	298.5 ± 16.9
	buffer + G-1-P	335.3 ± 11.3
GA_3 (1×10^{-4} M)	----	393.0 ± 7.5
	buffer only	268.5 ± 7.9
	buffer + G-1-P	300.0 ± 15.2

Table 4.32. Effect of GA_3 on starch synthesis in isolated segments exposed to 30 mM G-1-P for 3 hours.

48 hr. pretreatment	Incubation medium*	Mean starch content (± S.E.) (ug/250 mg fresh weight)
H_2O	buffer only	277.5 ± 16.5
	buffer + G-1-P	330.0 ± 17.3
	buffer + GA_3 (1×10^{-4} M)	282.0 ± 10.8
	buffer + G-1-P + GA_3	334.5 ± 7.5

*3 hour incubation period.

this observation, a GA_3 induced change in cellular membrane permeability was not pronounced (Table 4.33).

4.4 Discussion

Root regeneration in tomato leaf cuttings can be described accurately and consistently through the use of the developmental index, LPI. While maximum regeneration capacity occurs at approximately the point of maximum growth rate in length of the tomato leaf (i.e. LPI_3 2.0 - 3.0), hormonal application alters this situation. Treatment with natural or synthetic auxins enhances rooting optimally during the stage after maximum growth rate and prior to that leaf's completion of the exponential growth phase (i.e. LPI_3 3.0 - 4.0). A consistent, positive correlation between petiole or terminal leaflet and rachis growth with rooting response was unaltered by auxin treatment.

Localized application of GA_3 to the severed petiole bases consistently inhibited rooting regardless of LPI_3. Stimulation of rooting by high concentrations of the gibberellin biosynthetic inhibitors, CCC and AMO-1618, suggests a possible role for endogenous gibberellins in the restriction of root regeneration. However, although CCC and AMO-1618 are capable of selective inhibition of gibberellin biogenesis in higher plants, the present evidence is far from conclusive and may simply indicate increased organic nitrogen levels due to the breakdown of the inhibitors (Lange, 1970). A significant GA_3 mediated

Table 4.33. Changes in cellular membrane permeability of GA_3 treated petiole segments.

Treatment	Cell contents released (mean absorbance ±SE)		
	A 240 nm	A 260 nm	A 280 nm
distilled H_2O	.15 ± .003	.07 ± .005	.05 ± .004
GA_3 (1×10^{-4} M)	.16 ± .004	.08 ± .003	.06 ± .003
GA_3 (2×10^{-4} M)	.18 ± .009	.09 ± .004	.06 ± .002
GA_3 (4×10^{-4} M)	.15 ± .001	.07 ± .002	.05 ± .002
GA_3 (6×10^{-4} M)	.15 ± .006	.08 ± .001	.05 ± .001

increase in petiole growth over the control tissue could be related to rooting response. No significant GA_3 effects on growth were evident prior to or during root initiation. These results suggest that no diversion of essential nutrients takes place for either enhanced petiole or blade growth and would tend to rule out the nutrient diversion hypothesis through the GA_3 mediated formation of alternative 'metabolic sinks'. Unlike the cuttings from distilled water or auxin treatments, maximum rooting response in GA_3 treated material occurred prior to the stage of maximum leaf growth rate. In view of the high endogenous auxin levels observed in dicot leaves at the stage immediately preceding maximal leaf growth (Goodwin, 1937) and the observed ability of auxin treatment to reverse the GA_3 mediated inhibition of root regeneration in tomato leaf cuttings, it is tentatively suggested that the optimal rooting point in the GA_3 treated material may be primarly due to high endogenous auxin levels in the tomato leaves at this stage. Although the mode of GA_3 action (or "sequence of reactions leading to the physiological effect"; Paleg, 1965) can be modified (or "overridden") by auxins in an apparently independent manner (when considered from the point of view of timing sequence of auxin application relative to GA_3), ABA reversal of GA_3 inhibition is quite time and concentration specific, which suggests a direct interaction between the two growth substances. However, the precise nature of ABA action in the sequence of reactions

leading to decreased starch levels is unknown. At a low concentration (i.e. 1×10^{-8} M) kinetin had no effect on GA_3 inhibition regardless of time of application or the presence of ABA. Consequently, its role as a possible modifier of GA_3 action in the regeneration response of tomato leaf cuttings seems questionable. Interestingly, high exogenous sucrose concentrations cannot overcome the GA_3 inhibition of regeneration. This feature suggests that soluble carbohydrate levels may not be the primary candidate for GA_3 action. This conclusion is supported by the quantitative analyses of total soluble carbohydrates which demonstrate an increase in both the treated and untreated leaves prior to and during root regeneration. Similarly, histological evidence reveals an active vascular cambium under both treatment conditions. This aspect would tend to rule out a GA_3 mediated inhibition of mitotic activity in the tissues which will be associated with the organogenic event.

Biochemical and histochemical evidence tend to support Thorpe and Murashige's hypothesis (see section 2.4) that localized starch accumulation prior to and during primordia formation are a necessary prerequisite to an organogenic response. However, contrary to the suggestion put forth by Thorpe and his colleagues that GA_3 mediates localized starch levels primarily by altering amylase activity (Thorpe and Meier, 1974a; 1974b), the present results support the hypothesis that GA_3 initially inhibits localized starch

synthesis with no significant effect on starch breakdown by amylase. The present evidence of GA_3's role in the inhibition of localized starch synthesis may be summarized as follows:

1) GA_3 application has no significant effects on the breakdown of starch in tomato petioles when the leaf cuttings are placed under conditions favouring continuous starch breakdown (i.e. after 2-6 days in continuous darkness);

2) When GA_3 treated leaf tissue is placed under a 16 hour photoperiod, no active accumulation of starch takes place in the petioles although endogenous sugar levels increase. Increased sugar and starch levels are also observed in the leaflets of the treated and untreated tissues.

3) Total amylolytic activity increases in both the treated and untreated petioles during the initial four days with no significant treatment effect evident.

4) Amylolytic activity is localized primarily in the vascular bundles although inhibition of active starch accumulation in the GA_3 treated petioles is evident in the cortical parenchyma as well as the vascular tissues.

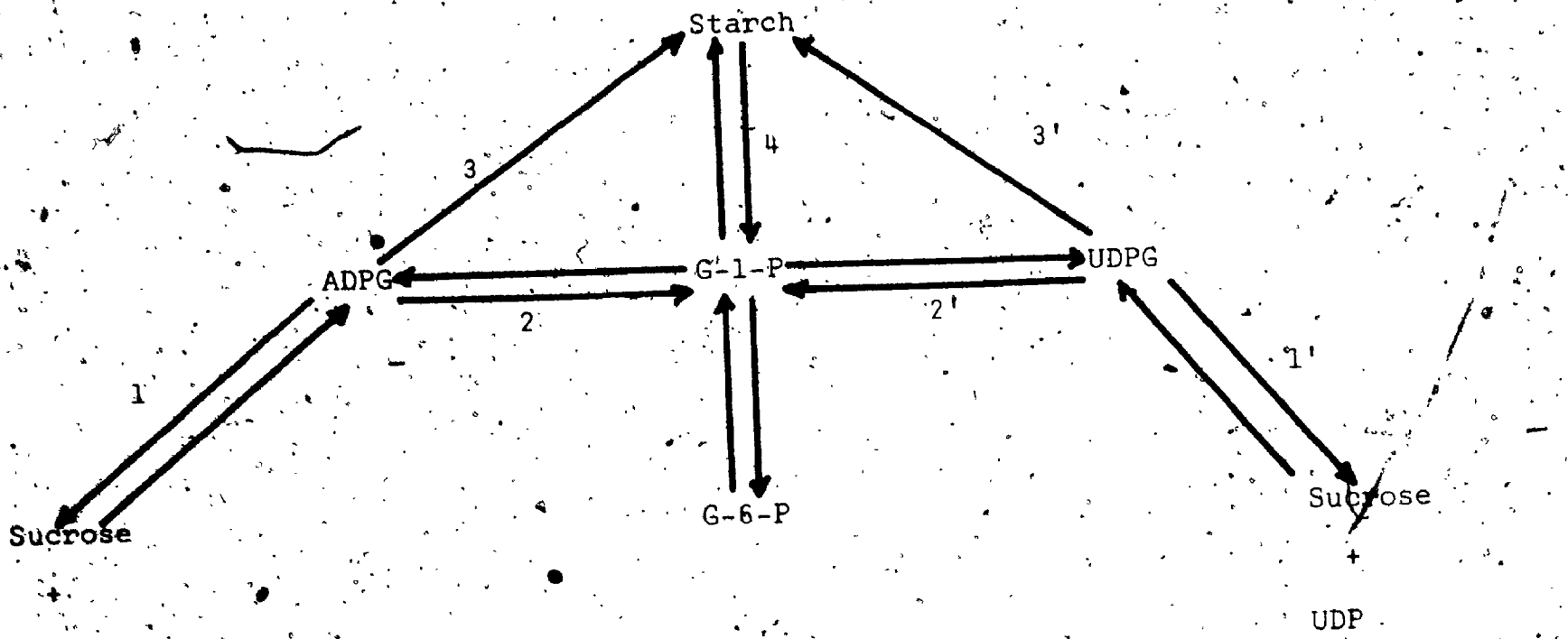
5) Net starch synthesis in the presence of the starch precursor, G-1-P, did not occur in GA_3 treated petiole segments.

On the basis of the present evidence, the mode of GA_3 action can be hypothesized as involving inhibition of the starch synthesizing mechanism within the plastids causing

the observed failure of the petioles to accumulate starch. This event would, in turn, block the formation and early development of the organ primordia through a limitation in the availability of locally high concentrations of carbohydrates in an osmotically inactive form which would be readily utilized for metabolic energy production and rapid cell wall synthesis via the phosphorylated sugars. In view of the apparent lack of GA_3 treatment effects on net starch synthesis from G-1-P in petiole segments when applied during the incubation period and the failure to detect a significant GA_3 modification of cellular permeability, the GA_3 mechanism of action (or specific metabolic change: Paleg, 1965) would not appear to involve a rapid and pronounced membrane modification effect (at least at the cellular level). Rather, the inability of GA_3 treated material to synthesize starch in the presence of G-1-P suggests that the mechanism of GA_3 action may reside in either 1) the inhibition of de novo or enhanced synthesis of starch synthesizing enzymes and/or 2) "destabilization" of pre-existing starch synthesizing enzymes. Since three routes have been identified for starch synthesis in the leaves of higher plants (Fig. 4.13) an examination of the specific activity changes of such enzymes as UDPG-pyrophosphorylase, phosphorylase, UDPG-starch synthetase, ADPG—pyrophosphorylase and ADPG-starch synthetase would appear to be a desirable first step. However, as noted by Fekete and Vieweg (1974), starch synthesis may take place

Figure 4.13. Proposed main pathways for starch synthesis (after Huber, Fekete and Ziegler, 1969; Vieweg and Fekete, 1972; Fekete and Vieweg, 1974). Key to the enzymes:

1. ADP glucose:fructose glucosyl transferase;
   1'. UDP glucose:fructose glucosyl transferase;
2. ADPG-pyrophosphorylase;
   2'. UDPG-pyrophosphorylase;
3. ADPG-starch synthetase;
   3'. UDPG-starch synthetase;
4. phosphorylase.


Starch
3
4
3'
ADPG
G-1-P
UDPG
2
2'
1
1'
Sucrose
+
ADP
G-6-P
Sucrose
+
UDP

during rapid starch degradation with the overall effects o starch metabolism "the sum of quite a number of different synthetic and degradation steps which all function at approximately the same time" (p. 135). These complex biochemical problems are beyond the scope of the present study. Moreover, it is apparent that a cautious approach is necessary. For example, although ADP-glucose pyrophosphorylase appears to be an important enzyme in the regulation of starch levels in leaf tissue (Turner and Turner, 1975), its' *in vivo* activity may be controlled by such factors as i) G-1-P levels, ii) P_i levels; iii) level of glycolytic intermediates (e.g. P-glycerate); iv) ATP levels within the chloroplasts and v) pH. Whether direct or indirect regulation of this enzyme or enzymes of the starch biosynthetic pathways is mediated by plant growth regulators is currently unknown. Furthermore, whether the postulated regulation of one or more of the starch synthes enzymes is under nuclear or organelle control is also unkn The limited evidence in the literature would suggest that the regulation of synthesis and turnover of starch metabolizing enzymes in plastids may be controlled primarily b the nucleus with a subordinate role played by the organell itself (Badenhuizen, 1969).

GROWTH, DEVELOPMENT AND REGENERATION OF THE TOMATO LEAF

by

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Doctor of Philosophy

Faculty of Graduate Studies
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CHAPTER 5

ROOT REGENERATION FROM LEAF DISCS CULTURED IN VITRO: PRELIMINARY OBSERVATIONS

5.1 Introduction

The tomato leaf cutting is a relatively autonomous system with regard to root regeneration. This feature no doubt arises due to a sufficient supply of endogenous metabolites (both hormonal and nutritional), although the levels of these factors fluctuate with the developmental stage of the leaf. As a consequence, the relative importance of various endogenous factors (e.g. carbohydrate substrate, vitamins and hormones) and their possible interactions in the regeneration process are unknown. A more ideal regeneration system should exhibit a total dependence on chemically defined, exogenous nutrients and plant growth regulators. In view of the previous work with tomato leaf cuttings (see Chapter 4), the present study centred around a) developing a regeneration system utilizing 5 mm diameter leaf discs cultured in vitro which would be totally dependent upon external stimuli and b) examining that system at the histochemical level (utilizing both light and electron microscopy) with a view to defining the regeneration response

in terms of cellular activities.

The present study is the first detailed account of anatomical/histochemical changes occurring during root formation which are induced in cultured leaf blade tissue by a specific hormonal stimulus. No comparable study with any species is evident in the literature. Finally, this study will serve as a reference point for subsequent physiological experiments dealing with the apparent multiple controls of organogenesis by gibberellins in cultured leaf material (see Chapters 6 and 7).

5.2 Materials and Methods

5.2.1 In vitro Culture

True leaf number 3 from the tomato cultivar 'Farthest North' was selected for all in vitro regeneration experiments. An average LPI_3 was calculated for each group of leaves used in the culture experiments on the basis of the measurements of 10 randomly selected plants (see section 2.2 for LPI calculations). Twenty to thirty leaves were severed at the petiole base, and the cut surface immediately sealed with liquid Paraplast. Leaves were surface sterilized with freshly prepared and filtered 5% calcium hypochlorite. After sterilization for 7 minutes, all subsequent operations were carried out in a sterile culture hood. The leaves were rinsed with four changes of sterile distilled water (total volume approximately 1 liter) and then placed, adaxial side

down, on a glass plate. Leaf discs were cut with a 5 mm (inside diameter) stainless steel cork borer. Two discs were removed from the basal half of each leaflet and included the midrib medially placed within the disc. The discs were temporarily stored (i.e. up to 1 hour) on moist filter paper in small (6 cm diameter) petri plates until all of the leaflets had been sampled. Subsequently, the leaf discs (one disc per vial) were placed abaxial side down on approximately 10 ml of .8% agar-based medium (pH 5.2 ± .1) in screw top vials (Richards Glass Co., Montreal) containing Murashige and Skoog (1962) mineral elements, 1×10^{-7} M thiamine and 2% sucrose. This medium is termed "basal" and is non-inductive with regard to organ regeneration. IAA at final concentrations of 5×10^{-6} M to 5×10^{-5} M was added to basal medium by filter sterilization using a Gelman Swinny type hypodermic filter attachment with Millipore filters (GA-8; pore size 0.2 u) after autoclaving the other constituents. This medium is termed the "auxin medium" and is inductive with regard to root regeneration. The discs were placed under continuous low intensity (200-250 f.c.) fluorescent (Sylvania Cool White) lighting at a constant 25°C for up to seven days unless otherwise noted.

5.2.2 Histological and Histochemical Techniques

5.2.2.1 Fixation and Embedding for Light Microscopy

At twelve hour intervals for a period of five days and

again on the sixth and seventh days, five leaf discs were removed from both the basal and auxin media and fixed immediately in cold 10% formalin in 0.1 M phosphate buffer (pH 7.1) for 2-3 hours using vacuum infiltration. The fixed discs were passed through a tertiary-butanol series and embedded in Paraplast wax (Jensen, 1962). Transverse and paradermal sections were cut 10 μ in thickness and mounted on slides coated with Haupt's gelatin adhesive (Jensen, 1962). The slides were dewaxed in xylene and hydrated through a graded ethanol series. The slides were washed in running tap water for 2-3 hours prior to staining. In the following histochemical procedures, the prepared slides were batch processed in order to minimize variability of the staining results. All histochemical treatments were repeated at least four times with serially cut sections (20 tissue sections per slide) for each time period. A total of approximately 10,000 to 15,000 tissue sections were examined in this histochemical study.

5.2.2.2 DNA and RNA Staining

DNA and RNA were localized using the Azure B method of Flax and Hines (1952). Control slides were treated with 5% TCA at 90-95°C for 15 minutes in order to hydrolyze the nucleic acids prior to staining (Jensen, 1962).

5.2.2.3 Total Protein Staining

Total proteins were localized by the Mercuric Bromphenol

blue method of Mazia, Brewer and Alfert (1953) as modified by Pearse (1968). No controls were included as preliminary experiments demonstrated that neither the Van Slyke deamination (Gifford and Dengler, 1966) or acetylation (100% acetic anhydride at 60°C for 4 hours; Pearse, 1968) blocked the staining reaction. Similar results were obtained by Quintarelli, Scott and Dellovo (1964).

5.2.2.4 Basic Protein Staining

Basic proteins were localized by the picric acid-bromphenol blue method of Block and Hew (1960,a;b); see also Ringertz and Zetterburg (1966) for discussion of the staining procedure. Controls included the following and preceded the staining:

i) modified Van Slyke deamination according to Block and Hew (1960a) - equal volumes of cold 5% TCA/5% sodium nitrite for 1/2 hour at 4°C. Two solution changes were made during this time period. This deamination step was used either with or without prior picric acid hydrolysis of the nucleic acids. This procedure blocks the α-amino groups and the ϵ-amino group of lysine. The guanidino group of arginine is relatively unaffected.

ii) acetylation - 100% acetic anhydride containing 1% acetic acid at 60°C for 1 hour (Block and Hew, 1960b). This procedure blocks the amino groups by acetylating primary and secondary amines and amino compounds (Pearse, 1968).

iii) absence of nucleic acid hydrolysis - as noted above, the picric acid hydrolysis of the nucleic acids was omitted in one control series in order to demonstrate basic amino groups which were not bound to the nucleic acids. A second control series was not hydrolyzed with subsequent deamination in order to demonstrate the involvement of terminal amino and/or the ϵ groups of lysine in the staining of the "unbound" basic protein fraction.

Extensive evaluation of the ammoniacal silver procedure of Black and Ansley (1964) lead to its rejection as a suitable stain for basic proteins due to its apparent non-specificity.

5.2.2.5 Starch Staining

Starch grains were localized using the Periodic Acid-Schiff's (PAS) reagent (Jensen, 1962) as well as a Periodic Acid-Ammoniacal Silver (PAAS) method which was developed by the author (see Appendix). The PAAS method was simpler than the PAS method while allowing starch grain morphology and distribution to be adequately described. IKI staining for starch (Jensen, 1962) was not possible with the fixed material.

5.2.2.6 NaOH-Basic Fuschin Clearing Technique

Method no. 1 of Fuchs (1963) was used in order to study the stages of xylem vascularization and Casparian strip formation within the developing primordia (see

section 3.2.2.1 for method outline).

5.2.2.7 FPA-Propiocarmine Clearing Technique

This clearing technique was developed by the author and is described in section 3.2.2.1. The procedure was useful for studying nuclear activity in the various tomato leaf tissues during primordia induction and development. Nuclear/nucleolar volumes for three different leaf tissue types (i.e. midrib cortical parenchyma, bundle sheath cells of the minor veins, and spongy mesophyll) were calculated using the formula for spheroidal volume:

$$V = \frac{4}{3} a^2 b$$

where a equals transverse radius and b equals longitudinal radius (List, 1963). A diploid, pre-DNA synthetic phase, G_1 nuclear volume has been determined at approximately 150-190 μ^3 on the basis of Van't Hof and Sparrow's work (Van't Hof, 1965; Van't Hof and Sparrow, 1965) with tomato root tissue.

Two complete experiments of 7 days duration and comprising induced and non-induced leaf discs were sampled at 12 hour intervals (20 samples/treatment) and processed by each of the above clearing methods.

All histological and histochemical observations were made primarily for leaf discs derived from maturing leaflets within the LPI_3 range of 3.0 to 4.0.

5.2.2.8 Histochemistry of Fresh Tissue

Free-hand sections of leaf discs aged 8 to 10 days on basal medium were stained according to Jensen (1962) and Johansen (1940) as follows: a) neutral red for vacuolar structures; b) Sudan IV for lipids; c) Janus green for mitochondria; d) IKI for starch; and e) 1% aqueous Methylene blue for general cellular morphology. Acid phosphatase was localized by the lead sulphide procedure (Gomori, 1952, as cited in Jensen, 1962) while peroxidase activity was located according to the benzidine reaction as modified by Van Duijn (1955, as cited in Jensen, 1962).

5.2.2.9 Electron Microscopy

Fresh leaf material and leaf discs aged for 8 days on basal medium were cut into 1 to 2 mm squares under cold 3% glutaraldehyde in .1 M phosphate buffer, pH 7.1, plus 2% sucrose. Tissue pieces were subsequently transferred to fresh glutaraldehyde fixative and repeatedly vacuum infiltrated until the tissue was essentially free of trapped air bubbles. Total fixation time in cold glutaraldehyde was 16 hours at 4°C. Tissue was rinsed in cold buffer and 2% sucrose five times over a 6 hour period and post-fixed for 3 hours in 2% osmium tetroxide buffered to pH 7.0 with .1 M phosphate. Using fresh buffer, the tissue sections were washed 4 times over a 14 hour period followed by staining the tissue blocks with saturated (approximately 8%) aqueous uranyl acetate for 30 minutes at room temperature.

Following whole tissue staining, the preparations were washed in distilled water and dehydrated through a graded acetone series at 4°C. Total dehydration time to pure acetone was 5 hours. The tissue blocks were subsequently infiltrated with Spurr's low viscosity epoxy resin medium (Spurr, 1969) over a 2 1/2 day period at room temperature with continuous agitation followed by embedding and polymerization for 2 days at 60°C. Sections were cut with a diamond knife and stained for 45 seconds to 1 minute in .35% aqueous lead citrate. Sections were observed with a Phillips electron microscope (Model 200).

5.2.2.10 Photomicrography

Prepared histochemical slides were photographed with a Zeiss Photomicroscope II. Black and white photos were taken and processed as previously described (section 3.2.4). Colour photographs were taken as follows:

i) Kodacolor-X 35 mm film for preparations staining red or yellow. Correct adjustments included an ASA setting of 80, 12 volt setting for the 60 watt illuminator and the use of a 3200°K compensating filter (Kodak 80A).

ii) Kodak Photomicrography colour film 2483 for preparations staining blue, green or purple. Correct adjustments included an ASA setting of approximately 4.0, 12 volt setting for the 60 watt illuminator and the use of a 3200°K compensating filter (Kodak 80A).

5.2.3 Statistical Analysis

Root count data was evaluated as described in section 4.2.6 using 70% ethanol cleared material in order to count internal as well as external roots. In the leaf-disc system, rooting response was divided into three categories depending on the vascular origins: i) midrib; ii) secondary vein and iii) minor vein origin. Two sub-categories were recognized for each one of the three categories: external or emerged roots and internal or presumptive root primordia. Fresh weight, dry weight and midrib lengths were transformed to log values prior to analysis (Sokal and Rolf, 1968; Wolf, 1968).

5.3 Results

5.3.1 Culture Requirements for Leaf Disc Regeneration in vitro.

Some of the variables associated with root regeneration in cultured tomato leaf discs were defined by extensive preliminary culture experiments. A few of the more important observations are summarized below.

5.3.1.1 Nutritional Factors

i) Preliminary experiments with White's mineral nutrients (White, 1963) proved unsuccessful in supporting root regeneration from the leaf discs. However, the inorganic medium of Murashige and Skoog (1962) was successful and, as a consequence, was used throughout this study. The omission

of any inorganic component (i.e. macroelement, microelement, or FeEDTA stock) blocked regeneration.

ii) A total dependence on exogenous carbohydrate source was necessary for regeneration by the leaf discs. All three carbohydrate sources tested (i.e. sucrose, glucose, and glucose/fructose) supported regeneration over the concentration range of .005 M to .10 M although different concentration optima were apparent in terms of root production or fresh weight changes. Filter sterilization instead of autoclaving the glucose/fructose component of the medium had no effect on root regeneration although a decreased fresh weight was evident in the autoclaved treatment.

iii) Thiamine was necessary for root regeneration in excised leaf discs. This effect was evident only in mature leaf material (i.e. mean LPI_3 = 6.4) and no effect could be demonstrated in younger (i.e. mean LPI_3 = 3.2) samples (Table 5.1). Other vitamin combinations tested, which included nicotinic acid, pyridoxine, biotin, calcium pantothenate and ascorbic acid, increased rooting significantly although no dependence on morphological age was evident.

5.3.1.2 Non-Nutritional Factors

i) Rooting appeared to be relatively independent of the amount of medium available to the leaf disc (i.e. between 2 and 16 ml).

ii) Growth and root regeneration from the leaf discs exhibited a broad ranged pH optima from 4.3 to 6.6 although

Table 5.1. Effects of LPI_3 on regeneration response of cultured tomato leaf discs to various vitamins.

Treatment	LRI_3	Mean number of roots per disc	
control	3.2	11.3	a
	6.4	2.1	c
thiamine	3.2	11.4	a
(1×10^{-7} M)	6.4	15.5	b
thiamine and vitamin	3.2	16.4	b
supplement I*	6.4	14.8	a,b
thiamine and vitamin	3.2	12.9	a,b
supplement II**	6.4	16.6	b
thiamine, supplements	3.2	16.6	b
I and II	6.4	16.6	b

* Vitamin supplement I contained nicotinic acid (1×10^{-6} M), pyridoxine (1×10^{-6} M) and glycine (5×10^{-5} M).

** Vitamin supplement II contained biotin (1×10^{-6} M), calcium pantothenate (1×10^{-6} M) and ascorbic acid (1×10^{-6} M).

Means followed by the same letter in a column not different at 5% level.

the media pH changes during the culture period tended towards mildly acidic conditions (Table 5.2).

iii) Leaf discs exhibited an apparent increased sensitivity to IAA in terms of number of roots regenerated when cultured in the dark in contrast to different light conditions (Fig. 5.1 A, B and C). Furthermore, increased light intensity caused a decrease in the number of internal or presumptive root primordia regardless of their origins. However, midrib elongation and mean root length were significantly greater in light grown discs (Table 5.3).

The possibility that the regeneration responses to IAA were related to either 1) the nature of the auxin and/or 2) light effects on the medium were explored. As indicated in Table 5.4, conditioning of the media by continuous light exposure had a significant effect on the number of regenerated roots regardless of the auxin source.

iv) Leaf discs taken from different aged material (expressed in terms of their mean LPI_3) showed no significant differences in their regeneration responses to low IAA concentrations. However, there was a significant difference in response at high IAA concentrations between young leaves (mean LPI_3 = 2.0) and leaf material taken from the end of phase one growth (mean LPI_3 = 3.6) or older leaves (LPI_3 = 5.7) (Table 5.5). This difference in response was due primarily to fluctuations in the numbers of primordia originating from the minor vein system.

v) Essentially similar results were obtained with different

Table 5.2. Effects of media pH on growth and regeneration of cultured tomato leaf discs.

Initial pH*	Final pH*	Mean number roots per disc		Mean fresh weight (mg)	
4.3	4.7	7.0	a	24.4	c
5.0	5.0	7.0	a	25.7	c
5.3	5.2	10.1	b	27.2	c
6.0	5.6	8.2	a,b	26.1	c
6.2	5.8	7.9	a,b	27.9	c
6.6	6.1	9.1	a,b	23.8	c

* Mean value of four determinations.

IAA 5 x 10^{-6} M

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.6

Figure 5.1. Effects of light intensity on root regeneration from tomato leaf discs cultured *in vitro* on media containing different IAA concentrations.
A. Continuous light, 800 f.c.; B. continuous light, 250 f.c.; and C. continuous darkness.
Mean LPI_3 = 4.2
Closed circles, roots of midrib origin; open circles, roots of secondary vein origin; closed squares, roots of minor vein origin.

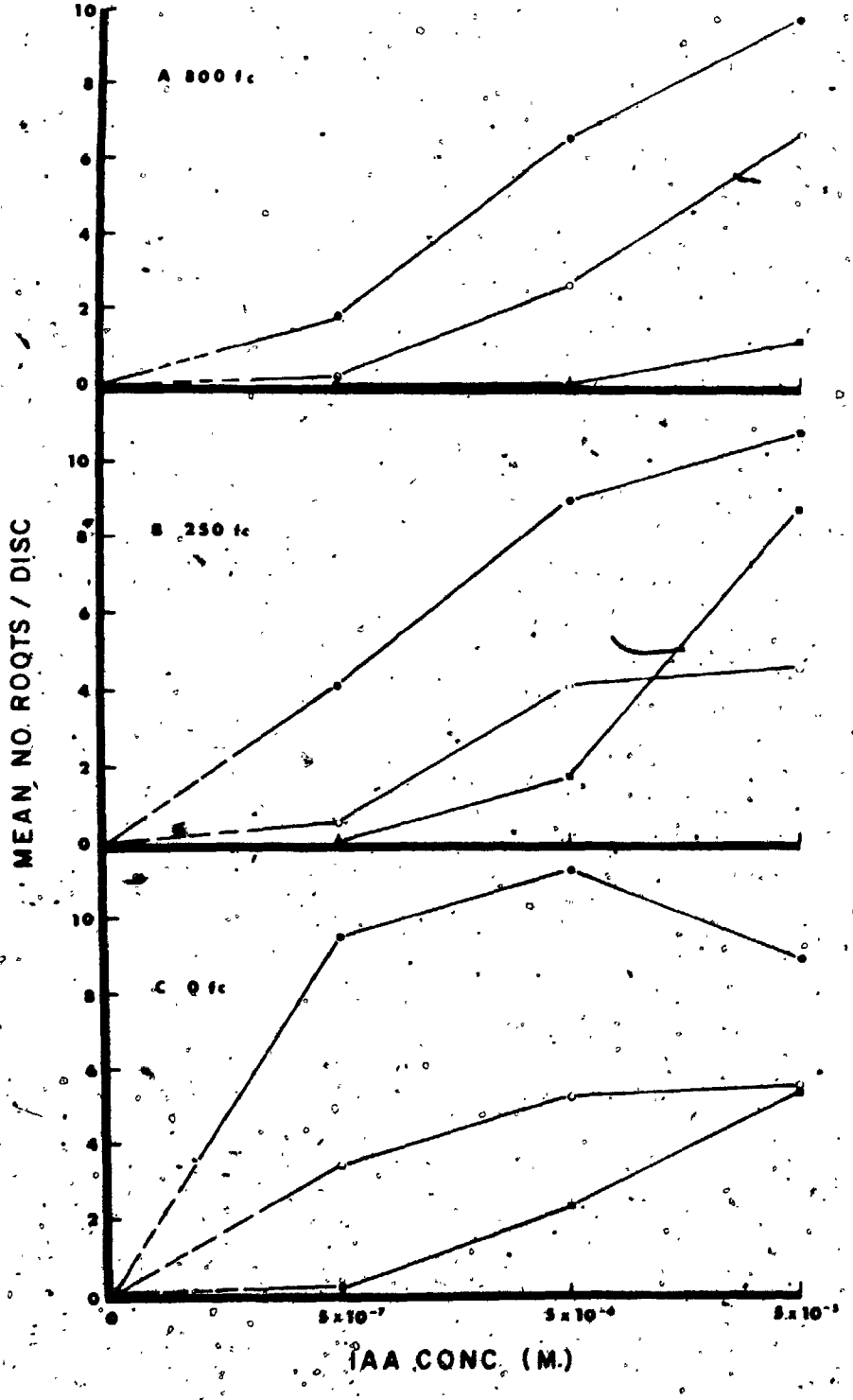

A 800 fc
B 250 fc
C 0 fc
MEAN NO. ROOTS / DISC
IAA CONC. (M.)

Table 5.3. Effect of light intensity on midrib and root elongation from regenerating leaf discs on media containing different levels of IAA.

IAA concentration	light intensity (foot candles)	Mean midrib length (mm) after 7 days (± S.E.)	Mean root length (mm) after 7 days (± S.E.)
0	0	5.7 ± .1	---
	250	7.1 ± .2	---
	800	7.2 ± .2	---
5×10^{-7} M	0	6.3 ± .1	3.0 ± .2
	250	7.1 ± .1	9.2 ± .9
	800	7.2 ± .2	15.1 ± .9
5×10^{-6} M	0	6.2 ± .1	1.9 ± .1
	250	7.0 ± .1	2.5 ± .1
	800	7.2 ± .2	13.1 ± 1.1
5×10^{-5} M	0	6.1 ± .1	.8 ± .1
	250	6.6 ± .1	2.0 ± .2
	800	6.8 ± .1	12.7 ± 1.1

Mean LPI_3 = 4.2

Table 5.4. Effect of light treatment of different auxin media on subsequent root regeneration.

Auxin*	Media pretreatment conditions**	Culture conditions**	Mean number of roots per disc
IAA	none	light	4.5 c,d,e
	none	dark	13.2 f
	light	light	.9 a
	dark	light	4.5 c,d,e
	light	dark	5.4 c,d,e
	dark	dark	16.7 f
IBA	none	light	3.6 c,d
	none	dark	8.2 e
	light	light	1.3 a,b
	dark	light	2.9 b,c
	light	dark	4.2 c,d,e
	dark	dark	8.1 d,e
NAA	none	light	7.3 d,e
	none	dark	14.5 f
	light	light	3.6 b,c
	dark	light	8.2 e
	light	dark	15.8 f
	dark	dark	16.4 f

* Auxin concentration: 5×10^{-6} M.

** Media were pretreated in continuous darkness or continuous light (800 fc) for 7 days at 25°C. Freshly cut leaf discs were then placed onto the media and subsequently cultured under the described conditions.

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.8

Table 5.5. Effects of LPI_3 on regeneration response of tomato leaf discs to different IAA concentrations.

IAA concentration	Mean LPI_3	Mean no. of roots per leaf disc	
0	2.0	.4	a
	3.6	.2	a
	5.7	0.0	a
5×10^{-7} M	2.0	4.5	b
	3.6	3.0	b
	5.7	3.2	b
5×10^{-6} M	2.0	10.0	d
	3.6	6.8	c
	5.7	8.1	c,d
5×10^{-5} M	2.0	22.1	e
	3.6	11.1	d
	5.7	8.4	c,d

Means followed by the same letter in a column not different at 5% level.

leaf positions as were obtained with different LPI_3.

vi) When leaf discs (true leaf #3) from different cultivars (i.e. Bonney Best, Marglobe, San Marzano, and Tiny Tim) at comparable stages of development were cultured at different IAA concentrations, regeneration was observed in all cases.

5.3.1.3 Hormonal Factors

The minimum level of IAA necessary for a regenerative response in maturing leaf discs was approximately 0.01 ug/ml medium for dark grown discs and 0.2 ug/ml for light grown ones (Table 5.6). Exogenous IAA application for the initial 4 to 5 days of the 7 day culture period was sufficient for maximum root production at each IAA concentration tested (Table 5.7). IAA concentration also regulated the root distribution (Fig. 5.2A). Root primordia were present in 72 hour material as presumptive or internal root primordia only. However, by the end of the 7 day period, most of the roots were external (Fig. 5.2B).

Root elongation was progressively inhibited by increased auxin exposure, regardless of IAA concentration while the vascular patterns of the emerging roots (which are normally diarch) possessed increasing numbers of triarch arrangement (Table 5.8).

Various synthetic auxins and structurally related (inactive members) of the substituted phenoxy class were tested for rooting activity in the presence or absence of IAA. In general, the substituted phenoxy compounds did not

Table 5.6. Rooting responses of maturing leaf discs to low concentrations of IAA.

IAA concentration	Mean number of roots per disc: continuous light (250 fc)	Continuous dark
0	0.0 a	0.0 a
5×10^{-8} M	.1 a	.7 a
1×10^{-7} M	0.0 a	1.6 a,b
1×10^{-6} M	4.1 b,c	6.4 c

Means followed by same letter in a column not different at 5% level.

Mean LPI_3 = 3.4

Table 5.7. Effect of IAA concentration and duration of treatment on rooting.

IAA concentration	Duration on IAA medium (days)*	Duration on basal medium (days)	Mean number of roots per disc	
5×10^{-6} M	2	5	.4	b
	3	4	4.3	c
	4	3	10.1	a,d
	5	2	12.0	a,d
	7	0	8.3	a
1×10^{-5} M	2	5	.7	b
	3	4	6.1	c
	4	3	14.5	d
	5	2	11.5	a,d
	7	0	14.0	d
5×10^{-5} M	2	5	12.3	a,d
	3	4	21.3	e
	4	3	29.2	e,f
	5	2	30.4	f
	7	0	26.4	e,f

* Discs were cultured on IAA media for varying times and then subcultured onto basal or non-inductive media for the remaining time period. Total culture time was 7 days.

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.5

Figure 5.2. IAA effects on root distribution within tomato leaf discs cultured *in vitro*.

A. Percent distribution of presumptive (hatched bars) and emergent roots (solid bars) after 7 days on different media containing different IAA concentrations. Mean LPI_3 = 3.5

B. Percent distribution of presumptive and emergent roots at different times on a medium containing 5×10^{-5} M IAA. Mean LPI_3 = 3.2

MR - midrib; SV - secondary vein; MV - minor vein.

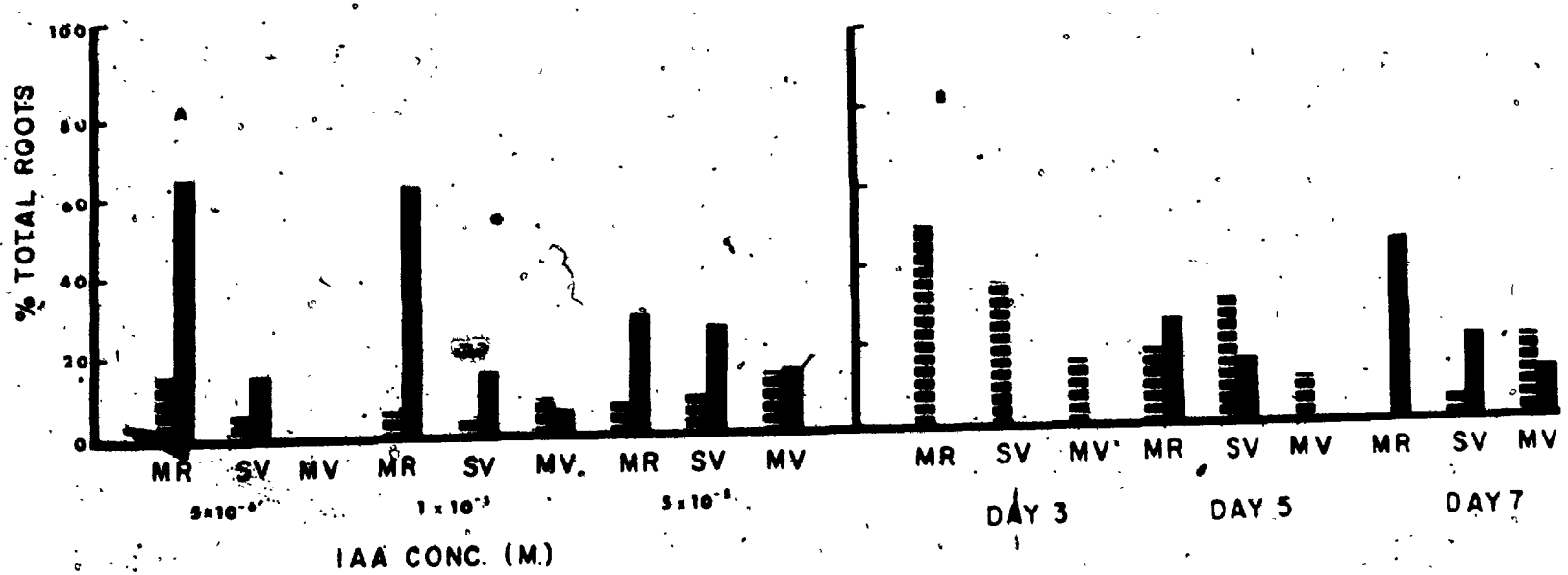

% TOTAL ROOTS
100
80
60
40
20
0
MR SV MV MR SV MV. MR SV MV MR SV MV MR SV MV MR SV MV MR SV MV
5x10⁻⁶
1 x 10⁻⁵
5 x 10⁻⁵
IAA CONC. (M.)
DAY 3
DAY 5
DAY 7

Table 5.8. Modifications of the primary vascular patterns of emerging root primordia and their subsequent growth.

Treatment and Duration* IAA (5×10^{-6} M)	basal medium	Total no. of roots per sample**	Initial root vascular pattern Diarch	Triarch	Tetrarch	Mean root length (mm)
3 days	4 days	55	91%	9%	0%	19.2 a
5 days	2 days	50	82%	18%	0%	13.8 b
7 days	--	69	53.6%	43.5%	2.9%	1.5 c

*Leaf discs were cultured on an inductive (IAA) medium for various time periods (i.e. 3, 5 or 7 days) and subsequently subcultured onto basal medium for a total of seven days.

**Sampled roots originated from the mid rib and secondary veins only. Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.5.

stimulate root formation appreciably and consistently inhibited IAA-induced rooting (Table 5.9). Histological abberations produced by the substituted phenoxy acids on IAA-induced rooting included disruption and callusing of the apical meristem of the root primordia and apparent inhibition of xylem element formation into the primordia although Casparian strip formation - which is normally observed a short time after xylem vascularisation in IAA-induced roots - was apparent.

One day pulses of IAA during the first four days of culture revealed a slightly greater sensitivity during the 48 hour to 72 hour period when evaluated after 7 or 14 days (Table 5.10). In vitro "aging" of the leaf discs on the basal medium for periods up to 10 days followed by the standard 7 day period on IAA medium revealed a similar increase in sensitivity by the second day followed by a progressive loss of regeneration capacity (Table 5.11).

The possibility that this loss of organogenic ability was due to specific endogenous physiological factors (and hence reversible) as opposed to irreversible genetic change, was explored. Leaf discs aged 8 days on basal medium were placed on IAA medium to which various nutritional and hormonal factors were added. The following factors were tested at a final concentration of 10 mg/l and found to be ineffective: catechol, resorcinol, coumarin, glutamine, phenylalanine, yeast RNA, yeast hydrolysate, yeast extract, kinetin and various vitamin combinations.

Table 5.9. Effects of different substituted phenoxy acids on root regeneration from cultured tomato leaf discs.

Auxin treatment	Mean number of roots per disc	
o-chlorophenoxyacetic acid (1×10^{-6} M)	0.0	a
o-chlorophenoxyacetic acid + IAA (1×10^{-5} M)	8.6	d
p-chlorophenoxyacetic acid (1×10^{-6} M)	0.0	a
p-chlorophenoxyacetic acid + IAA	1.6	a,b
2,4,5-trichlorophenoxyacetic acid (1×10^{-6} M)	.7	a
2,4,5-trichlorophenoxyacetic acid + IAA	1.8	a,b
2,4,5-trichlorophenoxypropionic acid (1×10^{-6} M)	3.1	b
2,4,5-trichlorophenoxypropionic acid + IAA	7.4	c
2,4-dichlorophenoxyacetic acid (1×10^{-6} M)	.6	a
2,4-dichlorophenoxyacetic acid + IAA	.6	a
IAA	13.3	e

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.6

Table 5.10. Changing sensitivity of cultured leaf discs to exogenous IAA applications.

Time of IAA pulse treatment (5×10^{-5} M)	Mean number of roots per disc after 7 days	After 14 days	
0 - 24 hrs.	.3	.6	a
24 - 48 hrs.	1.5	1.9	c
48 - 72 hrs.	.4	.6	a
72 - 96 hrs.	0.0	0.0	b

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.5

Table 5.11. *In vitro* aging and loss of regeneration potential.

Duration on non-inductive medium (days)	After 7 days on inductive medium* Mean number of roots per disc		Mean fresh wt. (mg)
0	9.6	a	22.0
1	11.1	a	27.5
2	12.8	a	37.8
3	10.4	a	38.1
4	5.9	b	33.6
6	2.0	b	33.8
8	.3	c	27.5
10	.1	c	27.3

* Leaf discs were cultured on the non-inductive or basal medium for the times indicated and subsequently transferred to an inductive medium (5×10^{-6} M IAA) for a seven day period.

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 4.4

However, 10% coconut milk significantly (at the 5% level) stimulated rooting in the aged discs in the presence of IAA although a large number of the primordia were derived from the callusing edges of the discs. High levels of IAA and IBA were capable of significantly increasing the regenerative response of the in vitro aged discs to a level comparable to low levels (approximately 1 to 5 x 10^{-6} M) of IAA supplied to freshly prepared discs (Table 5.12). Attempts to reverse or at least slow down the in vitro aging process leading to a progressive loss of organogenic capacity by culturing the discs on media containing cysteine, ascorbic acid, PCIB, or TIBA proved unsuccessful. GA_3, which is capable of retarding senescence in detached leaf tissue of such species as Taraxacum officinale, Tropaeolum majus, Rumex crispus and Rumex obtusifolius (Beevers, 1966; Goldthwaite and Laetsch, 1968), had no retarding influence on the loss of organogenic potential by cultured tomato leaf discs. Subsequent work indicated that wounding was essential to renewed regeneration provided that discs had previously formed one or two roots (Table 5.13).

5.4 Histochemical Aspects of Induced Root Formation

5.4.1 General Histological Changes

By twelve hours after culture initiation, nuclei and nucleoli had increased in volume on both the inductive and non-inductive media (Figures 5.3A, B, C and D). In general, the

Table 5.12. Effects of different auxins on root regeneration from *in vitro* aged tomato leaf discs.

Auxin	concentration*	Mean number of roots per disc	
IAA	1×10^{-6} M	.1	a,b
	1×10^{-4} M	6.3	f
IBA	1×10^{-6} M	.2	a,b
	1×10^{-4} M	4.6	e
NAA	1×10^{-6} M	.2	a,b
	1×10^{-4} M	2.8	c,d,e
2,4-D	1×10^{-6} M	1.7	c,d
	1×10^{-4} M	0.0	a
IAA + IBA	1×10^{-6} M + 1×10^{-6} M	.3	a,b
IAA + NAA	1×10^{-6} M + 1×10^{-6} M	.4	a,b
IAA + 2,4-D	1×10^{-6} M + 1×10^{-6} M	1.4	b,c

* Leaf discs were aged *in vitro* for eight days on basal medium prior to sub-culturing onto the different auxin media.

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.8

Table 5.13. Effects of wounding and kinetin on regeneration of *in vitro* aged leaf discs.

Treatment*		Mean number roots per disc	
days 0 to 8	days 8 to 15		
(rooted) basal	IAA (5×10^{-6} M)	5.1	a
(rooted) basal	IAA + Kn (1×10^{-8} M)	2.6	b
(unrooted) IAA	IAA	2.5	b
(unrooted) basal	IAA + Kn	1.1	c

* 10 mm discs which rooted spontaneously during the eight days on basal medium (designated 'basal, rooted') were cut into two 5 mm discs and subsequently cultured onto an inductive medium (with or without kinetin at 1×10^{-8} M) for 7 days (i.e. days 8 to 15). Failure to severely wound the *in vitro* aged tissue (rooted or unrooted) lead to no regeneration response when subsequently placed on an inductive medium.

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.7

Figure 5.3. Nuclear and nucleolar volume changes in three different tissues of tomato leaf discs cultured on inductive or non-inductive media.

A. Nuclear volume changes on inductive medium.

B. Nuclear volume changes on non-inductive medium.

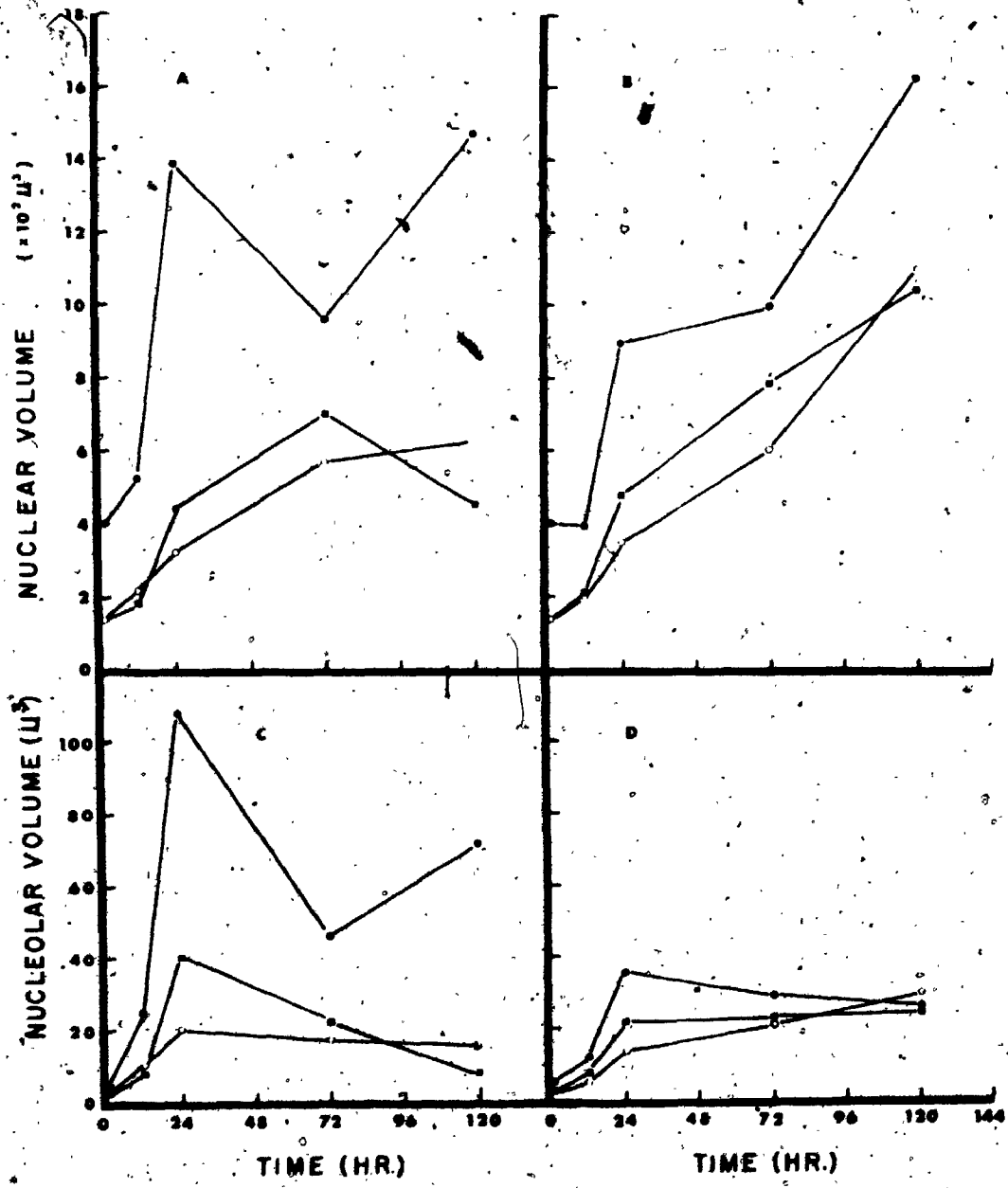
C. Nucleolar volume changes on inductive medium.

D. Nucleolar volume changes on non-inductive medium.

Midrib cortical parenchyma, closed circles.

Spongy mesophyll parenchyma, open circles.

Minor vein sheath parenchyma, closed squares.


A
B
C
D
NUCLEAR VOLUME (×10³ μ³)
NUCLEOLAR VOLUME (μ³)
0
2
4
6
8
10
12
14
16
18
0
20
40
60
80
100
0
24
48
72
96
120
144
TIME (HR.)
TIME (HR.)

nuclei of the 3 different tissues studied continued to increase in volume during the initial 24 to 120 hours regardless of the medium. The nucleoli appeared slightly vacuolated by 12 hours. Nucleolar volume increases were most pronounced during the first 24 hours in the leaf tissues cultured on IAA medium (Plate 5:1;3,4 and 5) with volume increases recorded up to 27 x for the cortical parenchyma of the midrib (Fig. 5.3C).

After 24 hours on the inductive medium, numerous cell divisions were evident in the following tissues:

i) xylem and phloem parenchyma - anticlinal divisions with some periclinal figures;

ii) cortical parenchyma - anticlinal divisions with most mitotic figures oriented transversely to the long plane of the veins;

iii) sheath parenchyma of the minor veins - anticlinal with some periclinal figures.

The following cells and tissues were essentially non-mitotic:

i) palisade and spongy mesophyll

ii) mature sieve tubes and xylem elements or tracheids; and,

iii) epidermal cells (including the stomata and hair cells).

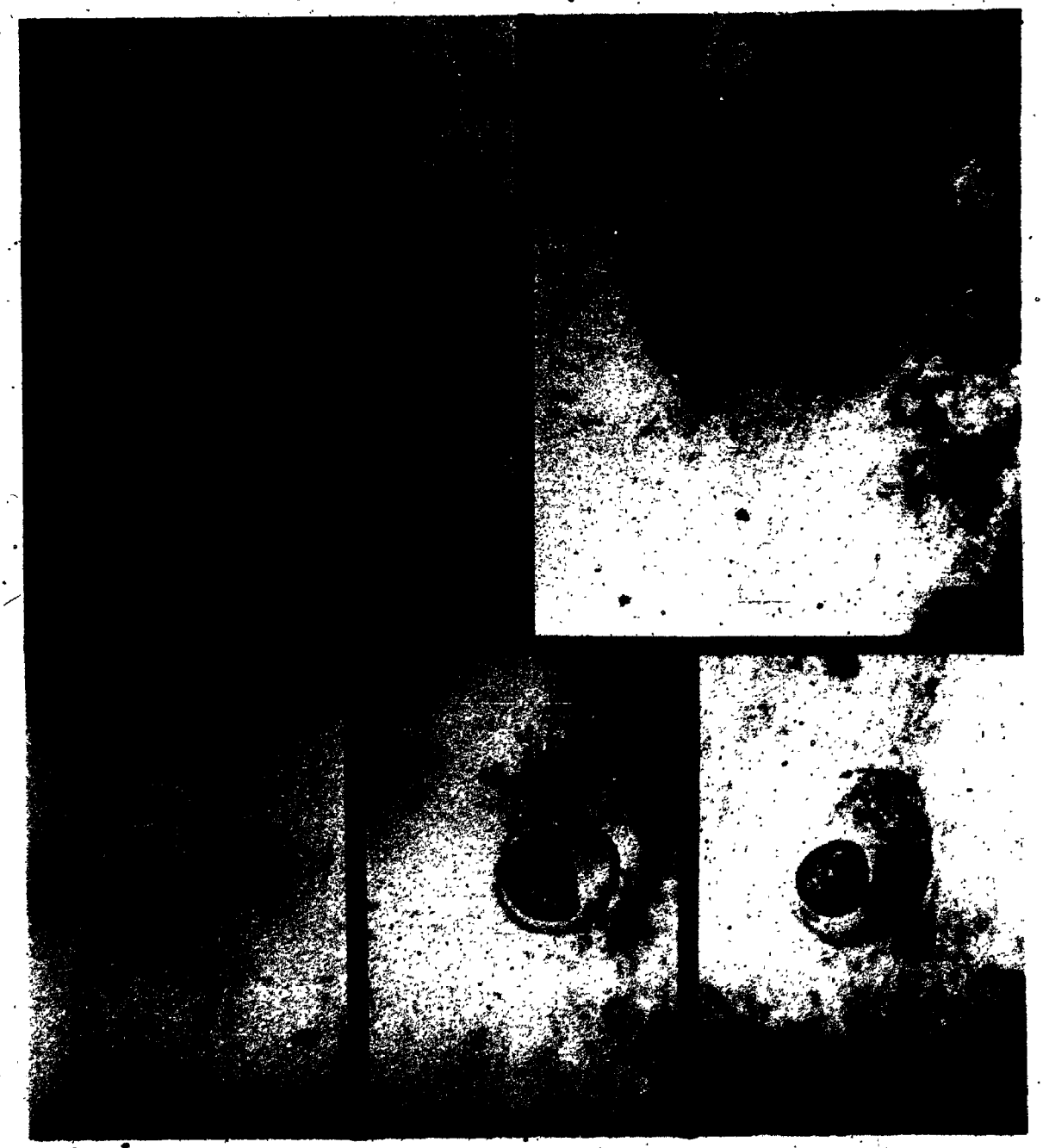
Similar mitotic responses were observed in leaf discs cultured on the basal medium although to a lesser degree.

Data for nuclear volume changes and observation of metaphase plate diameters of the major vein cortical parenchyma and minor vein sheath parenchyma supported the view that the

Plate 5.1. Cytological aspects of IAA treated leaf discs cultured *in vitro*.

1. Diploid metaphase figure in a FPA cleared minor vein sheath parenchyma cell after 36 hours on an IAA medium. (x 1600)
2. Polyploid metaphase figure in a FPA cleared midrib cortical tissue after 36 hours on an IAA medium. (x 1600)

3, 4 and 5. Large and highly vacuolated nucleoli in FPA cleared midrib cortical tissue after 24 hours on an IAA medium. (x 1280)



two tissues initially possess predominantly polyploid (i.e. tetraploid) and diploid nuclei respectively (Plate 5.1 and Fig. 5.3A).

After 36 hours, small meristematic centers were observed forming from the external phloem parenchyma cells of the primary and secondary veins. Very short vessel elements were also observed to be differentiated from the xylem parenchyma located abaxially and in continuity with the youngest pre-existing xylem strands.

By 48 hours, the leaf discs on the inductive medium differentiated small primordia from their primary and secondary veins. It is apparent from Plate 5.6;2 that the root cap is derived from the overlying endodermal cells which initially divide anticlinally followed by one periclinal division step leading to a 2-cell layered cap stage.

The sheath parenchyma of the minor veins appeared to respond in a variety of ways when exposed to inductive conditions. These cells exhibited the following developmental patterns in an unpredictable manner:

i) starch sheath formation
ii) Casparian strip formation
iii) wound vessel member (WVM; Roberts, 1971) formation
iv) root primordium formation

Primordia on minor veins appeared to be derived solely from dedifferentiated sheath parenchyma derivatives (Plates 5.2; 5.3).

On the basal medium, considerably less mitotic activity

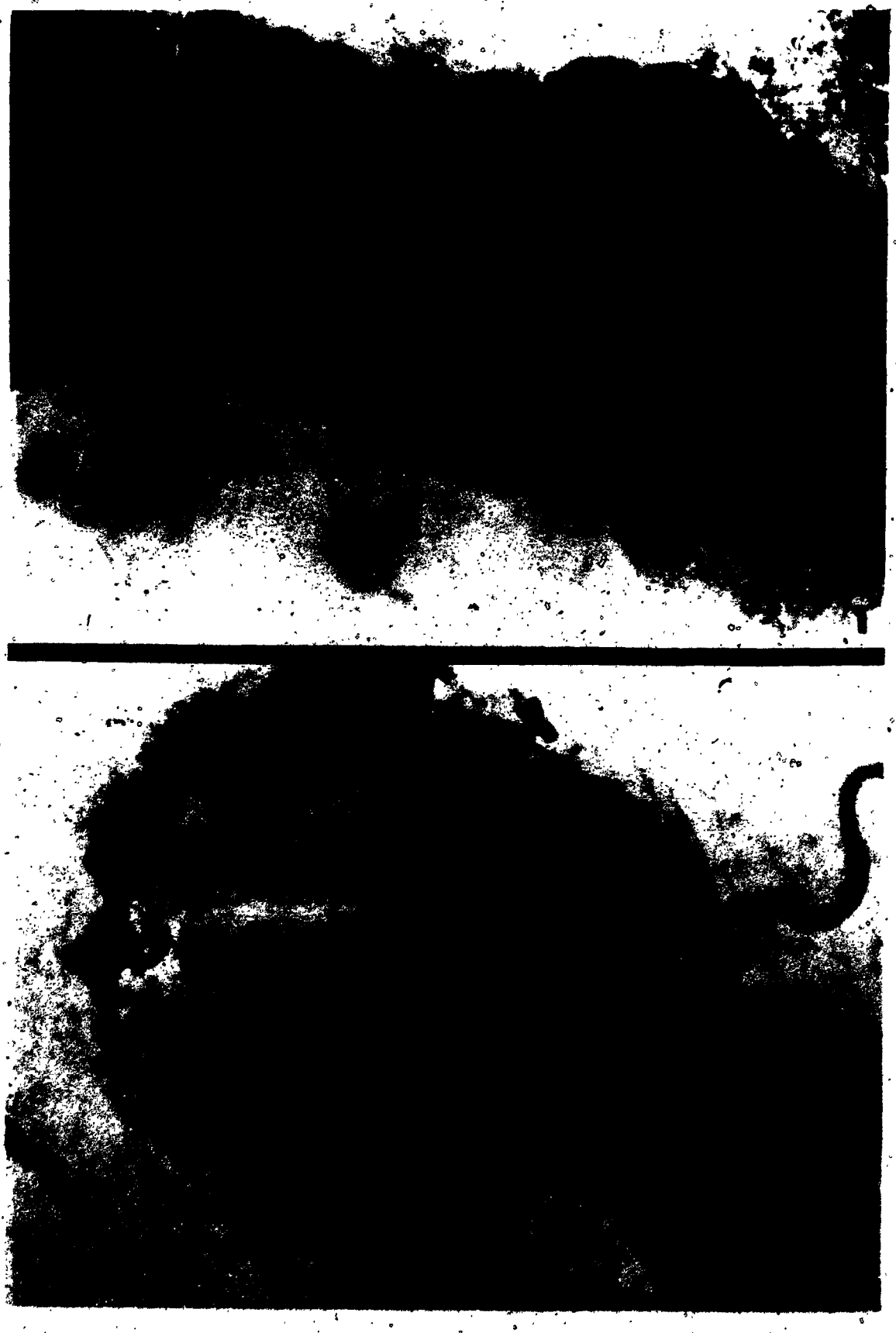
Plate 5.2. Early stages in root primordia development from minor veins.

1. Recent periclinal divisions in FPA cleared minor vein sheath parenchyma adjacent to external phloem after 36 hours on an IAA medium. (x 800)
2. Meristemoid development from derivatives of the minor vein sheath parenchyma after 72 hours on an IAA medium. (x 200)


1

Plate 5.3. Late stages in root primordia development from minor veins.

1. Well developed meristemoidal regions derived from minor vein sheath parenchyma after 72 hours on an IAA medium. (x 800)
2. Three roots with xylem vascularization developed from a minor vein after 120 hours on an IAA medium. (NaOH cleared disc; x 256)



was apparent in the vascular parenchyma. The sheath parenchyma of the minor veins were capable of all the above developmental patterns except root primordium formation. In fact, no meristematic cell clumps were evident although mitotic activity was apparent.

From 60 to 72 hours, the differentiating primordia formed procambial strands which, in turn, differentiated into the root vasculature. Xylem maturation began initially from xylem parenchyma adjacent to the youngest xylem elements and consisted of reticulate to scalariform elements being laid down at right angles to the existing xylem.

Unlike the vasulature of spontaneously formed adventitious roots in tomato stem and leaf cuttings which (initially) is normally diarch (Plate 5.4), the induced primordia often possessed triarch (and occasionally tetrarch) xylem patterns. As pointed out in section 5.3.1.3, this xylem patterning could be related to duration of auxin application. While the initial xylem elements within the developing root mature acropetally, subsequent additional xylem development may be basipetal. Discontinuous xylem maturation and extensively pitted vessel elements were also in evidence (Plate 5.5).

Casparian strip formation within the developing root primordia became evident at a later stage than the xylem vascularization and was continuous with the newly formed Casparian strip in the surrounding midrib or secondary vein endodermis. It matured in an acropetal direction and

Plate 5.4. Normal ontogeny of adventitious roots in intact tomato seedling hypocotyls.

1. Early stage in a NaOH cleared seedling showing a developing meristematic area (arrow) which causes the overlying endodermis to bulge into the surrounding cortical tissue (x 307)
2. Initial xylem vascularization stage with the short vessel elements forming at right angles to the hypocotyl vasculature (arrows). (x 307)
3. Well developed root vasculature with typical diarch pattern. (x 307)

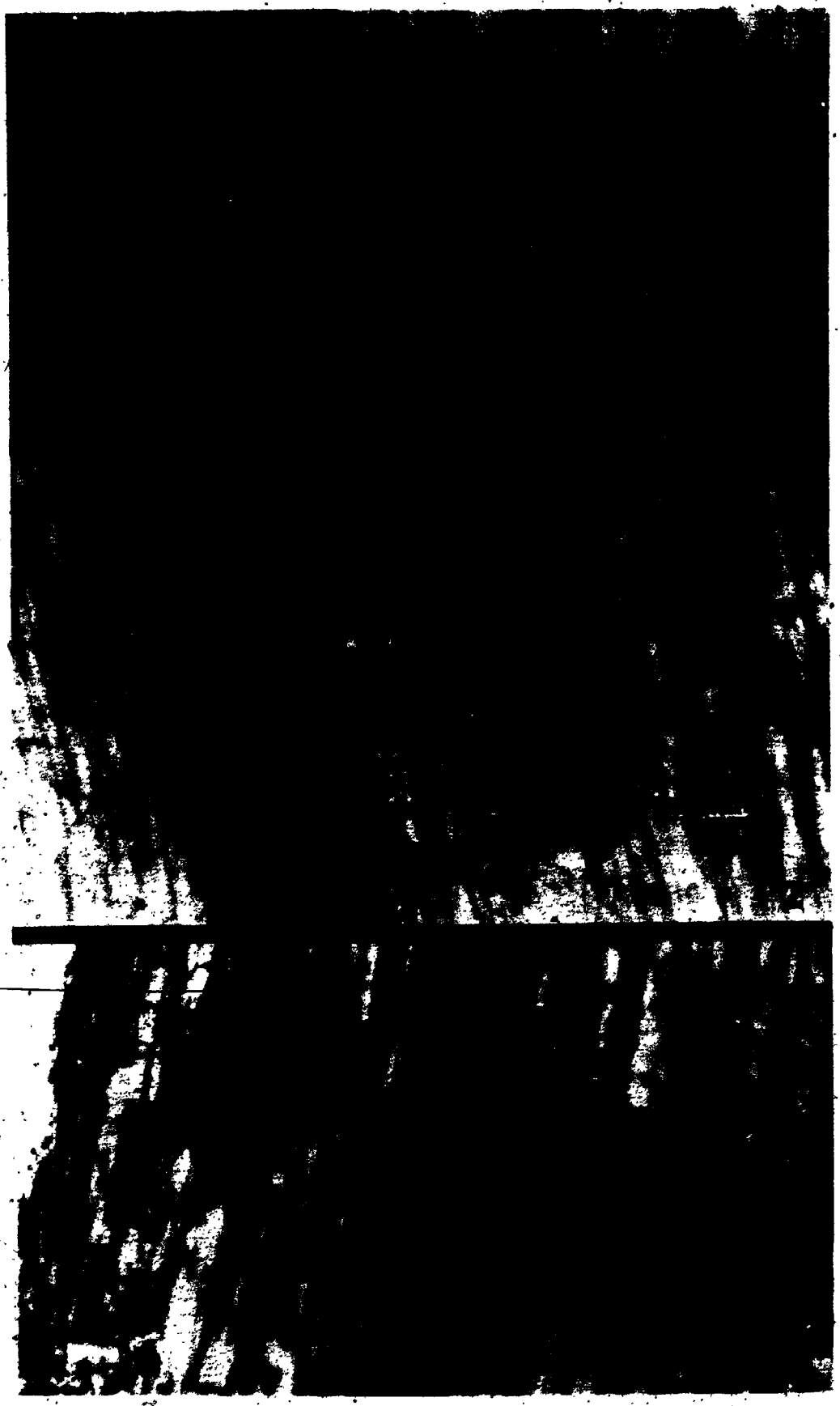


Plate 5.5. Primary xylem patterns in roots induced from tomato leaf discs cultured on an IAA medium.

1. NaOH cleared root tip region showing the well developed Casparian strips (CS) of the endodermis and triarch xylem pattern (arrow). (x 256)
2. Anomolous maturation and development of the primary root xylem. Note the isolated vessel elements (arrows). (x 200)


1
2

usually approached the root primordium apex more closely than the xylem elements (Plate 5.5;1). The phloem appeared to precede the xylem during root primordia development and also matured in an acropetal direction.

On the non-inductive medium, extraxylary fibers associated with the external phloem of the major veins became apparent during this period (i.e. 60-72 hours). Casparian strip formation also became evident in the midrib and secondary vein endodermal cells and in minor vein sheath parenchyma in an apparently random manner.

By 120 hours, a number of roots derived from the primary and secondary veins had broken through the epidermal leaf tissue. At this stage, the roots had a well defined root apex and internal differentiation had occurred to delineate the normal tissues of a mature root. Small vascularized roots (approximately one half the diameter of roots from the midrib or secondary veins) were apparent along the minor veins (Plate 5.3;1).

While cell division was still evident, its distribution now appeared restricted to the developing root tips as well as the midrib and 2° vein cortical parenchyma. Unlike the initial figures, however, the mitotic activity within the cortical parenchyma appeared to be more random in its orientation with high activity apparent at the basal and apical ends of the major veins.

By seven days, a well developed vascular cambium was evident in the midrib and secondary veins of both the induced

and non-induced leaf material with numerous external roots visible on the induced discs.

5.3.2.2 Nucleic Acids

Initially, the leaf discs exhibited no cytoplasmic staining with Azure B. Nuclei were small and light greenish-blue while isolated heterochromatin clumps around the inner periphery of the nuclei stained dark blue. Nucleoli were small and pale greenish-blue. Chloroplasts (especially in the palisade mesophyll) appeared pale blue while the lignified vessels and tracheids stained a green colour. TCA hydrolysis removed all nuclear and chloroplast staining without affecting the xylem colouration.

After 24 hours on the inductive medium, the cortical parenchyma and external phloem parenchyma of the major veins possessed large blue, highly vacuolated nucleoli. Pale blue cytoplasmic staining also became evident in these tissues at this stage. The chloroplasts maintained a pale blue colouration. On the non-inductive medium, similar staining characteristics were observed (Plate 5.6;1).

Green amorphous deposits were observed at the wound sites along the disc edges on both media. They were TCA resistant and were believed to be phenolic in nature. In FPA cleared material, calcium oxalate crystals were also observed in the wound regions. They appeared to be derived from ruptured idioblasts. Similar accumulations of calcium oxalate crystals in wound regions have been reported by

Plate 5.6. Azure B staining for nucleic acids in cultured tomato leaf discs.

1. Cross section of the midrib from a disc cultured for 24 hours on basal medium. Nucleolar and cytoplasmic staining is evident in the vascular parenchyma. (x 480)
2. Paradermal view of a secondary vein after 48 hours on an inductive medium. Two densely staining root primordia are evident. Note the initial biseriate cell layer of endodermal cell derivatives which will form the root cap. (x 240)

Bloch (1941). By 36 to 48 hours, small meristematic centres were apparent in the external phloem parenchyma. The cells composing these centres were characteristically small with large nuclei and nucleoli and intensely stained from RNA.

After 48 hours, the developing primordia were intensely stained for nucleic acids (Plate 5,6;2). Similar intense cytoplasmic RNA staining was observed in young sieve tubes and their companion cells.

By 84 to 96 hours on the inductive medium, the vascular parenchyma of the midrib and secondary veins showed considerably reduced nuclear and cytoplasmic staining. Intense cytoplasmic RNA staining was restricted to the developing primordia on the major veins and the meristems of the young roots from the primary and secondary veins. However, on the non-inductive medium, phloem and cortical parenchyma still possessed moderately intense cytoplasmic RNA staining.

By seven days, the nucleic acid staining of the leaf discs on both the inductive and non-inductive media had become restricted to the nuclei. The root apices were the only areas to exhibit intense cytoplasmic RNA staining.

5.3.2.3 Total Proteins

Initial protein staining was restricted to the nuclei and small nucleoli as well as the cytoplasm of the sieve tubes and companion cells. The mesophyll chloroplasts also showed moderate staining. By 24 hours, cytoplasmic staining

was evident in the vascular and cortical parenchyma which paralleled the increased cytoplasmic RNA staining. In general, the total protein staining pattern showed similar trends to the RNA staining as to intensity and cell localization in both the induced and non-induced leaf discs (Plates 5.7, 5.8 and 5.9;1). However, small, intensely staining crystalline particles within the cytoplasm of non-meristematic cells became evident by 60 to 72 hours. This particulate staining was quite evident in the non-induced discs and tended to increase both in particle size (from approximated 0.2 u to 1.5 u diameter) as well as distribution (from vascular parenchyma and minor vein sheath parenchyma to cortical parenchyma and finally, mesophyll parenchyma). Further observations on their staining characteristics are presented below.

5.3.2.4 Basic Protein

Freshly cut leaf discs showed intense basic protein staining of the palisade mesophyll chloroplasts as well as the midrib parenchyma. Nuclear staining was also evident. Both deamination and acetylation reduced the staining in the chloroplasts and nuclei. By 12 hours, sieve tubes and companion cells (especially in the external phloem) showed considerable nuclear and cytoplasmic staining in both induced and non-induced leaf discs. Both deamination and acetylation were able to reduce considerably the staining intensity.

At 24 hours, chloroplast staining was reduced in both

Plate 5.7. Mercuric bromphenol blue staining for total proteins in the cultured tomato leaf discs.

1. Cross-sectional view of the midrib of an induced leaf disc after 60 hours. Note the large and darkly staining nuclei of the external phloem parenchyma. (x 120)
2. Cross-sectional view of a region of the external phloem in an induced leaf disc after 72 hours. Note the small and intensely staining phloem parenchyma derivatives adjacent to the external phloem strands. (x 480)

Plate 5.8. Mercuric bromphenol blue staining for total proteins in cultured tomato leaf discs.

1. Cross-sectional view of the midrib of an induced leaf disc after 84 hours. Note the intense nuclear and cytoplasmic staining of the root primordium in comparison with the highly vascuolated mesophyll and cortical parenchyma. (x 120)
2. Paradermal view of the midrib of an induced leaf disc after 96 hours. The intense cytoplasmic staining is primarily localized in the developing root meristem. (x 120)

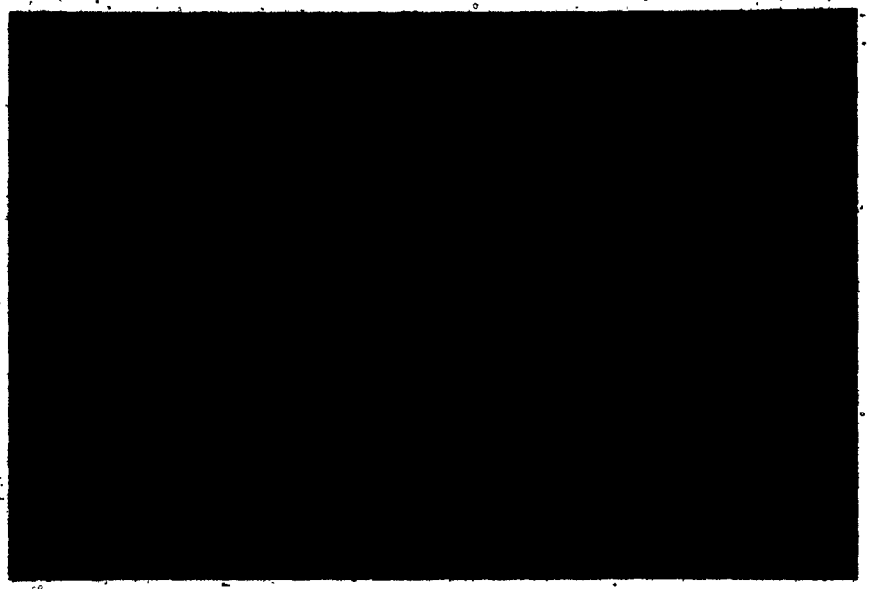


Plate 5.9. Protein staining in tomato leaf discs cultured on a basal medium.

1. Cross-sectional view of the midrib of a leaf disc cultured on basal medium for 48 hours and subsequently stained for total proteins. Note the absence of any densely staining meristematic centers. (x 240)
2. Cross-sectional view of the mesophyll region after 168 hours. The protein microbodies (i.e. the small blue "dots") are intensely stained for basic proteins and are quite abundant in the non-meristematic regions of both the induced and non-induced leaf discs. (x 480)

types of leaf discs while intense staining was observed in the nuclei and nucleoli of the vascular and cortical parenchyma of the primary and secondary veins as well as the sheath parenchyma of the tertiary veins. Faint cytoplasmic staining was also observed in these tissues.

By 48 hours, intense cytoplasmic staining was apparent at the sites of the root primordia. This staining pattern was unaffected by deamination or acetylation. Furthermore, the omission of nucleic acid hydrolysis by picric acid only slightly reduced the staining intensity, regardless of whether it was subsequently deaminated or not.

In the non-induced discs, the omission of picric acid hydrolysis had no effect on chloroplast or cytoplasmic staining of the sieve tubes or companion cells. However, subsequent deamination abolished the chloroplast as well as cytoplasmic staining of the phloem cells.

By 60 hours, small intensely staining particles of a crystalline nature became evident in both types of discs where they were localized in the non-meristematic regions of the tertiary vein sheath parenchyma and the vascular parenchyma of the primary and secondary veins. In the non-induced discs, they were also evident in some of the surrounding palisade cells. These particles did not require prior nucleic acid hydrolysis and the staining reaction was reduced considerably by deamination and acetylation. In both size and pattern of distribution, these particles closely resembled the particles giving an intense total

protein stain (Plate 5.9;2).

From 72 to 168 hours, the root primordia showed intense nuclear and cytoplasmic staining although the omission of prior nucleic acid hydrolysis or deamination only slightly reduced the staining intensity.

In the non-induced discs, the staining of the phloem cell cytoplasm and cytoplasmic particles was abolished by deamination.

Further attempts at the characterization of the protein microbodies or crystals using leaf discs aged for 8 or 10 days on basal medium was carried out at the light and EM levels (Plates 5.10 to 5.13). A summary of the results is presented in Table 5.14.

5.3.2.5 Starch

Initially, the freshly cut leaf discs possessed a few, small PAS or PAAS positive granules in the plastids of the starch sheath surrounding the midrib and the guard cells. By 12 hours small, irregular starch grains were apparent under both culture conditions in the sieve tube plastids and the chloroplasts present within the midrib and sheath parenchyma of the major and minor veins. Faint nuclear staining by the PAAS reaction was also evident at this stage.

By 24 hours, small starch grains were apparent within the cortical parenchyma chloroplasts surrounding the primary and secondary veins as well as the adjacent mesophyll. The

Plate 5.10. Histochemical characteristics of protein microbodies in tomato leaf discs aged *in vitro* for eight days.

1. Peroxidatic activity localized in protein microbodies (PMB; arrows). Fresh tissue preparation. (x 800)
2. Acid phosphatase localized in vascular parenchyma. Some particulate staining is evident (arrows). However, the staining was restricted to specific cells and not to the protein microbodies. (x 800)

2

Plate 5.11. Prominent organelles of fresh and *in vitro* aged tomato leaf tissue.

1. Fresh mesophyll tissue preparation from a mature leaflet showing a well developed chloroplast (C) with small plastoglobuli and an interphase nucleus (N). Both organelles are appressed to the cell wall (CW) of a highly vacuolated cell. (approximately x 25,000)
2. Protein microbody (PMB) from *in vitro* aged leaf tissue showing the surrounding single membrane as well as the 100 Å lattice structure. Note the irregular appearance of two sides of the crystal (arrows) which may indicate sites of protein incorporation. (x 159,120)

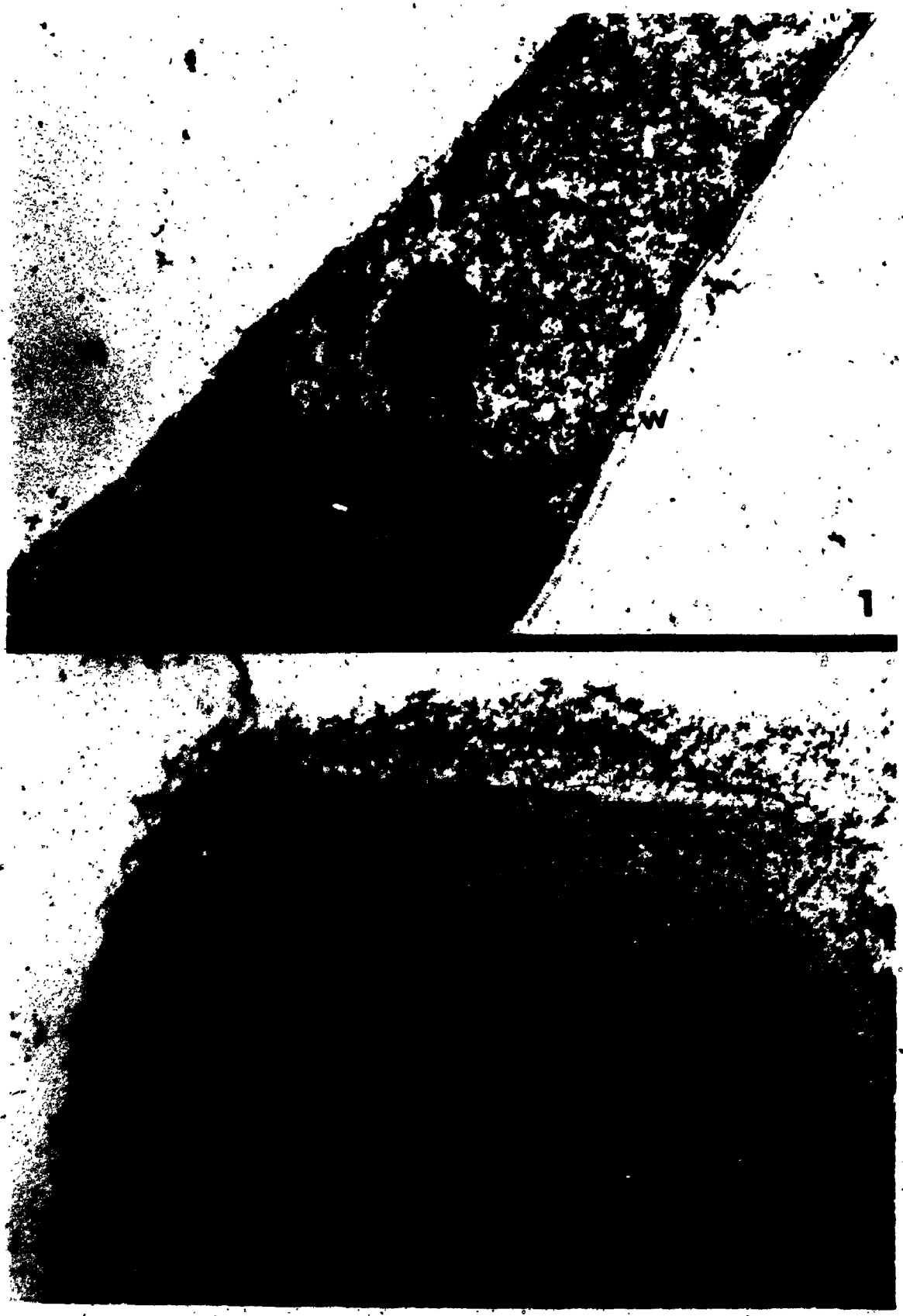

W
1

Plate 5.12. Subcellular aspects of *in vitro* aged tomato leaf tissue. The nucleus (N) displays an irregular margin with very little evidence of endoplasmic reticulum (ER). Chloroplasts (C) possess abundant and large plastoglobuli. Mitochondria (M), a dictyosome (D) and a protein microbody (PMB) are also evident in these highly vacuolated mesophyll cells. (x 22,000)

Plate 5.13. Further subcellular aspects of *in vitro* aged tomato leaf tissue. The protein microbody (PMB) displays a very prominent lattice pattern of approximately 100 Å wide repeating units. Associated with the microbody are chloroplasts (C), a mitochondrion (M) and a multivesicular body (MVB). (x 57,920)

Table 5.14. Characteristics of protein microbodies from tomato leaf discs cultured in vitro.

1. Size range: approximately 0.5 to 1.5 μ (after 8 days on basal medium).
2. Distribution: non-meristematic leaf tissue aged at least 60 hours in vitro.
3. Lattice spacing: approximately 100Å.
4. Birefringence: nil
5. General staining characteristics*:

	++	Remarks
a) Total protein		
i) after .02 N HCL extraction 30 min. at 2°C	++	no effect on crystal morphology
b) Basic protein		
i) after deamination	+	
ii) after acetylation	+	
c) Nucleic acids	0	
i) after TCA hydrolysis	0	
d) Insoluble polysaccharides (PAS)	0	
e) Lipids (Sudan IV)	0	

Table 5.14 (cont'd.)

6. Specific enzyme staining*:	
a) Acid phosphatase	0
i) minus sodium glycerophosphate	0
ii) complete mixture: repeated freezing and thawing (3x)	0
b) Peroxidase	++
i) minus H_2O_2	0
ii) complete mixture: tissue heated to 90°C for five minutes	0

*

++ Intense staining

+ very weak staining

0 no staining

non-induced discs also showed starch accumulations within the xylem parenchyma.

At 36 hours, starch grains were present within the epidermis (especially the stomatal guard cells) and a more wide-spread distribution of grains within the mesophyll tissue was evident. These grains appeared larger in the non-induced discs. In the induced discs, the small meristematic cells derived from the external phloem parenchyma were devoid of starch grains although heavy accumulations of starch were evident in the surrounding tissue. Similar observations were made at 48 to 72 hours in the induced discs for meristemoidal areas from the major and minor veins (Plate 5.14;1). The non-induced discs appeared to have larger starch grains, especially in their vascular parenchyma. In general, starch was distributed throughout the tissues of the non-induced discs (Plate 5.14;2).

By 84 hours, some of the developing primordia from the primary and secondary veins possessed starch grains in the root cap cells prior to their emergence. Differentiating phloem was observed in the basal region of these developing primordia.

The starch grains in the root cap cells were of a considerably different morphology than the grains in the surrounding mesophyll. The cap cell starch occurred in multigrained amyloplasts and appeared rounded, pitted and uniform in size in any particular cap cell layer while the mesophyll starch grains were localized within the chloroplasts and appeared angular and heterogenous in size (Plate 5.15;1 and 2).

At 7 days, the induced discs possessed no starch grains in the well-developed vascular cambium which was present in

Plate 5.14. PAAS staining of starch grains in cultured tomato leaf discs.

1. Cross-sectional view of a meristemoidal region forming from the minor vein sheath parenchyma derivatives of a leaf disc cultured on an inductive medium for 72 hours. Note the starch accumulations in the surrounding mesophyll and their absence from the meristemoidal region. Similar accumulations are not as pronounced in the non-meristematic regions of the induced discs. (x 480)
2. Cross-sectional view of a minor vein from a leaf disc cultured on an inductive medium for 72 hours. The starch accumulations are typically spread throughout the mesophyll tissues with no preferentially localized deposits as observed in the induced leaf discs. (x 480)

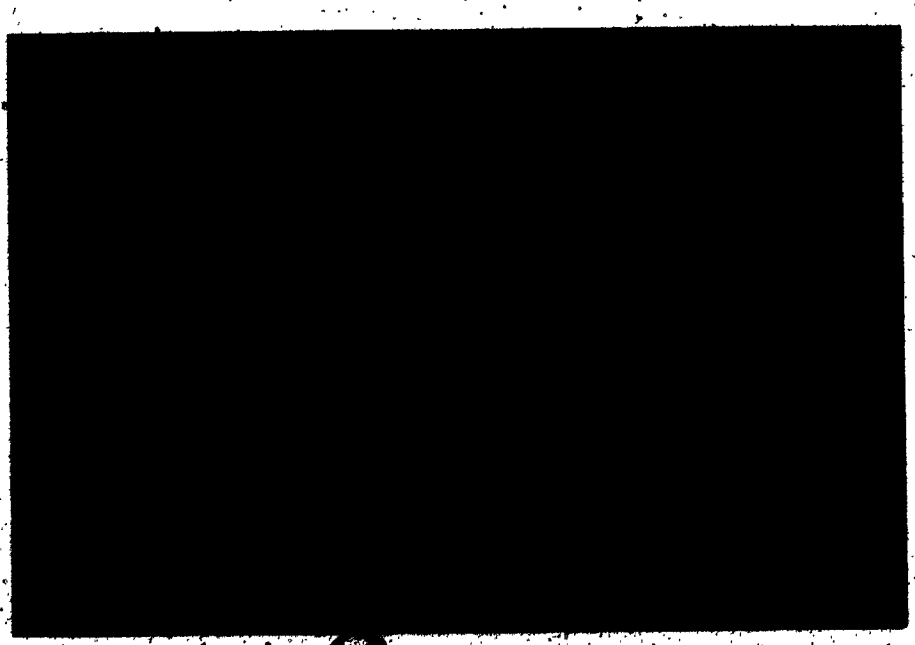


Plate 5.15. PAAS staining of starch grains in cultured tomato leaf discs.

1. Cross-sectional view of an induced leaf disc at 120 hours showing localized starch deposits within the cap cells of developing root primordium as well as the surrounding mesophyll. (x 120)
2. Similar to 1. A close-up view of the root cap cells containing starch grains which are morphologically distinct from the grains within the adjacent mesophyll cells. (x 480)

the primary and secondary veins. Starch grains were confined to the xylem parenchyma, the cortical parenchyma, mesophyll and root cap cells.

On the non-inductive medium, however, the discs possessed abundant starch grains in the vascular parenchyma while the mesophyll was essentially without any starch grains. The apparent loss of starch from the mesophyll tissue was evident as early as 84 hours after culturing the discs.

5.4 Discussion

A comprehensive study of the various tissue culture parameters has substantiated the hypothesis that 5 mm diameter tomato leaf discs possess a total dependence on external stimuli (such as organic and inorganic nutrients and hormones of the auxin class) in order for root regeneration to occur. When discs from maturing leaves (LPI_3 3.0 - 4.0) are cultured in continuous darkness, their sensitivity to exogenous IAA is comparable to the Avena coleoptile segment elongation bioassay with the lowest detectable response level in the range of 0.005 to .010 µg IAA/ml of medium. However, the various substituted phenoxy acid auxins and their structurally related, inactive members displayed little or no ability to induce root regeneration. The reasons for this lack of organogenic induction are unknown but the observations are compatible with similar ones made on rooting effects of 2,4-D from tomato leaf cuttings (see Chapter 4). The observations may

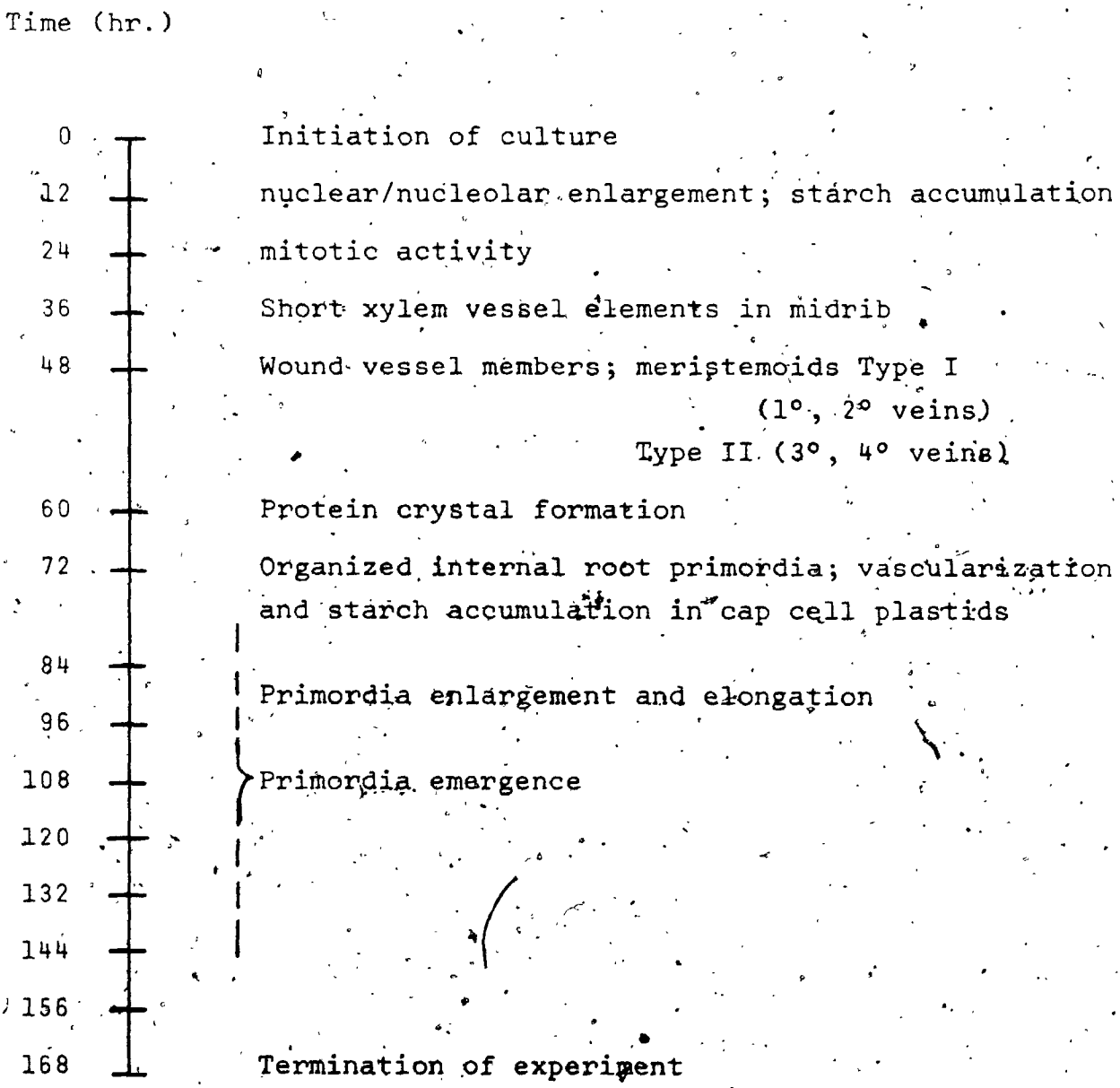
indicate: i) a high degree of structural specificity in terms of auxin type for induction of regeneration; ii) an exceptionally efficient detoxification process which renders molecules such as 2,4-D inactive; or iii) an incapacity to take up the substituted phenoxy acids.

Prolonged culture (i.e. up to 10 days) on a non-inductive medium leads to a progressive decline in organogenic capacity of the leaf tissue when subsequently placed on inductive media. Correlated with this decline were increasing nuclear volumes in non-organ forming and potential organ forming tissues (which may indicate increasing ploidy levels) and declining nuclear and cytoplasmic staining for RNA, total and basic proteins. This in vitro aging phenomena is quite typical for subcultured tissues (e.g. see review by Reinert, 1973) and has been correlated with irreversible genome instability (e.g. polyploid cell formation and possible somatic mutations). However, the loss of capacity for organogenesis in tomato leaf tissue may be reversed (at least partially) by culturing the aged tissues on high auxin media, inductive media supplemented with coconut milk, or a combination of both. Furthermore, the additional factors of wounding and the presence of one or two spontaneously formed roots are beneficial for maintenance and subsequent expression of organogenic capacity in long term cultures. Reinert (1973) has observed similar reversibility in the embryogenic capacity of in vitro aged carrot cell cultures. The loss of the capacity for embryogenesis by the carrot

cells could be reversed by subculturing these cells onto an inductive medium with a high nitrogen level. Consequently, in vitro aging and associated loss of organogenic capacity would appear to encompass both genome alterations and presently unknown changes in endogenous physiological factors.

Tomato leaf tissue, which is essentially mitotically inactive at the LPI_3 used in the histochemical observations (i.e. LPI_3 between 3.0 and 4.0) responds rapidly to culture conditions by undergoing diverse histochemical and developmental changes (summarized in Fig. 5.4). Unfortunately, comparison with similar leaf disc culture systems is impossible as none have been described. In general, the induced and non-induced leaf discs show similar histological and histochemical changes over the 7 day culture period, except in terms of the events which surround root primordium initiation and subsequent development. The auxin induced tomato leaf discs are unique in their ability to give rise to root primordia simultaneously from two different mature tissue types. The initial steps of root primordia induction within primary and secondary veins (type I root primordia) consisted of anticlinal with subsequent periclinal mitotic activity leading to dedifferentiation of the phloem parenchyma. Accompanying the initial mitotic activity, a localized build up of RNA (especially within the cytoplasm) became evident in the small, isodiametrically shaped, phloem parenchyma derivatives. This localized increase in RNA

Figure 5.4. Time sequence of developmental events in cultured tomato leaf discs.


Time (hr.)
0
12
24
36
48
60
72
84
96
108
120
132
144
156
168
Initiation of culture
nuclear/nucleolar enlargement; starch accumulation
mitotic activity
Short xylem vessel elements in midrib
Wound vessel members; meristemoids Type I (1°, 2° veins)
Type II (3°, 4° veins)
Protein crystal formation
Organized internal root primordia; vascularization and starch accumulation in cap cell plastids
Primordia enlargement and elongation
Primordia emergence
Termination of experiment

staining was paralleled by increased total and basic protein staining of the nucleolus and cytoplasm. While acropetal differentiation of the root primordia's vasculature is consistently observed, auxin-induced primordia show abnormal vascular archy and xylem maturation. Both features appear to be related to the duration of exposure to auxin media.

Root primordia induction within the minor veins (type II root primordia) consisted of the development of small meristemoidal areas from derivatives of the bundle sheath parenchyma. Some of these meristemoidal areas eventually develop into small, yet essentially normal, root primordia and subsequently, mature roots. Similar changes in RNA, total and basic protein staining patterns were observed in the type II primordia as were found in the type I. Although these general histochemical changes are similar to those observed in other root regenerating systems with regard to timing and localization of organ formation, specific features of protein histochemistry are unique to the regenerating tomato leaf discs.

The basic proteins change both with regard to location and susceptibility to deamination. Initially, basic protein staining is susceptible to deamination and is restricted to the nucleoli and chloroplasts. However, by 36 to 48 hours, it is apparent that meristematic leaf disc tissue and developing root primordia have a high content of basic protein in both the cytoplasm and nucleus which is only

slightly hindered in its stainability by the high nucleic acid levels present within the same cells. Most of this protein is not susceptible to deamination suggesting that the resistant guanidyl groups of arginine are responsible for the observed staining. While histones probably contribute to the staining patterns observed, it is likely that the majority of nuclear and cytoplasmic basic proteins are ribosomal in origin (Busch and Smetana, 1970).

The basic proteins of the sieve tube and phloem parenchyma cytoplasm, and mesophyll chloroplasts are susceptible to deamination, suggesting a different type of protein. Furthermore, prior nucleic acid hydrolysis had no effect on this staining pattern in both induced and non-induced leaf tissue. Since the chloroplasts, sieve tube and companion cell cytoplasm contain considerable total protein, it is possible that the basic protein staining within these areas is due to the terminal amino groups of the proteins. The modified Van Slyke deamination procedure used in this study would not distinguish the terminal amino groups and the ϵ-amino groups of lysine (Pearse, 1968).

By 60 hours, particulate protein staining within the cytoplasm was observed in non-meristematic, highly vacuolated cells (e.g. mesophyll cells and most of the minor vein sheath parenchyma in leaf discs from the basal medium). These protein particles increased both in size and distribution and took on a more crystalline shape with time.

As outlined in Table 5:14, the protein crystals do not appear to contain any acid phosphatase activity which would have suggested lysosomal functions similar to certain forms of crystal containing lysosomes found in animal tissues. Rather, the presence of high peroxidase activity suggests that these crystals may be peroxisomal in nature (Tolbert, 1971). It should be noted, however, that the histochemical presence of peroxidatic activity detected by the benzidine reaction may be due to the catalase component as no peroxidase enzyme per se has been isolated from them (Tolbert, 1971). Similar crystalline inclusions have been found in the non-meristematic regions of shoot-forming and non-shoot forming tobacco callus tissues (Ross et al., 1973). These inclusions were assumed to be protein bodies on the basis of their configuration. It was suggested that these crystals were a form of storage protein which was subsequently broken down into amino acids and utilized in the synthetic processes of meristemoid formation and development. However, Matsushima and his co-workers (Matsushima, Wada, and Takeuchi, 1969; Matsushima, 1971) had previously described these crystalline microbodies in organogenic and non-organogenic tobacco callus. On the basis of histochemical observations at the electron microscope level, they concluded that the crystals were proteineous and contained high peroxidatic activity of catalase within the crystalloid cores. Similarly, morphological and histochemical evidence at the light and EM levels suggests

that the crystalline protein bodies observed in cultured tomato leaf tissue are probably not relatively inert protein storage bodies; rather, they appear to be enzymatically active microbodies of a peroxisomal nature which may be associated with photorespiratory activity (Tolbert, 1971). Structurally similar crystal-containing bodies have been found in detached tomato leaves (Shumway, Rancour and Ryan, 1970) tobacco leaf cells (Frederick and Newcomb, 1969), virus free leaf cells of Verbesina, Sanvitalia, Zinia, Calendula and Dahlia (Petzold, 1967), pedicels of tobacco and tomato (Jensen and Valdovinos, 1967), cells of Avena coleoptile (Cronshaw, 1965; Thornton and Thimann, 1964) and bud cells of potato tubers during dormancy (Marinos, 1965). It has been suggested that these inclusions may represent a specialized type of microbody characteristic of cells with low metabolic activity (Frederick, Newcomb, Vigil and Wergin, 1968). Their presence in the non-meristematic tissue areas of both induced and non-induced leaf discs and the ability of in vitro aged discs to be experimentally induced to form root primordia suggests that these microbodies play no direct role in organ regeneration. It is presently unknown whether a peroxidatic oxidation of IAA is possible.

Starch grains, which first became evident at 12 hours within vascular parenchyma chloroplasts, continued to accumulate in the leaf tissue. However, by 48 hours, starch was conspicuously absent from the primordial initials.

Rather, it preferentially accumulated in tissue surrounding the developing primordia and subsequently, morphologically distinct starch grains became evident within the cap cells of the developing root. This sequence of starch changes in the vicinity of the developing primordia is in agreement with Borthwick's study (Borthwick, et al., 1937) on IAA stimulated rooting in tomato stem cuttings as well as previous studies on bud initiation in tobacco callus (Thorpe and Murashige, 1968; 1970). Furthermore, it would tend to support the latter's hypothesis that starch plays a definite role in organogenic processes. However, a non-specific accumulation of starch grains was evident in the non-induced discs. Similar starch accumulations have been observed in detached and photosynthesizing leaves and cells cultured in the presence of exogenous carbohydrates where no obvious organogenic events are occurring. As a consequence, a direct relationship between organogenesis and localized starch accumulation would be obscured in terms of gross quantitative analysis. Whether this non-specific starch synthesis would alter the GA_3 induced inhibition of root regeneration as found in leaf cuttings (Chapter 4) will be explored in the following chapter.

CHAPTER 6

TOMATO LEAF DISCS CULTURED IN VITRO: INHIBITION OF ROOT REGENERATION BY GIBBERELLIC ACID

6.1 Introduction

In vitro culture techniques allow a far greater control over external and internal variables affecting organ regeneration than do leaf or shoot cutting systems. The cultured tomato leaf disc system has a rapid regeneration time, as well as histologically and temporally defined primordia from two distinct tissue types. Moreover, the factors necessary for regeneration in this system have been delineated (see Chapter 5). These factors include a complex, yet defineable group of specific inorganic and organic nutrients, vitamins and auxin-type growth regulators.

The study of regenerating tomato leaf cuttings in Chapter 4 suggests that localized starch synthesis is a necessary prerequisite for root regeneration. A GA_3 induced inhibition of starch synthesis appears to be the basis for GA_3 inhibition of regeneration although the specific biochemical mechanism is unknown. The present chapter attempts a similar analysis of GA_3 effects on root regeneration in cultured tomato leaf discs in terms of the following questions:

1) Can GA_3 inhibition of root regeneration be related to an earlier effect on internal starch levels?

2) Does the concentration or type of soluble external carbohydrate source modify the GA_3 effect?

3) Does GA_3 preferentially affect Type I or Type II primordia in terms of initiation and/or development?

In view of the divergent GA_3 effects on the growth and metabolism of leaf discs in other systems (e.g. Kuraishi and Hashimoto, 1957; Goldthwaite and Laetsch, 1968) and probable growth modifications in the present system, three more questions are evident:

4) What effect does GA_3 have on disc growth?

5) Is the growth effect tissue specific and/or auxin dependent?

6) Does the growth response modify the regeneration response and can the two events be experimentally separated?

6.2 Materials and Methods

Culture techniques and biochemical analyses are essentially similar to those outlined in Chapters 5 and 3 respectively. Histological procedures for cleared leaf disc preparation are outlined in section 3.2.2.1.

6.3 Results

GA_3 inhibited auxin-induced root regeneration from cultured tomato leaf discs in the presence or absence of light (Table 6.1). A study of the time course of root

Table 6.1. GA_3 inhibition of auxin-induced root regeneration from tomato leaf discs cultured in continuous darkness or continuous light.

GA_3 concentration	Mean number roots per disc			
	Continuous darkness		Continuous light	
0	12.1	a	5.3	c
10^{-10} M	9.2	a,b	5.6	c
10^{-9} M	9.2	a,b	5.2	c
10^{-8} M	9.1	a,b	2.1	d
10^{-6} M	8.6	b,c	2.0	d
10^{-4} M	7.6	b,c	2.0	d

Means followed by same letter in a column not different at 5% level.

IAA concentration: 5×10^{-6} M.

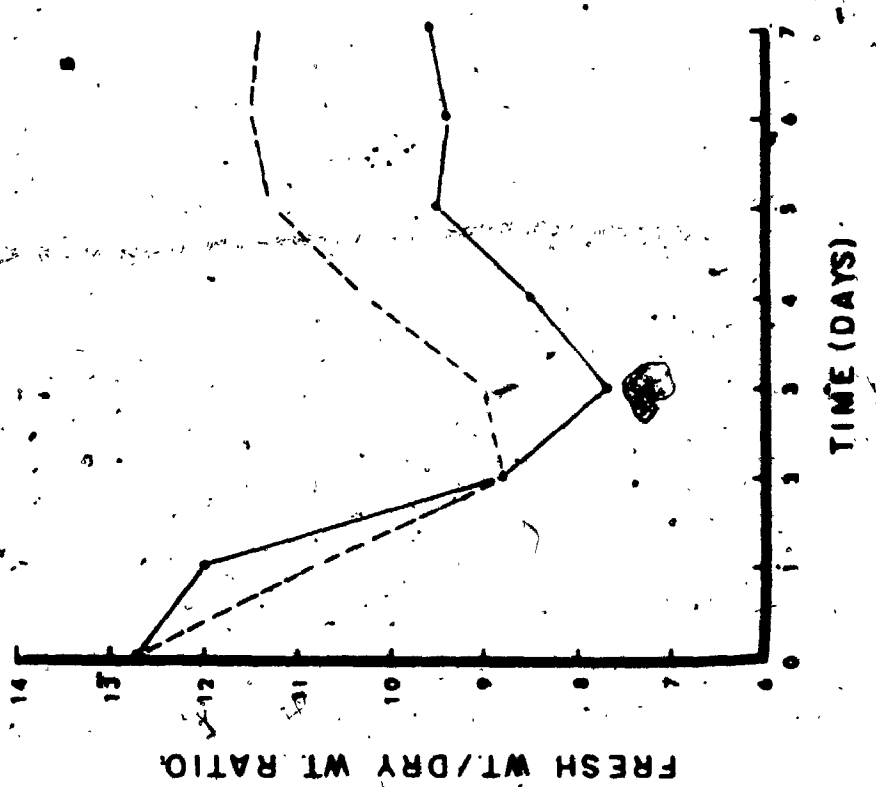
Mean LPI_3 = 4.3

formation revealed a delay as well as a reduction in the total number of organs formed (Fig. 6.1A). However, fresh weights and midrib elongation of the GA_3 treated discs were significantly greater than the control tissues (Table 6.2) with an initial difference becoming noticeable during the 48-72 hour period in terms of fresh/dry weight ratio changes (Fig. 6.1B). Callus proliferation from the midrib and secondary veins was evident in the GA_3 treated leaf material cultured in the light.

Sequence treatment and GA_3 pulsing experiments were carried out in an attempt to determine the time of maximum sensitivities for regeneration inhibition and growth stimulation. GA_3 inhibited organ regeneration if applied for the first 1 to 5 days followed by the control inductive medium (Table 6.3). However, maximum inhibition occurred after 7 days on an organogenic medium containing GA_3. A GA_3-induced growth stimulation was also apparent during the first five days of culture with a maximum effect apparent after an initial 48 hour treatment period. When GA_3 was applied during the later stages of the culture period (i.e. from days 3 to 7), no inhibitory effect on organ regeneration was apparent (Table 6.4). Maximum GA_3-induced growth was observed if GA_3 was applied from 48 to 72 hours after the initiation of the culture experiment. A pulsing experiment, in which 24 hour treatments of GA_3 were applied to the tomato leaf discs, was inconclusive (Table 6.5). However, the observation that maximum inhibition of root regeneration

Figure 6.1. A. Time course of root regeneration from tomato leaf discs cultured *in vitro* in the presence or absence of GA_3 (1×10^{-4} M).

B. Fresh weight/dry weight ratio changes during root regeneration from tomato leaf discs cultured *in vitro* in the presence or absence of GA_3 (1×10^{-4} M).


FRESH WT/DRY WT RATIO
TIME (DAYS)

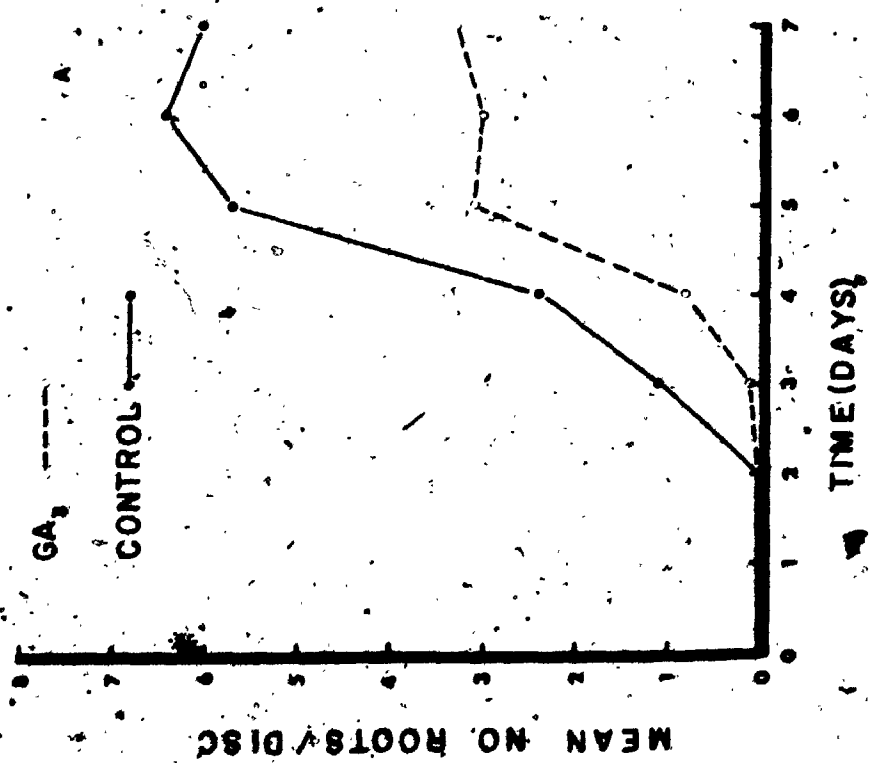

MEAN NO. ROOTS/DISC
TIME (DAYS)
CONTROL
GA3

Table 6.2. GA_3 stimulation of growth in cultured tomato leaf tissue in the light.

Treatment	Mean final fresh weight (mg)*		Mean final dry weight (mg)*		Mean final midrib length (mm)*	
IAA (5×10^{-6} M)	25.9	a	2.7	c	6.9	e
IAA + GA_3 (1×10^{-4} M)	35.3	b	3.1	d	7.5	f

*Initial fresh weight, approx. 2 mg; initial dry weight, approx. .2 mg; initial midrib length, 5.0 mm.

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 4.3

Table 6.3. Effect of initial GA_3 treatment period on subsequent root regeneration from tomato leaf discs cultured *in vitro*.

Treatment duration (hr)*		Mean number of roots per treatment		Mean fresh weight (mg)	
IAA + GA_3	IAA				
-	0 - 168	8.2	c,d,e	19.3	f
0 - 24	24 - 168	5.5	b,c	33.4	g
0 - 48	48 - 168	6.5	b,c,d	37.0	g
0 - 72	72 - 168	5.5	b,c	32.3	g
0 - 96	96 - 168	6.3	b,c,d,e	32.1	g
0 - 120	120 - 168	4.7	b	32.9	g
0 - 168	-	2.6	a	22.2	f

* IAA 5×10^{-6} M; GA_3 1×10^{-4} M

Means followed by the same letter not different at 5% level.

Mean LPI_3 = 3.6

Table 6.4. Effect of late GA_3 treatment period on subsequent root regeneration from tomato leaf discs cultured *in vitro*.

Treatment duration (hr)*		Mean number of roots per treatment		Mean fresh weight (mg)	
IAA	IAA + GA_3				
-	0 - 168	2.6	a	22.2	f,g,h
0 - 24	24 - 168	6.3	b,c,d	22.2	f,g,h
0 - 48	48 - 168	6.5	b,c,d,e	29.2	h,i
0 - 72	72 - 168	9.3	d,e	30.7	i
0 - 96	96 - 168	8.7	d,e	26.9	g,h,i
0 - 120	120 - 168	9.8	e	20.3	f,g
0 - 168	-	8.2	c,d,e	19.3	f

* IAA 5×10^{-6} M; GA_3 1×10^{-4} M

Means followed by the same letter not different at 5% level.

Mean LPI_3 = 3.6

Table 6.5. Effect of short GA_3 treatments at different time periods on subsequent root regeneration from tomato leaf discs culture *in vitro*.

Duration of GA_3 treatment (hr)*	Time interval during the 168 hour culture period when GA_3 treatment (hr) applied	Mean number of roots per treatment	
IAA control	---	7.7	a
IAA + GA_3 control	---	4.4	b
24	0 - 24	6.6	a,b
24	24 - 48	5.9	a,b
24	48 - 72	6.2	a,b
24	72 - 96	9.5	a
48	0 - 48	5.9	a,b
48	24 - 72	7.6	a
48	48 - 96	8.7	a

* IAA 5×10^{-6} M; GA_3 1×10^{-4} M.

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.4

5 6

OF/DE

took place if GA_3 was applied over the entire culture period suggested that the inhibition might involve 2 effects: i) an early inhibition (i.e. during the initial 48 hours) and, ii) a late inhibition possibly associated with earlier effects of GA_3 (e.g. growth modification).

In view of the earlier results with tomato leaf cuttings which suggested that GA_3 inhibition of organogenesis was associated with an altered starch metabolism, experiments using different concentrations of various soluble carbohydrate sources (i.e. sucrose, glucose/fructose, glucose and maltose) were carried out. The results indicated (Fig. 6.2A, B, C and D) that the type of carbohydrate substrate had no effect on GA_3-induced inhibition of organ initiation. However, under conditions of low carbohydrate concentrations, GA_3 exhibited no significant effect on organ regeneration although growth was significantly enhanced (Table 6.6). An examination of total soluble sugar and starch levels within the GA_3 treated and untreated leaf discs revealed consistent differences (Fig. 6.3A and B) in starch levels during the initial four days. In the control tissue, maximum starch levels occurred at 72 hours while GA_3 treated tissue exhibited a slower accumulation of endogenous starch reserves with maximum levels at 96 hours. It is significant that no GA_3 was applied on day 3 or later (Table 6.4) when maximum starch levels were achieved in the organogenic tissues. Total soluble carbohydrates increased

Figure 6.2. Effect of exogenous, soluble carbohydrate type and concentration on GA_3-induced inhibition of root regeneration. Open circles, GA_3 1×10^{-4} M; closed circles, control.

A. Sucrose concentration. Mean LPI_3 = 2.6

B. Maltose concentration. Mean LPI_3 = 3.0

C. Glucose concentration. Mean LPI_3 = 3.1

D. Glucose/fructose concentration. Mean LPI_3 = 3.1

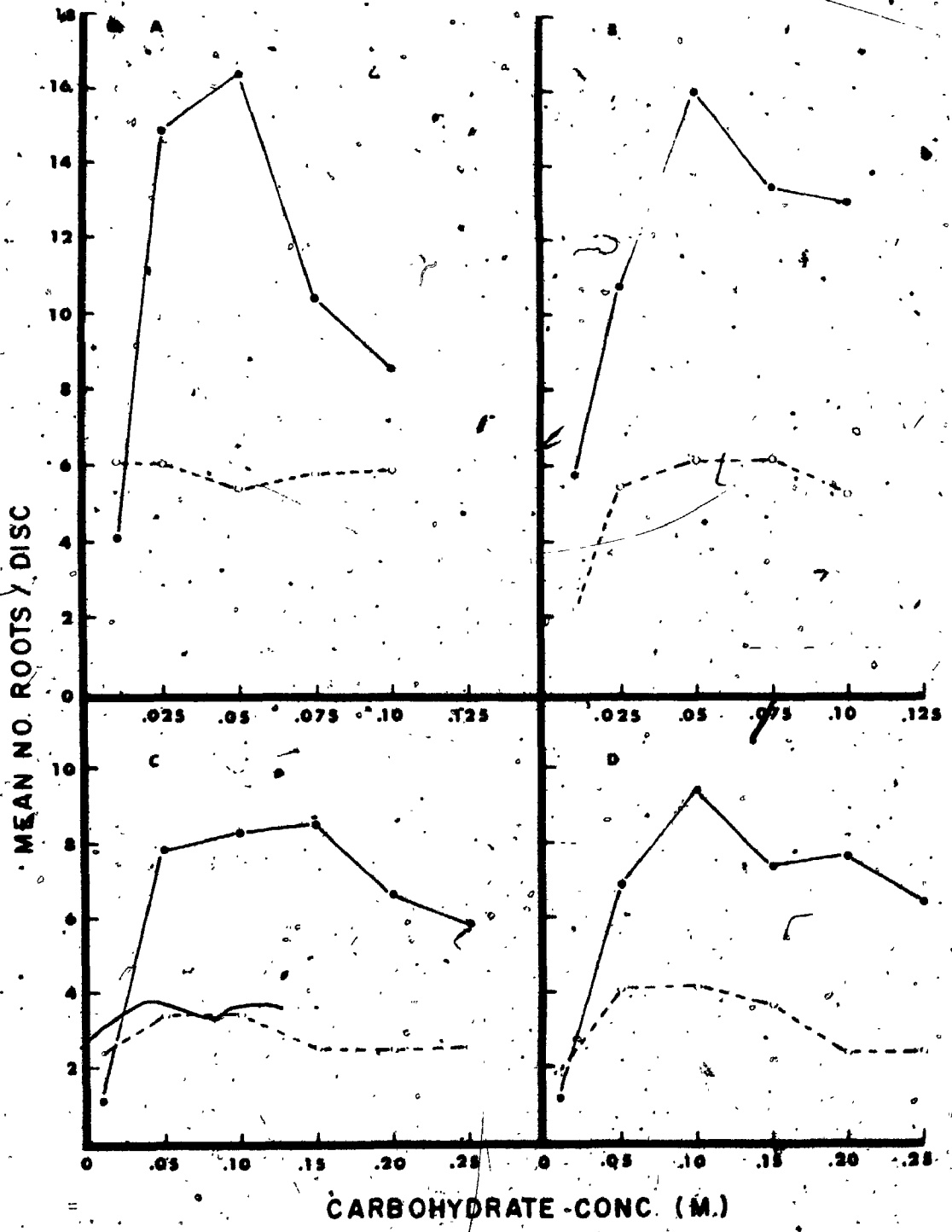

A
B
C
D
MEAN NO. ROOTS / DISC
CARBOHYDRATE - CONC. (M.)
0
2
4
6
8
10
12
14
16
.025
.05
.075
.10
.125
.15
.20
.25

Table 6.6. Effects of sucrose concentration on growth and regeneration of tomato leaf discs as modified by simultaneous GA_3 application.

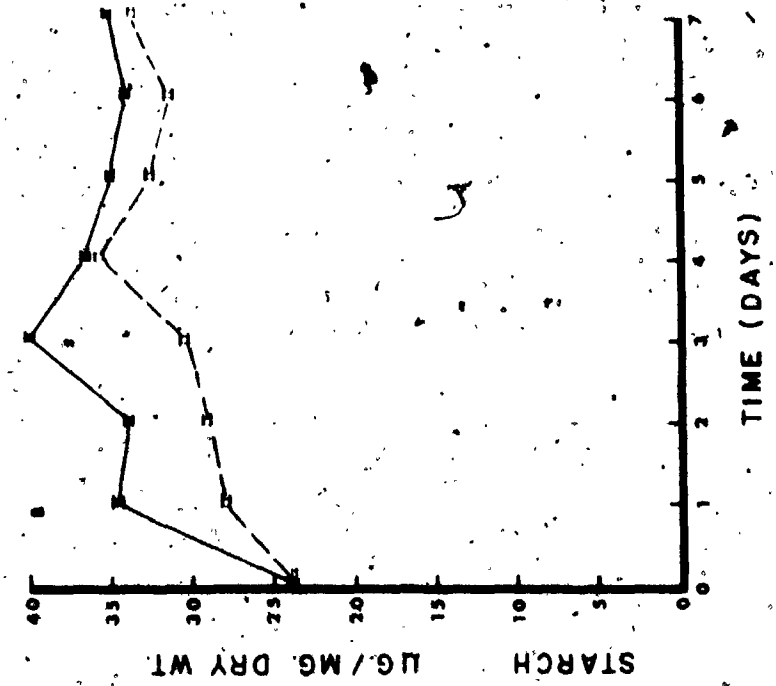
Treatment	Mean number of roots per disc		Mean fresh weight (mg)		Mean midrib length (mm)	
.01 M sucrose	4.1	a	17.0	e	7.1	f
.01 M sucrose + GA_3 (1×10^{-6} M)	6.2	a	21.4	c	8.2	g
.05 M sucrose	16.4	b	19.3	c,e	7.1	f
.05 M sucrose + GA_3	5.3	a	30.1	d	8.1	g

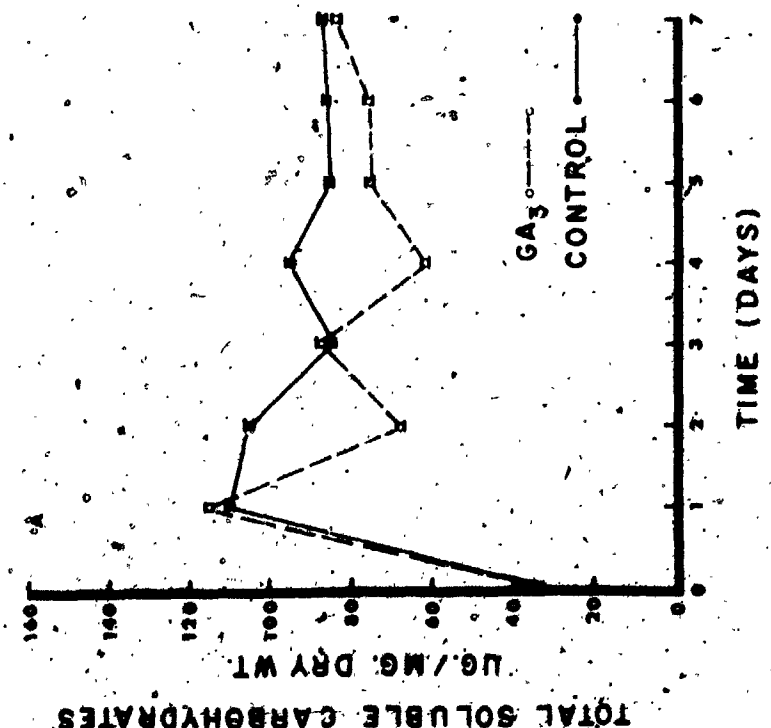
Means followed by same letter in a column not different at 5% level.

Mean LPI_3 = 2.6

Figure 6.3. A. Changes in total soluble carbohydrates during root regeneration from tomato leaf discs cultured *in vitro* in the presence or absence of GA_3 (1×10^{-4} M). Vertical lines ± S.E.

B. Changes in starch content during root regeneration from tomato leaf discs cultured *in vitro* in the presence or absence of GA_3. Vertical lines ± S.E.


STARCH
UG./MG. DRY WT
40
35
30
25
20
15
10
5
0
0
1
2
3
4
5
TIME (DAYS)

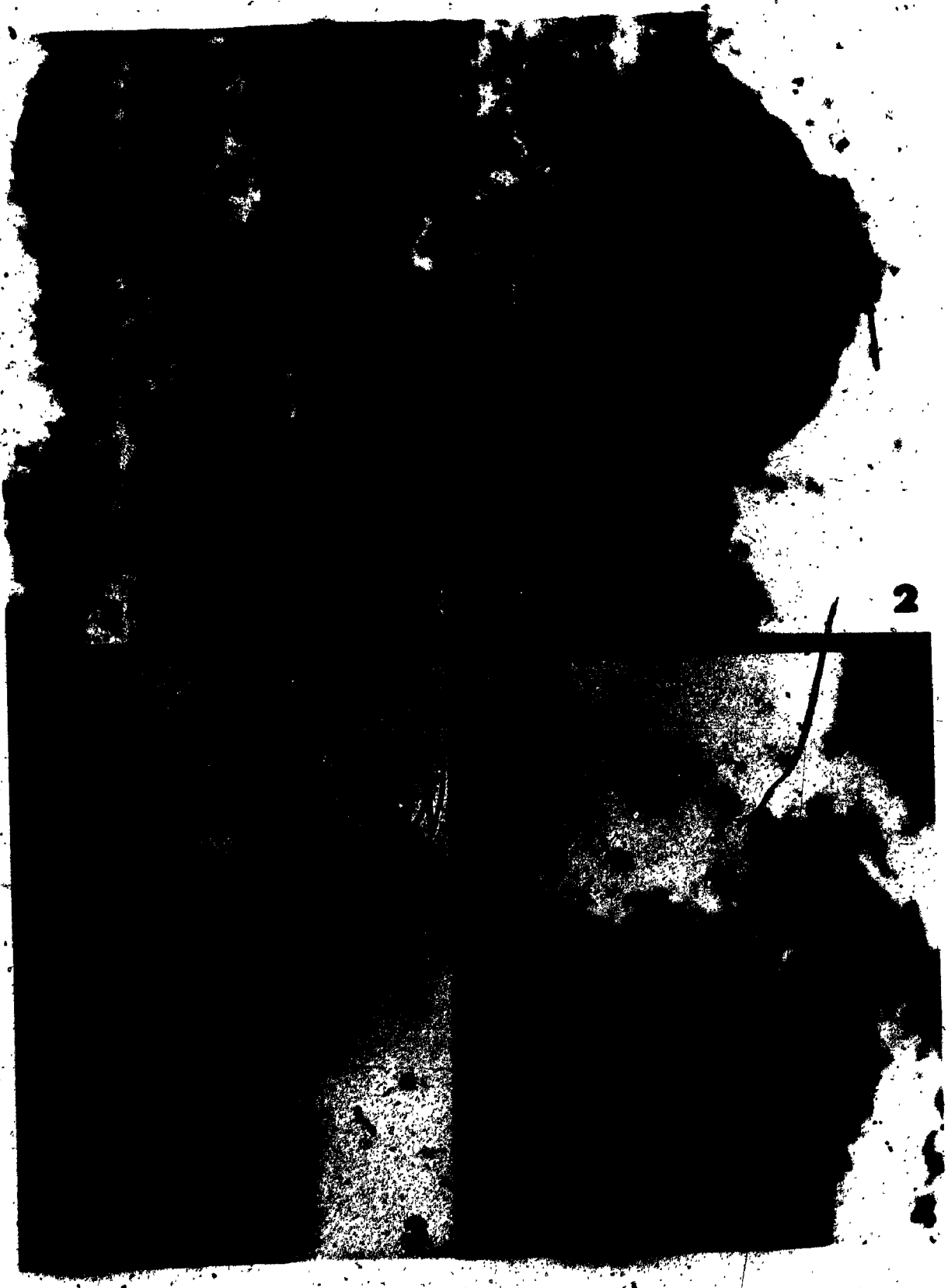

TOTAL SOLUBLE CARBOHYDRATES
UG./MG. DRY WT
160
140
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
TIME (DAYS)
GA3
CONTROL

four fold during the initial 24 hour culture period followed by relatively gradual declines during the remaining 6 days. Initial inflection points in the soluble carbohydrate levels of the GA_3 and control treated tissues at day 2 and day 3 respectively, coincided with similar inflections in the fresh weight/dry weight ratio curves. Consequently it appears that decreased starch levels in the GA_3 treated leaf discs may account for the initial inhibitory effects of GA_3 on organ regeneration.

In order to explore more closely the possible late effects of GA_3 on organ regeneration, a histological examination of GA_3 treated and untreated leaf discs was carried out using the two whole tissue clearing techniques. Leaf tissues cultured on an organ inducing medium containing GA_3 were observed to form callus proliferations at the severed ends of the primary and secondary veins. Callus was derived primarily from the cortical parenchyma tissues. By 120 hours, a noticeable cortical cell enlargement at right angles to the vascular traces was evident in the GA_3 treated material. In a number of cases this marked polarization of cell enlargement included the endodermal cells involved in root cap formation of late forming primordia (Plate 6.1). Subsequent cell enlargement appeared to include the entire primordium. This apparent destruction of the late forming primordia by a parenchymatization process beginning in the endodermal derivatives of the root cap cells would explain the late effects of GA_3 on

Plate 6.1. Histological changes induced in late forming root primordia by GA_3.

1. FPA cleared midrib tissue with developing primordia from leaf discs cultured on IAA medium for 120 hours. (x 120)
2. Similar to 1. A close-up of IAA-induced primordium showing the typical flat, hemispherical, bulging morphology with rectangular shaped cells (arrow) forming the root cap. (x 200)
3. GA_3 treated leaf tissue after 120 hours showing a typical, late forming primordium with sharp, beak-like apex of enlarging cells. Note the large, highly vacuolated cortical parenchyma cells around the primordium. (x 200)
4. GA_3 treated leaf tissue after 120 hours showing a later primordium stage where the cap cells appear to be elongating in a direction parallel to the surrounding cortical parenchyma cells. (x 200)


2

inhibition of organogenesis. In some cases masses of proliferating cortical cells possessed vascular traces similar to ones found in root primordia (Plate 6.7;1,2). Whether these traces were initially developed within discrete primordia which were subsequently destroyed by parenchymatization or were formed in response to localized callus proliferations is unknown. However, their strong resemblance to root vasculature suggests that the former hypothesis may be correct.

At 120 hours, meristemoidal areas in endodermal derivatives of the minor veins were virtually absent in GA_3 treated material (mean number meristemoids = .1) although quite abundant in the control tissue (mean number = 5.3). At the LPI_3 and auxin concentrations used in these experiments, however, no type II primordia would be formed from these meristemoids.

Attempts were made to modify the GA_3 responses by auxins or ABA. Regardless of auxin type, high concentrations of GA_3 inhibited root regeneration (Table 6.7) at high auxin concentrations. GA_3 inhibition of root regeneration was most pronounced at high auxin concentrations regardless of the presence or absence of light (Tables 6.8 and 6.9). GA_3 alone induced slight yet reproducible rooting responses in the dark. Interestingly, when high concentrations of IAA were applied in the presence or absence of a low GA_3 concentration, no inhibition of organogenesis was observed although a growth stimulation was evident (Table 6.10). No

Plate 6.2. Abnormal vascularization of late forming roots induced by GA_3.

1. NaOH cleared leaf tissue after 120 hours on IAA medium showing 3 root traces (arrows) joining with the mid-rib vasculature. Note the long vessel elements which compose the root trace. (x 200)
2. NaOH cleared leaf tissue after 120 hours on a GA_3 + IAA medium showing the apparent effects of primordium destruction on the vascularization process of the root. The vessels (arrows) are short, irregular and discontinuous in appearance. (x 200)

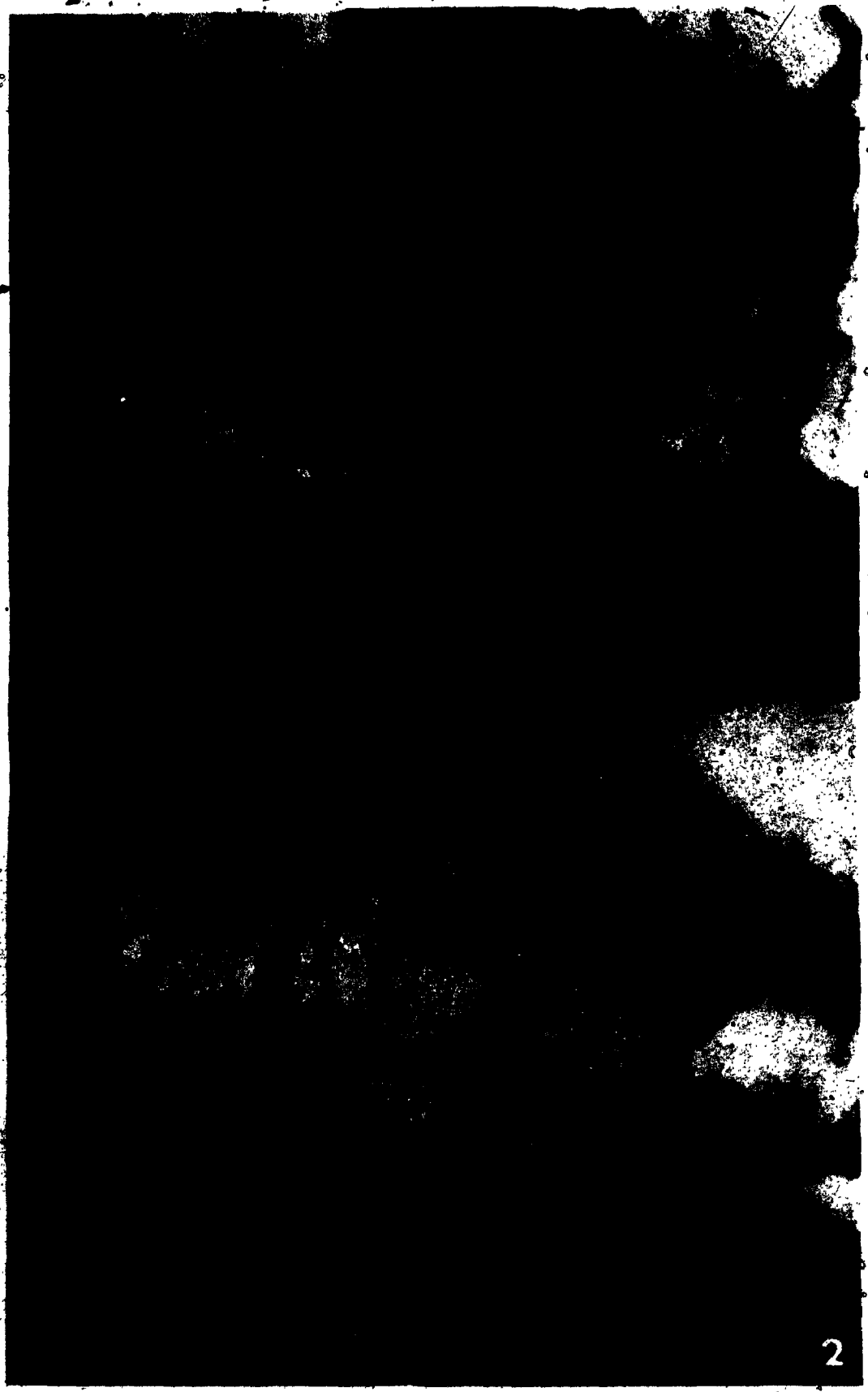

2

Table 6.7. Effects of GA_3 on rooting induced by different types of auxins in the dark.

Treatment	Mean number of roots per disc	
Control	.1	a
Control + GA_3 (1×10^{-4} M)	.1	a
IAA (5×10^{-7} M)	5.7	b
IAA + GA_3	1.9	d
IAA (5×10^{-6} M)	10.4	c
IAA + GA_3	6.0	b
IBA (5×10^{-7} M)	.4	a
IBA + GA_3	.1	a
IBA (5×10^{-6} M)	6.9	b,c
IBA + GA_3	3.2	d
PAA (5×10^{-7} M)	0.0	a
PAA + GA_3	0.0	a
PAA* (5×10^{-6} M)	0.0	a
PAA + GA_3	0.0	a

*Phenylacetic acid

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.9

Table 6.8. Effect of GA_3 concentration on rooting response in the light of tomato leaf discs cultured *in vitro* at different IBA concentrations.

Treatment	Mean number of roots per discs	
IBA (1×10^{-6} M)	1.0	a
IBA + GA_3 (1×10^{-8} M)	.8	a
IBA + GA_3 (1×10^{-6} M)	.5	a
IBA + GA_3 (1×10^{-4} M)	.2	a
IBA (1×10^{-5} M)	7.5	b
IBA + GA_3 (1×10^{-8} M)	6.2	b
IBA + GA_3 (1×10^{-6} M)	3.5	c
IBA + GA_3 (1×10^{-4} M)	1.3	a

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.7

Table 6.9. Effect of IAA concentration on the GA_3-induced rooting inhibition from tomato leaf discs cultured *in vitro* in continuous darkness.

Treatment	Mean number of roots per disc			
	$-GA_3$		$+GA_3$ (1×10^{-4} M)	
Control	0	a	.1	a
IAA (5×10^{-8} M)	1.3	b	.2	a
IAA (1×10^{-7} M)	2.3	c	1.4	b
IAA (5×10^{-7} M)	7.1	d	6.2	d
IAA (1×10^{-6} M)	10.5	e	7.9	d
IAA (5×10^{-6} M)	10.5	e	7.6	d

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.8

Table 6.10. Effect of a low GA_3 concentration on IAA-induced root regeneration and growth of tomato leaf discs cultured *in vitro*.

Treatment	Mean number of roots per disc		Mean fresh weight (mg)	
IAA (2.5×10^{-5} M)	8.9	a,b	32.4	c,d
IAA + GA_3 (1×10^{-8} M)	9.8	a,b	41.0	e
IAA (5.0×10^{-5} M)	11.0	a,b	29.6	c
IAA + GA_3	11.5	b	38.9	d,e

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.6

significant growth effect was evident in GA_3 treated material without the presence of an auxin (Table 6.11). ABA had no effect on GA_3 inhibition of rooting when applied simultaneously although an inhibition of growth was apparent at high concentrations (Table 6.12). An initial twenty-four hour pulse of ABA at different concentrations were also unable to overcome the GA_3-induced inhibition of organogenesis (Table 6.13).

6.4. Discussion

The present set of experiments has revealed multiple inhibitory roles of GA_3 in the control of *in vitro* root regeneration when the leaf discs are cultured in continuous light. Furthermore, the GA_3 inhibition of organ regeneration is apparent only at or above threshold concentrations of externally applied soluble carbohydrates or auxins although the type of soluble carbohydrate or auxin had no effect on the inhibitory response.

The early or initial inhibitory effect on rooting (i.e. during the first 48 hours) can be related to a partial inhibition of endogenous starch synthesis. Both tissue analysis of starch levels and GA_3 timing experiments support this hypothesis. The conclusions are also in agreement with the previous leaf cutting experiments of Chapter 4.

However, it is apparent from histological observations that meristemoid formation from the minor vein sheath parenchyma is also inhibited. Under conditions of low

Table 6.11. Effect of GA_3 on tomato leaf disc growth in the light or dark.

Treatment*	Mean fresh.weight (mg)	
continuous light	16.1	a
continuous light + GA_3 (1×10^{-4} M)	15.3	a
continuous dark	10.0	b
continuous dark + GA_3	11.2	b

* Basal medium without auxin.

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.2

Table 6.12. Effect of ABA on GA_3 rooting inhibition and growth stimulation from tomato leaf discs cultured *in vitro*.

Treatment	Mean number of roots per disc		Mean fresh weight (mg)	
control	6.4	b	25.6	c
ABA (1×10^{-4} M)	5.9	b	10.0	d
ABA + GA_3 (1×10^{-8} M)	3.8	a	10.8	d
ABA + GA_3 (1×10^{-4} M)	3.9	a	12.9	d

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 2.8

Table 6.13. Effects of an initial 24 hour ABA pulse on GA_3 inhibition of root regeneration.

Treatment*	Mean number of roots per disc	
Control	14.0	a
GA_3 (1×10^{-6} M)	10.7	b,c
GA_3 (1×10^{-4} M)	7.9	c,d
ABA (1×10^{-6} M)	12.4	a,b
ABA (1×10^{-4} M)	15.2	a
GA_3 (1×10^{-6} M) + ABA (1×10^{-6} M)	8.8	c,d
GA_3 (1×10^{-4} M) + ABA (1×10^{-6} M)	7.1	d
GA_3 (1×10^{-6} M) + ABA (1×10^{-4} M)	7.1	d
GA_3 (1×10^{-4} M) + ABA (1×10^{-4} M)	8.2	c,d

* Discs were initially cultured on the different treatment media and then subcultured onto an inductive medium for the remaining 144 hours.

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 2.7

LPI_3 and/or high exogenous auxin levels, this GA_3-mediated meristemoid inhibition would be expressed in terms of a reduced number of type II root primordia. Whether this inhibition of meristemoid formation from the sheath parenchyma derivatives of the minor veins is causally related to reduced starch levels in the GA_3 treated leaf discs or to a direct effect on the mitotic and enlargement processes of the potential meristemoidal cells is unknown.

A third inhibitory effect occurs relatively late in the culture period (i.e. during days 4 to 6) and involves the destruction of late-forming type I primordia by a process of gradual parenchymatization. This inhibition appears to be related to an earlier, GA_3-induced mitotic activation and subsequent elongation of the cortical parenchyma cells at right angles to the underlying vascular tissues. The destruction of the root primordium begins with the endodermal derivatives which form the cap cells and may involve vascularized primordia as well as younger stages. This growth related inhibition of primordia development may account for up to 50% of the GA_3-induced inhibition and may help to explain why auxins are unable to reverse the inhibition as was found in the leaf cutting system. However, the GA_3 effects on rooting and growth can be experimentally separated under conditions of high auxin levels and low GA_3 concentration where no inhibitory effect on rooting was apparent although a significant growth stimulation occurred. Similarly, although ABA was capable of suppressing the GA_3

growth stimulation, it had no effect on a GA_3-induced inhibition of root primordia initiation or development. The consequences of ABA suppression of GA_3-induced growth as it affects the late GA_3 effect on root inhibition (i.e. the parenchymatization process) are unknown as no detailed histological study was made. It is conceivable, however, that the proliferating cells of the developing root primordium may be selectively modified through the action of GA_3 with a consequent destruction of the primordium. This concept of primordium destruction by GA_3 differs from Haissig's observations on GA_3 inhibition of preformed root primordium development (Haissig, 1972). Based on histological observations of cell numbers in median longitudinal sections of GA_3 treated and untreated, preformed root primordia in brittle willow seedlings, Haissig concluded that GA_3 inhibited development by a direct inhibition of mitotic activity within the developing primordium. Although the present study does not rule out GA_3 inhibited intraprimordium cell division leading to limited root primordium development, it does present histological evidence for a second inhibitory effect of GA_3 on root primordium development which is based upon the actual destruction of the primordium.

Although GA_3 appears to exhibit multiple inhibitory roles in the control of root regeneration when the leaf discs are cultured in continuous light, a consistent observation was the slight stimulation of rooting by GA_3 when the leaf

discs were cultured in continuous darkness. This observation is pursued in the following chapter.

CHAPTER 7

TOMATO LEAF DISCS CULTURED IN VITRO: STIMULATION OF ROOT REGENERATION BY GIBBERELLIC ACID

7.1 Introduction

This chapter explores the ability of cultured tomato leaf discs to form a small, yet reproducible, number of roots when the discs are incubated in the presence of GA_3 in continuous darkness. Previous work (see section 6.3) has established that GA_3 inhibits IAA-induced rooting in the dark regardless of auxin concentration. Consequently, the consistent yet slight stimulation of rooting in the dark by high concentrations of GA_3 alone appeared paradoxical unless one hypothesized a GA_3 effect on the levels of endogenous auxin. A number of researchers have demonstrated that GA_3 increases the amount of endogenous auxin in various plant materials. For example, Sastry and Muir (1963) observed that GA_3 treatment of tomato fruits lead to increased diffusible auxin levels from the developing fruits. Thimann (1972) suggested that tomato pollen could contain gibberellin which could, in turn, stimulate auxin production in the ovary. Further work by Muir and his associates (Kuraishi and Muir, 1963; 1964) established that GA_3 treated

stem tips of the rosette plant, Hyoscyamus, contained 43 times as much auxin as in controls. Gibberellic acid has also been reported to increase the endogenous auxin levels of bean seedlings 200 times (Nitsch and Nitsch, 1963). Similar reports are present in the literature (e.g. Nitsch and Nitsch, 1959; 1961; Bastin, 1967; Varga and Bitó, 1968 as cited in Varga and Humphries, 1975). Two mechanisms have been hypothesized for these observations: 1) GA_3 inhibition of auxin oxidases leading to increased auxin levels by a sparing effect; and 2) GA_3 stimulation of endogenous auxin synthesis. Although both systems have been supported by experimental evidence (see section 2.4), the current study suggests that the latter hypothesis presents a more plausible explanation of the observations. As noted in Figure 2.1 (see section 2.4), IAA may be synthesized in tomato shoots by one of two pathways:

$$TPP \rightarrow TNH_2 \rightarrow IAAld \rightarrow IAA \qquad (1)$$

$$TPP \rightarrow TPyA \rightarrow IAAld \rightarrow IAA \qquad (2)$$

Since GA_3 is capable of apparently stimulating endogenous IAA synthesis by pathway (1) in a number of non-regenerating systems (see section 2.4) the following study examines this effect in a root regenerating system and involves two interrelated aspects:

i) culture studies involving GA_3 effects on root regeneration in the presence or absence of possible IAA precursors (see Fig. 2.1);

ii) radioactive incorporation studies with the IAA precursor,

tryptamine-2-^{14}C bisuccinate, in the presence or absence of GA_3 over a 24 hour period.

7.2 Materials and Methods

7.2.1 Culture Techniques

Similar procedures were followed as outlined in section 5.2.1. The indole derivatives, GA_3 and labile components such as the accessory factors, α-ketoglutaric acid and pyridoxal phosphate, were added by filter sterilization after autoclaving the media. All indole compounds and accessory factors were purchased from Sigma Co., St. Louis, Mo.

7.2.2 Radiotracer Techniques

7.2.2.1 Tissue Incubation

Duplicate samples of 100 discs per treatment from young expanding tomato leaves (LPI_3 2.0 to 3.0) were incubated in 25 ml Erlenmeyer flasks containing Murashige and Skoog (1962) inorganic nutrients, .06 M sucrose, 5×10^{-7} M thiamine, 5×10^{-6} M α-ketoglutaric acid and 2 µc of tryptamine-2-^{14}C bisuccinate (final concentration 8.6×10^{-6} M; specific activity 47 .0 mCi/m mole; New England Nuclear, Boston, Mass.) in a total volume of 5.0 ml. Radiochemical purity of the TNH_2-2-^{14}C was greater than 98.5% when purchased approximately six months before the

experiment and TLC separation of a 5 µl sample revealed a single homogenous radioactive peak which co-chromatographed with authentic tryptamine. Gibberellic acid was added to one half of the samples at a final concentration of 1×10^{-5} M. After surface sterilization of the whole leaves, one hundred 5 mm diameter leaf discs were punched out with a stainless steel cork borer, added to each flask and subsequently incubated for 6, 12 or 24 hours on a linear shaker (120 cycles per minute) in continuous darkness at 25°C.

7.2.2.2 Extraction of Indole Metabolites

All of the following steps were carried out in dim light at 4°C to prevent decomposition of the indole compounds. In order to effect "coprecipitation" of the labelled compounds, one hundred micrograms each of TNH_2, IAA and TOL in 80% methanol were added to each fraction associated with the extraction procedure (i.e. liquid medium, washings and methanol tissue extract). This step is based on the assumption that the minute amounts of 'hot' compound will be carried along with the relatively large amounts of 'cold' carrier, allowing easy detection and collection. At the indicated times, the liquid medium was pipetted off, made up to [redacted] methanol and stored in the dark at -15°C for subsequent analysis. Each sample of 100 leaf discs was rinsed onto a sintered glass filter with 200 ml cold distilled deionized water containing approximately

1×10^{-5} M unlabelled tryptamine. The aqueous washings were stored at +4°C. The leaf discs were ground with a prechilled mortar and pestle containing .5 ml 80% methanol and .02% sodium diethyldithiocarbamate (DIECA) for 4 minutes (Mann and Jaworski, 1970). The methanol/DIECA extraction solution which contained the ground tissue was made up to 40 ml (final volume) with the extraction solution, rinsed into a centrifuge tube of 50 ml capacity and stored at 4°C for 30 minutes with occasional stirring. The tubes were centrifuged for 10 minutes at 23,000 x g and the supernatant decanted off and saved. The pellet was reextracted for an additional 30 minutes in 40 ml cold 80% methanol and .02% DIECA and subsequently centrifuged at 23,000 x g for 10 minutes. The supernatants were combined and the pellets stored at -15°C.

The methanol extracts were evaporated to near dryness at 35°C using a Buchner flash evaporator. The soluble extract residues were taken up repeatedly in 5.0 ml (final volume) of methanol. The resulting extracts were stored in the dark at -15°C under a nitrogen atmosphere until subsequent analysis by TLC. Total time for extraction and TLC analysis was under 72 hours.

7.2.2.3 TLC Separation of Indole Metabolites

Precoated Eastman Chromagram sheets (20 cm x 20 cm) with a 100 mu layer of silica gel (without fluorescent indicator) were activated at 110°C for 30 minutes before use in order to provide a highly active adsorption layer. The following

solvent systems were evaluated for separatory properties and running times (in brackets):

i) ethyl acetate-isopropanol-water 65:24:11 v/v (75 minutes) (Ballin, 1964)

ii) isopropanol-ammonia-water, 85:5:15 v/v (180 minutes) (Kaldewey, 1968)

iii) chloroform-methanol-acetic acid, 75:20:5 v/v (55 minutes) (Pillay and Mehdi, 1968)

iv) chloroform-96% ethanol, 65:35 v/v (60 minutes) (Ballin, 1964)

v) chloroform-acetic acid, 95:5 v/v (Pillay and Mehdi, 1968)

vi) carbon tetrachloride-ethanol, 1:1 v/v (80 minutes) (Sherwin and Purvis, 1969)

Indole compounds were detected on the thin layer plates using Van Urk's reagent (Kaldewey, 1968). One gram of 4-dimethylaminobenzaldehyde was dissolved in 50 ml of HCl (density approx. 1.19) and 50 ml ethanol added. Immediately after development, the plates were sprayed with this reagent and then exposed to aqua regia vapors (HCl-HNO_3; 3:1 v/v).

The neutral solvent system (i) of Ballin (1964) was selected since it gave excellent, reproducible separations of IAA from TNH_2 and TOL with no evidence of decomposition products (Table 7.1). Further steps used to avoid decomposition of indole derivatives included spotting the TLC sheets under a gentle stream of nitrogen and running the thin layer plates in the dark at room temperature (ca. 25°C).

Table 7.1. R_f values and colour reactions of simple indole derivatives.

Indole derivative*	R_f value	Van Urk colour reaction wet plate	dry plate
1. Indole-3-lactic acid	.11	blue	dark blue
2. Indole-3-glyoxylic acid	.14	colourless	orange-yellow
3. Tryptamine	.15	colourless	light blue
4. Trytophan	.20	colourless	light blue
5. Indole-3-acetic acid	.38	red-purple	light blue
6. Indole-3-acetaldehyde	.78	red-purple	purple
7. Tryptophol	.81	dark purple	dark blue
8. 3-indole-aldehyde	.82	colourless	orange

* 2 ug of each indole were run in ethyl acetate-isopropanol-water, 65:24:11 v/v for 75 minutes at 25°C. Running distance was 10 cm. Results presented from one representative run.

Fifty microliters of each tissue or media extract containing the labelled indole compounds were spotted at 2 cm intervals, run for 10.0 cm and then dried at room temperature. A standard solution containing 2 µg each of TNH_3, IAA and TOL was routinely run adjacent to the labelled samples. Preliminary experiments indicated that neither DIECA nor the tissue extracts affected the R_f values of the indole compounds.

No attempts were made to identify IPyA among the labelled products of TNH_2. The reasons for this decision included the pronounced instability of IPyA and, more importantly, the failure of IPyA to incorporate a ^{14}C label from TNH_2-2-^{14}C during previous, and extensive, studies by Wightman (e.g. Gibson et al., 1972). Similarly, although the R_f zone corresponding to TOL could include IAAld, the latter substance is unlikely to be present due to its instability whereas TOL is quite stable and has been readily demonstrated in tomato extracts (Schneider et al., 1972; Gibson et al., 1972).

7.2.2.4 Liquid Scintillation Counting

The individual spots on the developed thin layer plates were cut out as 2 cm x 10.5 cm strips. Individual sections 2 cm x .8 cm were cut out at R_f values from -.05 to 1.00 and individually placed in low background glass scintillation vials (20 ml capacity) with .5 ml methanol. The capped vials were shaken for 1 hour in the dark at room temperature

on a linear shaker (180 cycles per minute) in order to elute the labelled indole derivative. Following the solubilization step, 12 ml of toluene based scintillation cocktail (Omnifluor; New England Nuclear Corp.) were added to the individual samples and subsequently counted as counts per minute (cpm) in a Beckman 230 liquid scintillation counted for 10 minutes per sample or until the 2 sigma counting error displayed fell to .2% of the gross counting rate. Quantitative determinations of label distribution were done on the basis of totalling the net cpm for each radioactive peak over the R_f values of the various indole components. Preliminary experiments were carried out in which the labelled TLC spots were eluted and counted for 10 minutes. The silica gel support was subsequently removed and the vial recounted for 10 minutes. From the two sets of count data, an estimate of label solubilization into the scintillation fluid was determined as:

$$\frac{\text{total net sample count rate without support}}{\text{total net sample count rate with support}} \times 100 = \% \text{ solubilization}$$

Although a consistently high solubilization of over 90% was indicated, the experimental results possess an unknown error factor due to the biphasic nature of the counting system.

Prior to counting, a quench correction was made by adjusting the gain mode so that the external standard ratio

(S value) was close to .750. No appreciable colour or chemical quenching was observed in the samples with the S values varying between .75 and .77. The background counting rate was subtracted from the gross counting rate to give net sample counts per minute. The per cent efficiency for counting at the gain setting used was approximately 74%.

In view of the fact that each treatment consisted of two samples which were, in turn, sampled twice, for a total of four determinations per treatment, the non-parametric Mann-Whitney U-test (Siegel, 1956; Sokal and Rolf, 1968) was used in preference to the calculation of a two sigma error factor based upon net sampling counting rate. This choice of statistical evaluation was preferable due to the complexities of the latter analysis for dealing with samples within samples. The Mann-Whitney U-test is the non-parametric equivalent of the conventional t-test for analysing two independent samples without the obligatory requirement for normally distributed data or homogeneity of variances which are demanded of the latter test. Furthermore, the Mann-Whitney U-test is ideally suited for small sample sizes.

7.3 Results

7.3.1 Culture Studies

Since GA_3 is suspected of increasing endogenous IAA levels in non-regenerating systems by a mechanism which may

involve enhanced synthesis from IAA precursors, a series of experiments was carried out in order to determine GA_3 effects on the rooting response induced by various IAA precursors. When TPP was evaluated, a high concentration of GA_3 significantly enhanced the TPP-induced regeneration response (Table 7.2). A similar observation was made for GA_3 effects on TNH_2-induced rooting (Table 7.3). Further work with TNH_2 revealed that the GA_3 response was consistently greater in young expanding leaves than in mature leaves (Table 7.4) with an apparent requirement for such accessory factors as thiamine, pyridoxal phosphate and α-ketoglutaric acid (Table 7.5). GA_3 was progressively more effective in enhancing TNH_2 induced rooting up to a concentration of 1×10^{-5} M GA_3 (Table 7.6). GA_7 gave essentially similar results (Table 7.7). Attempts to replace or modify the GA_3 effect by ABA or Kn were unsuccessful (Table 7.8). When IPyA was evaluated, however, no GA_3 enhanced rooting was evident regardless of IPyA concentration (Table 7.9) or the presence of accessory factors (Table 7.10). TOL, which occurs on a branch pathway from IAAld (see fig. 2.1) gave a slight stimulation at high concentrations and this effect was enhanced by GA_3 (Table 7.11).

ILA, which occurs on a possible branch pathway from IPyA (see Fig. 2.1), has been described as a native compound only in tomato with very weak biological activity in the oat, wheat and maize coleoptile tests and in the pea stem test (Schneider and Wightman, 1974). Surprisingly, ILA had very high root inducing capabilities and they were considerably

Table 7.2. GA_3 effects on rooting in the dark in presence or absence of tryptophan.

Treatment		Mean number of roots per disc.	
Tryptophan	GA_3		
0	0	0.0	a
0	1×10^{-8} M	.1	a
0	1×10^{-6} M	.4	a
0	1×10^{-4} M	.3	a
1×10^{-4} M	0	.1	a
1×10^{-4} M	1×10^{-8} M	.3	a
1×10^{-4} M	1×10^{-6} M	1.2	b
1×10^{-4} M	1×10^{-4} M	1.6	b

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 3.3

Table 7.3. GA_3 effects on rooting in the dark in the presence or absence of tryptamine.

Treatment	Mean number of roots per disc	
Control	0.0	a
GA_3 (1×10^{-4} M)	0.0	a
TNH_2 (1×10^{-4} M)	.1	a
TNH_2 (2×10^{-4} M)	.3	a
TNH_2 (1×10^{-4} M) + GA_3	.3	a
TNH_2 (2×10^{-4} M) + GA_3	2.9	b

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 2.7

Table 7.4. Effect of leaf age on GA_3 stimulation of root regeneration at different TNH_2 concentrations.

Treatment	Mean number of roots per disc	
	LPI_3 = 2.0	LPI_3 = 4.6
Control	.1 a	0.0 a
TNH_2 (1×10^{-4} M)	1.1 b,c	.4 a,b
TNH_2 (2×10^{-4} M)	.7 a,b	.3 a,b
TNH_2 (3×10^{-4} M)	.4 a,b	.4 a,b
GA_3 (1×10^{-4} M)	.5 a,b	0.0 a
GA_3 + TNH_2 (1×10^{-4} M)	2.1 c,d	.8 a,b
GA_3 + TNH_2 (2×10^{-4} M)	2.5 d	.6 a,b
GA_3 + TNH_2 (3×10^{-4} M)	1.3 b,c	.4 a,b

Means followed by the same letter in a column not different at 5% level.

Table 7.5. Effect of GA_3 and various accessory factors on TNH_2 stimulation of rooting.

Treatment*	Mean number roots per disc	
Control	0.0	a
TNH_2 (2×10^{-4} M)	0.0	a
GA_3 (1×10^{-4} M)	0.0	a
TNH_2 + GA_3	0.0	a
TNH_2 + GA_3 + accessory factors	.2	a
TNH_2 + GA_3 + vitamins (1×10^{-6} M)	1.6	b
TNH_2 + GA_3 + accessory factors + vitamins	2.3	b
TNH_2 + accessory factors (5×10^{-4} M)	.1	a
TNH_2 + vitamins	.3	a
TNH_2 + accessory factors + vitamins	.3	a
TNH_2 + accessory factors + vitamins + glycine (5×10^{-6} M)	.5	a
TNH_2 + GA_3 + accessory factors + vitamins + glycine	2.0	b

*accessory factors: 5×10^{-6} M α-ketoglutaric acid, 5×10^{-6} M pyridoxal phosphate; vitamins: 1×10^{-6} M each of thiamine, pyridoxine and nicotinic acid. A subsequent experiment revealed that thiamine alone could replace the vitamin mixture.

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 2.1

Table 7.6. GA_3 concentration effects on TNH_2 enhancement of root regeneration.

Treatment	Mean number of roots per disc	
	$-TNH_2$	$+TNH_2$ (2×10^{-4} M)
Control	0.0 a	.2 a
GA_3 (1×10^{-8} M)	0.0 a	1.1 b
GA_3 (1×10^{-7} M)	.3 a	1.6 b
GA_3 (1×10^{-6} M)	.2 a	1.9 b,c
GA_3 (1×10^{-5} M)	.2 a	2.7 c
GA_3 (1×10^{-4} M)	.4 a	2.2 b,c

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 1.9

Table 7.7. Effect of GA_3 and GA_7 on the enhancement of root regeneration.

Treatment	Mean number of roots per disc	
	$-TNH_2$	$+TNH_2$ (2×10^{-4} M)
Control	.2 a	.6 a
GA_3 (1×10^{-6} M)	.9 a,b	3.5 c
GA_3 (1×10^{-4} M)	2.9 c	4.3 c
GA_7 (1×10^{-6} M)	.5 a	3.2 c
GA_7 (1×10^{-4} M)	2.1 b,c	4.1 c

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 1.6

Table 7.8. Effects of kinetin and ABA on the TNH_2 enhancement of root regeneration.

Treatment	Mean number of roots per disc	
TNH_2 (2×10^{-4} M)	1.4	a
TNH_2 + GA_3 (1×10^{-4} M)	3.7	b
TNH_2 + ABA (1×10^{-8} M)	1.4	a
TNH_2 + ABA + GA_3	3.3	b
TNH_2 + Kn (1×10^{-6} M)	0.0	c
TNH_2 + Kn + GA_3	0.0	c
TNH_2 + Kn (1×10^{-8} M)	1.0	a,c
TNH_2 + Kn + GA_3	3.2	b

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 1.3

Table 7.9. GA_3 effects on IPyA stimulation of root regeneration.

Treatment	Mean number of roots per disc	
	$-GA_3$	$+GA_3$ (1×10^{-4} M)
Control	0.0 a	0.0 a
IPyA (1×10^{-8} M)	0.0 a	0.0 a
IPyA (5×10^{-8} M)	.3 a	.4 a
IPyA (1×10^{-7} M)	.7 a,b	1.1 b
IPyA (5×10^{-7} M)	3.6 c	3.8 c
IPyA (1×10^{-6} M)	5.1 d	5.1 d

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 2.3

Table 7.10 Effects of accessory factors on IPyA stimulation of root regeneration.

Treatment*	Mean number of roots per disc	
IPyA (5×10^{-7} M)	2.6	a
IPyA + accessory factors	3.8	a
IPyA + GA_3 (1×10^{-4} M)	4.1	a
IPyA + GA_3 + accessory factors	3.7	a
IPyA (1×10^{-5} M)	11.4	b
IPyA + accessory factors	11.7	b
IPyA + GA_3 (1×10^{-4} M)	10.4	b
IPyA + GA_3 + accessory factors	12.7	b

*Accessory factors include α-ketoglutaric acid (5×10^{-6} M) and pyridoxal phosphate (5×10^{-6} M).

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 2.6

Table 7.11. TOL concentration effects on root regeneration in the presence or absence of GA_3.

Treatment	Mean number of roots per disc	
	$-GA_3$	$+GA_3$ (1×10^{-4} M)
Control	0.0 a	.2 a,b
TOL (2×10^{-5} M)	0.0 a	.6 a,b,c
TOL (4×10^{-5} M)	0.0 a	.6 a,b,c
TOL (6×10^{-5} M)	0.0 a	.7 a,b,c
TOL (8×10^{-5} M)	.1 a	.8 b,c
TOL (1×10^{-4} M)	.5 a,b,c	1.3 c
TOL (2×10^{-4} M)	.9 b,c	2.4 d

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 2.5

enhanced by GA_3 (Table 7.12). Unlike the TNH_2/GA_3 response system, the GA_3 promotion of rooting in the presence of ILA was not enhanced further by such accessory factors as pyridoxal phosphate and α-ketoglutaric acid (Table 7.13). However, GA_3 was progressively more effective in enhancing ILA-induced rooting up to a concentration of 1×10^{-5} M GA_3 (Table 7.14). No interaction was apparent between ILA and TNH_2 in terms of the presence or absence of GA_3 (Table 7.15). TLC separation of the commercial ILA preparation (Sigma, St. Louis, Mo.) with 3 different solvent systems (see section 7.2.2.3) gave a single homogenous spot in terms of UV fluorescence and a positive colour reaction with the Van Urk reagent. This result indicated a pure ILA sample and suggested that no indole impurities could account for the observations.

7.3.2 Radiotracer Studies

Although ILA gave a more pronounced response in the presence of GA_3 than TNH_2 and GA_3, its effect was totally unexpected in view of the lack of response with IPyA and the fact that tomato is the only species known to contain ILA. Furthermore, very weak biological activity was accorded to ILA and the bulk of the literature suggested a GA_3 mediated effect on auxin biosynthesis by pathway (1). As a result, an evaluation of GA_3 effects on the metabolism of TNH_2-2-^{14}C in tomato leaf discs cultured *in vitro* over a 24 hour period was carried out as outlined in section 7.2.2.

Table 7.12. ILA concentration effects on root regeneration in the presence or absence of GA_3 (1×10^{-4} M).

Treatment	Mean number of roots per disc	
	$-GA_3$	$+GA_3$
Control	0.0 a	.6 a
ILA (1×10^{-6} M)	.2 a	1.1 a,b
ILA (5×10^{-6} M)	1.9 b	4.4 c
ILA (1×10^{-5} M)	4.2 c	5.4 c
ILA (2×10^{-5} M)	5.3 c	12.3 e
ILA (4×10^{-5} M)	8.0 d	10.9 d,e
ILA (6×10^{-5} M)	11.9 e	9.6 d,e

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 2.1

Table 7.13. Effects of accessory factors on ILA stimulation of root regeneration.

Treatment*	Mean number of roots per disc	
ILA (1×10^{-6} M)	.2	a
ILA + accessory factors	.5	a
ILA + GA_3 (1×10^{-4} M)	1.1	a,b
ILA + GA_3 + accessory factors	1.7	b
ILA (1×10^{-5} M)	4.2	c,d
ILA + accessory factors	3.6	c
ILA + GA_3 (1×10^{-4} M)	5.4	d
ILA + GA_3 + accessory factors	5.1	c,d

*Accessory factors include α-ketoglutaric acid (5×10^{-6} M) and pyridoxal phosphate (5×10^{-6} M).

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 2.1

Table 7.14. GA_3 concentration effects on ILA enhancement of root regeneration.

Treatment	Mean number of roots per disc	
	-ILA	+ILA (2×10^{-5} M)
Control	0.0 a	5.7 b
GA_3 (1×10^{-8} M)	0.0 a	6.7 b,c
GA_3 (1×10^{-7} M)	.1 a	8.0 b,c
GA_3 (1×10^{-6} M)	0.0 a	8.2 b,c
GA_3 (1×10^{-5} M)	.1 a	8.5 c
GA_3 (1×10^{-4} M)	0.0 a	8.2 b,c

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 2.3

Table 7.15. Effect of high TNH_2 concentration on the enhancement of rooting by ILA in the presence or absence of GA_3.

Treatment	Mean number of roots per disc -GA_3	+GA_3 (1×10^{-5} M)
Control	0.0 a	.1 a
ILA (1×10^{-6} M)	.2 a	.3 a,b
ILA (1×10^{-5} M)	3.4 d	5.5 e,f
ILA (2×10^{-5} M)	6.6 f	8.7 g
TNH_2 (1×10^{-4} M)	.2 a,b	1.6 c
TNH_2 + ILA (1×10^{-6} M)	.5 a,b	1.2 b,c
TNH_2 + ILA (1×10^{-5} M)	3.6 d,e	5.2 e,f
TNH_2 + ILA (2×10^{-5} M)	6.4 e,f	9.7 g

Means followed by the same letter in a column not different at 5% level.

Mean LPI_3 = 2.5

A preliminary experiment confirmed the suspicion that TNH_2-2-^{14}C could be converted into IAA within the leaf tissue during a 24 hour incubation period. Control flasks without tissue gave no evidence of any spontaneous breakdown of TNH_2-2-^{14}C into IAA during the 24 hours. Consequently, duplicate samples of 100 discs per 5 ml incubation medium containing 2 μc of TNH_2-2-^{14}C were prepared as outlined in section 7.2.2 and sampled at 6, 12 and 24 hours after the start of the experiment. The results indicated that IAA was synthesized in the tissue within 6 hours after exposure to TNH_2-2-^{14}C (Fig. 7.1). Increased label incorporation into IAA was apparent at progressively later times with no apparent effect of GA_3 on the incorporation (Fig. 7.2 and 7.3). However, an analysis of the net count rate data did indicate a GA_3 mediated decrease in the level of incorporation at 12 hours into the radioactive peak at R_f .70 - .85 which includes the indole metabolites TOL, IAAld, and Iald (Table 7.16). No evidence was found to support the idea of extracellular synthesis or leaching of IAA from the tissues (Fig. 7.4). Over 90% of the applied radioactivity was recovered after 24 hours incubation in the presence or absence of GA_3 (i.e. incubation medium, 35-40%; distilled water washing, 20-25%; methanol soluble tissue extract, 30-37%; and pellet, 1%).

7.4 Discussion

The culture experiments indicate that the enhancement of rooting by GA_3 can be related to specific IAA precursors

Figure 7.1. TLC distribution of labelled tryptamine metabolites in tissue extracts after 6 hour incubation in the presence (---) or absence (——) of 1×10^{-5} M GA_3. The radioactivity is expressed as counts per minute (cpm) and is uncorrected for a background count of 36.5 cpm.

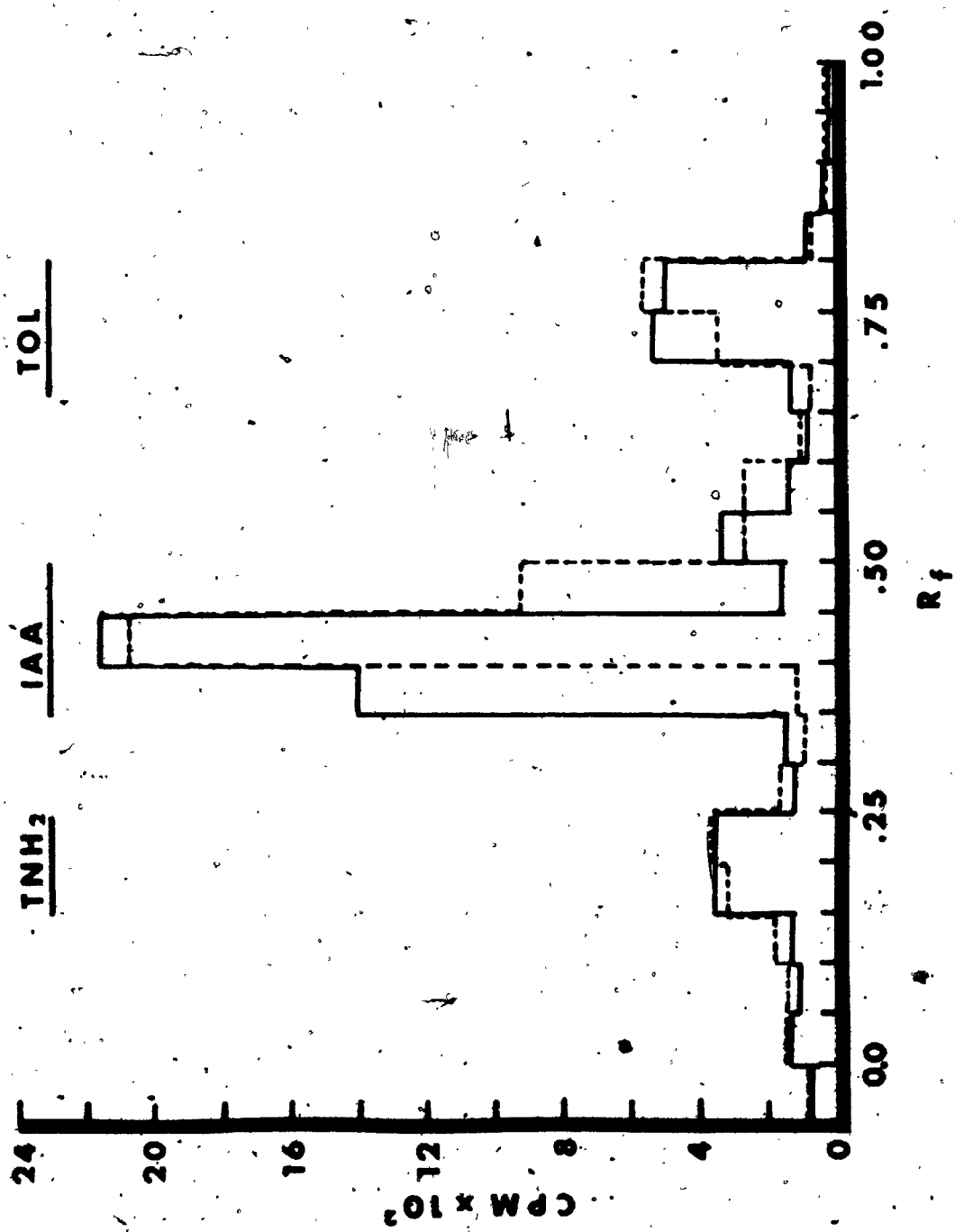

TOL
IAA
TNH2
24
20
16
12
8
4
0
CPM x 10³
.00
.25
.50
.75
1.00
Rf

Figure 7.2. TLC distribution of labelled tryptamine metabolites in tissue extracts after 12 hour incubation in the presence (---) or absence (——) of 1×10^{-5} M GA_3. Radioactivity (cpm $\times 10^2$) is uncorrected for background of 38.9 cpm.

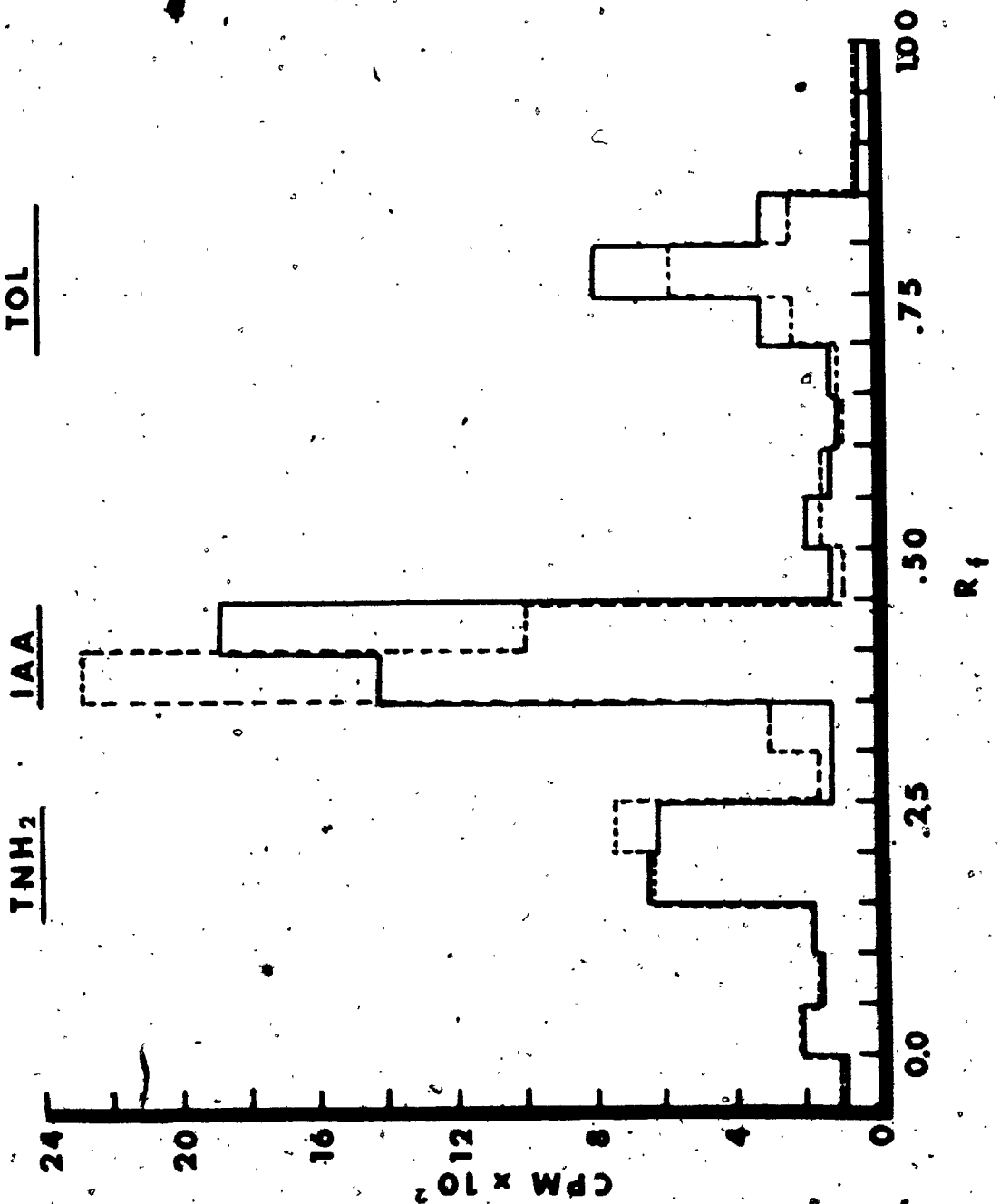

TOL
IAA
TNH2
24
20
16
12
8
4
0
CPM x 10²
0.0
.25
.50
.75
1.00
Rf

Figure 7.3. TLC distribution of labelled tryptamine metabolites in tissue extracts after 24 hour incubation in the presence (---) or absence (——) of 1×10^{-5} M GA_3. Radioactivity (cpm $\times 10^3$) is uncorrected for background of 38.6 cpm.

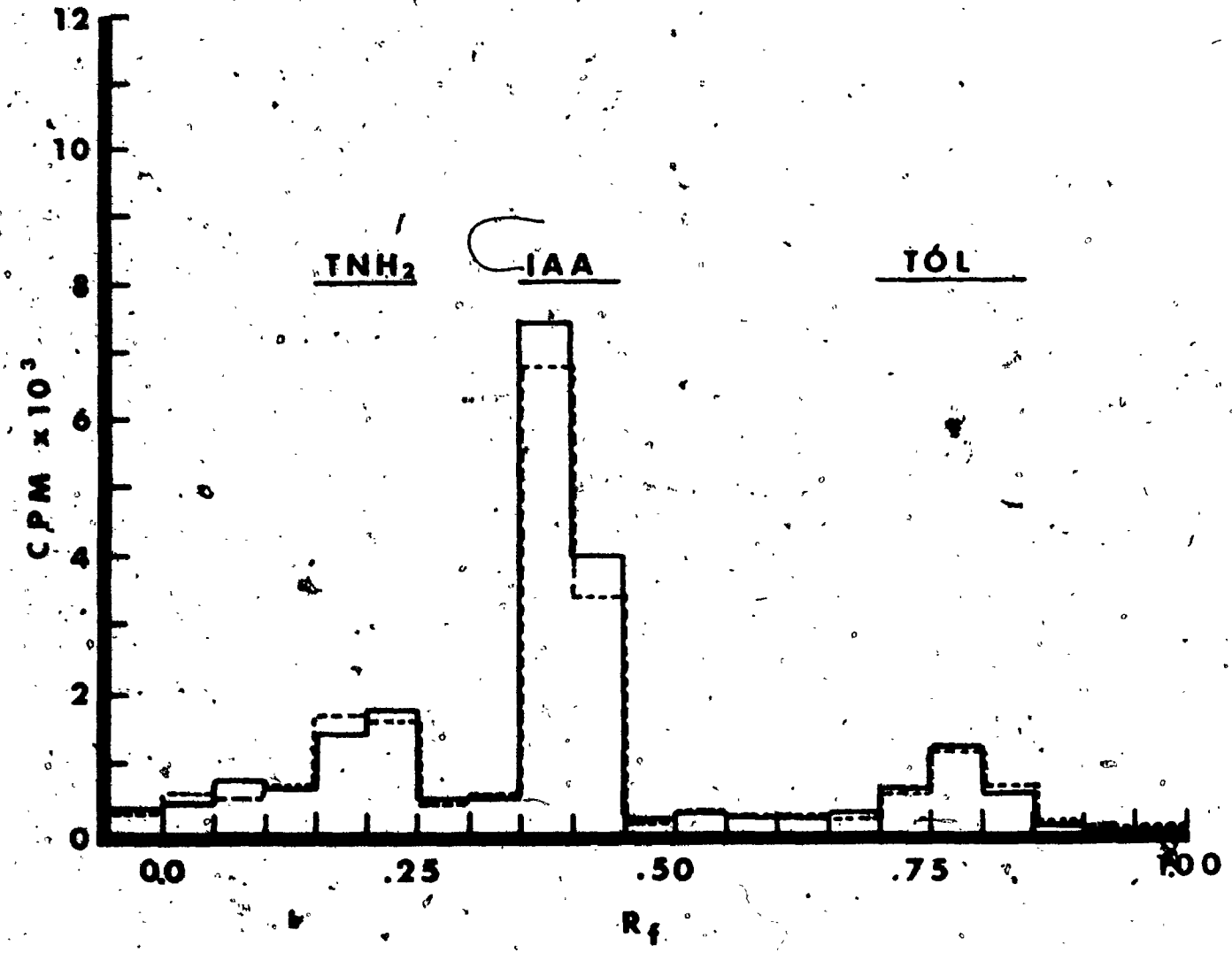

12
10
8
6
4
2
0
CPM x10³
TNH₂
IAA
TÓL
0.0
.25
.50
.75
1.00
Rf

Table 7.16. GA_3 effects on the TNH_2 metabolism within tomato leaf-discs cultured *in vitro* over a 24 hour period.

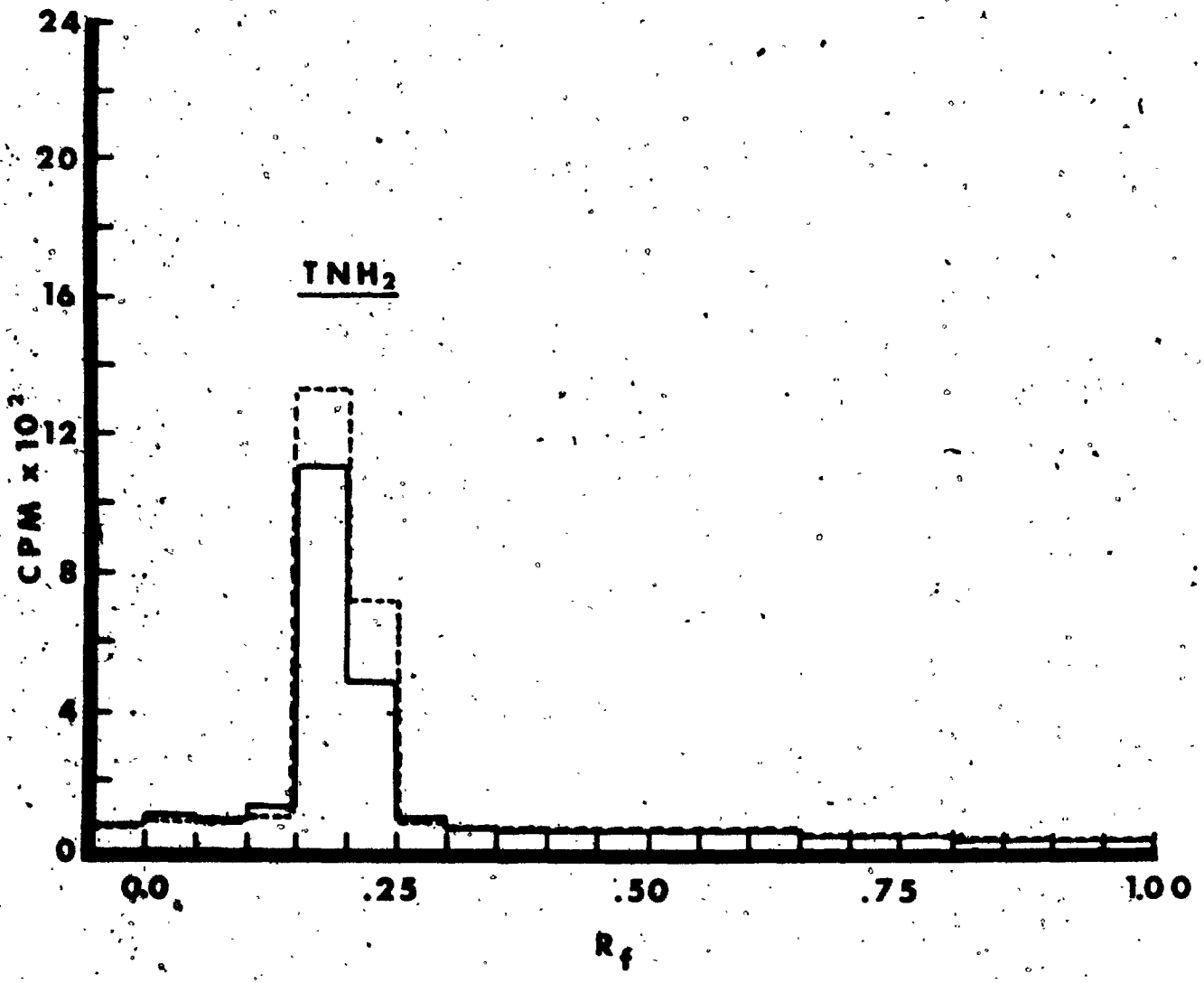
Sample time	Treatment	Mean Net cpm[a] TNH_2		IAA		TOL		Relative amounts of IAA formed[b]
6 hrs.	TNH_2 (8.6×10^{-6} M)	651.9		2896.9		1131.4		61.8%
	$TNH_2 + GA_3$ (1×10^{-5} M)	746.5	NS	2811.9	NS	1070.1	NS	60.7%
12 hrs.	TNH_2	1300.3		3438.1		1368.2		56.3%
	$TNH_2 + GA_3$	1207.1	NS	3596.3	NS	930.7	*	62.7%
24 hrs.	TNH_2	3736.0		12279.8		2310.4		67.0%
	$TNH_2 + GA_3$	3958.5	NS	10775.4	NS	2148.5	NS	63.8%

[a]Mean net cpm for 6 hrs and 12 hrs based upon two samples which were, in turn, sampled twice for a total of four determinations per treatment. Mean net cpm for 24 hour based upon one sample which was, in turn, sampled three times for a total of three determinations per treatment. TNH_2 activity may include the IAA breakdown product, indole-3-glyoxylic acid. Similarly, TOL activity may include the IAA precursor, IAAld, and the IAA breakdown product, Iald. Each pair of mean net cpm for each metabolite at the various times were individually compared by the Mann-Whitney U-test outlined by Siegel (1956) and Sokal and Rolf (1968). No comparisons were made among different times or different metabolites.

*significant at 5% level; NS - not significant

[b]Based on the total amount of labelled indole metabolites present in the methanol soluble fraction.

Figure 7.4. TLC distribution of labelled tryptamine metabolites in tissue media after 24 hour incubation in the presence (---) or absence (——) of 1×10^{-5} M GA_3. Radioactivity (cpm x 10^2) is uncorrected for background of 43.0 cpm.

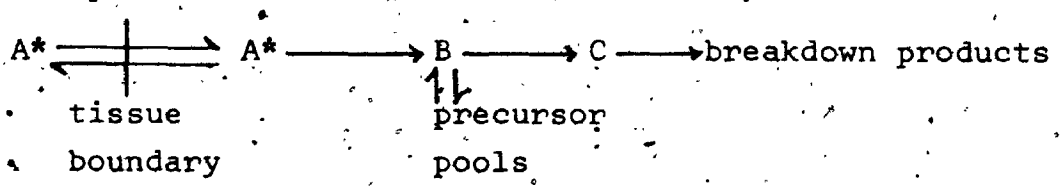

24
20
16
12
8
4
0
CPM x 10²
TNH₂
0.0
.25
.50
.75
1.00
Rf

such as TPP, TNH_2 and ILA and, hence, could involve a GA_3 mediated increase in endogenous auxin levels. The present study did not demonstrate any stimulatory effects of GA_3 on IPyA induced rooting. Although GA_3 stimulated TOL induced rooting, the response appeared essentially additive with no indication of a direct interaction between the two substances. Furthermore, GA_3 has no effect on tryptophol oxidase isolated from cucumber shoots which suggests (barring a species specific response) that the GA_3 mechanism of action does not reside directly in TOL metabolism (Percival, Purves and Vickery, 1973 as cited in Schneider and Wightman, 1974).

The results with TPP and TNH_2 agree with results from non-regenerating systems in the sense that the GA_3 effect appears to be related to pathway (1). Subsequent experiments dealing with the GA_3 enhancement of TNH_2 induced rooting have indicated the following: (i) the GA_3 induced response is concentration dependent; (ii) increased rooting is further enhanced by such accessory factors as α-ketoglutaric acid and pyridoxal phosphate which are known to be co-factors for a number of enzymes involved in IAA biosynthesis (e.g. tryptophan decarboxylase, amine oxidase); (iii) GA_3 can be replaced by GA_7; and (iv) the GA_3 effect cannot be replaced by either Kn or ABA. Although radiotracer studies confirm the suggestion by Wightman (1973) that active IAA synthesis can take place within young tomato tissue from TNH_2, the present experiment failed to confirm my hypothesis

that the GA_3 effect on the stimulation of rooting in cultured tomato leaf discs can be related to enhanced conversion of TNH_2 to IAA. Furthermore, no extracellular synthesis or leaching of labelled IAA was evident in either the GA_3 treated or control materials after 24 hour incubation. These results differ from Black's observations that extracellular synthesis of IAA from TPP did occur after 48 hours in media which had previously supported the growth of soybean callus tissue for 2 to 14 days (Black, 1970).

However, the experiment outlined above cannot be considered as definitive proof for a lack of GA_3 involvement in pathway (1) as no consideration was given to such aspects as uptake rates, intracellular compartmentation of indole compounds and turnover of metabolites. Glasziou (1969) has outlined the problems faced with radiotracer incorporation studies and gives the following example for a hypothetical labelled precursor A*.



According to Glasziou (1969), "the labelled precursor A* is added to the medium with and without regulatory substance and a difference in radioactivity in C is taken to indicate an effect on its synthesis or turnover. In fact, if the regulatory substance affected the rates of any of the reactions by any means, it would almost certainly change

the amount of radioactivity appearing in C. For example, assuming it accelerated the formation of B from intracellular pools, it could conceivably double the pool size of B, decrease its specific activity to half or considerably less, and change the rate of synthesis of C not at all or up to double. The amount of radioactivity in C could easily register a decrease under circumstances in which synthesis of C increased". Consequently, the present results indicate only the presence of intracellular biosynthesis of IAA from TNH_2. Further experiments with a "pulse chase" design are necessary in order to evaluate problems of uptake, compartmentation and turnover, and the effect that GA_3 has upon these factors.

Wightman has suggested that the IPyA route to IAA could be the main auxin pathway in tomato (Wightman, 1973). However, the present evidence from radiotracer work indicates a rapid (i.e. within 6 hours) and high incorporation rate of ^{14}C label from TNH_2-2-^{14}C into IAA in young leaf material. This result suggests that the TNH_2 route could be as important as the IPyA route in tomato - especially in young expanding tomato leaves where high levels of IAA are known to occur (Schneider et al., 1972; Wightman, 1973). Furthermore, exceptionally high levels of TNH_2 (i.e. 1000 µg/kg fresh wt. shoot tissue) have been recorded in tomato which suggests that fluctuations in the levels of either endogenous gibberellins or TNH_2, or both, could have important consequences on endogenous IAA synthesis and, ultimately, plant

development. It should be noted that the main pathway to IAA in at least two other plant species (i.e. cucumber and oats; Sherwin and Purves, 1969) proceeds via TNH_2 rather than IPyA. Extensive studies by Muir and Lantican (1968) indicate that GA_3 promotes the conversion of both TPP and TNH_2 to IAA by an enzyme system from oat coleoptile tissue. However, the same workers noted that GA_3 appeared to promote the conversion rate of TPP to IAA in pea apices (cv. Little Marvel) but not the conversion of TNH_2. They suggested that the GA_3 effect involved the decarboxylation of TPP to TNH_2 and not the oxidation of TNH_2 to IAAld. This conclusion has been supported by Valdovinos and Ernest (1966) who noted that GA_3 caused an increase in the evolution of labelled CO_2 from TPP-1-^{14}C supplied to macerates of Little Marvel apical tissue. Although the Avena curvature test was used as the auxin assay in the experiments of Lantican and Muir, subsequent work with TPP-1-^{14}C has supported the observation that GA_3 doubled the initial conversion rate of TPP to IAA when GA_3 was applied to cell-free extracts of oat coleoptiles (Valdovinos and Sastry, 1968). However, the latter authors suggest that the GA_3 effect on auxin formation probably involves the TNH_2 to IAA step although the precise site of action is unknown.

The use of in vitro or cell free extracts to demonstrate GA_3 enhancement effects on IAA synthesis from TPP or TNH_2 indicate a direct, although presently unknown, mode of action. Although Valdovinos and Sastry (1968) have

speculated that the site of action may be at the ribosomal level, no substantive evidence has appeared (e.g. that protein synthesis occurs in their systems). An interesting, although invariably ignored observation, has been made by Lantican and Muir (1967) which may provide some insight into the possible site of GA_3 action. Using a cell-free enzyme preparation from oat coleoptiles which was capable of synthesizing IAA from TPP, they observed that GA_3 additions to the enzyme extract restored the enzyme's activity which was lost by dialysis. In view of their use of the Avena curvature bioassay to monitor IAA synthesis in the enzyme preparations, further work with labelled precursors is highly desirable with a view to 1) extracting and characterizing the tryptophan decarboxylase and amine oxidase enzymes; and 2) evaluating the possible role of GA_3 as a post-translational effector substance (e.g. an enzyme cofactor molecule).

The results obtained with ILA were surprising and unfortunately, were made too late in this study to fully evaluate the problem. Although this indole derivative has been described only in tomato, Wightman suggests that it may be present in other species as well (Schneider, Gibson and Wightman, 1972). The similar R_f value between ILA and IAA in a number of PC and TLC solvent systems and the tendency to monitor indole compounds on the basis of colorimetric reaction and biological assay only may be responsible for its lack of detection. The pronounced activity of ILA in

this root regenerating system and the apparent enhancement of ILA induced regeneration by GA_3 suggests that GA_3 may directly affect the metabolism of ILA either i) by preventing its further metabolism and causing it (and possibly IPyA) to accumulate within the tissues which would in turn lead to increased endogenous IAA levels; or ii) by enhancing the metabolism of ILA into a more active (and presently unknown) indole auxin. With regard to ii), it is important to note that the auxin literature over the past 25 years has consistently pointed out the possibility of other naturally occuring indole auxins such as IPA and IBA which may be derived from different indole intermediates in the IAA pathway (e.g. see individual papers by Blommaert, 1954; Fischer, 1954; Linser, Mayr and Maschek, 1954; Melchior, 1958; Bayer, 1969; and review by Fawcett, 1961). In the tomato root regeneration system it has been shown experimentally that both IPA and IBA are highly active in stimulating root regeneration if supplied exogenously. Removal of the hydroxyl group from the carbon 2 position of the 3 carbon side chain would change ILA to IPA. Whether such a reaction is possible in this species is presently unknown. Nevertheless, the natural occurrence of other indole auxins with biological activities as great or greater than IAA, warrants further study in order to delineate their biosynthetic pathways and ultimate relationship to the plant's development.

CHAPTER 8

SUMMARY AND CONCLUSIONS

T. H. Morgan is reputed to have said, in a lighter moment, that since he had been unable to solve the problem of regeneration, he had decided to try something easier such as the problem of heredity (Bonner, 1963). Similar feelings are prevalent today. Nevertheless, although the problem of regeneration is quite complex, attempts continue to be made towards delineating regenerative processes with the ultimate goal of describing (and explaining) developmental events in biochemical terms. A necessary preliminary step in this direction must encompass the development and evaluation of a suitable regeneration system which will be amenable to biochemical probing. In this regard, the usefulness of the morphological index, LPI, for describing tomato leaf development and root regeneration capacity from leaf cuttings cannot be over-emphasized.

In the present study of tomato leaf development, the LPI has provided a precise and reproducible yardstick by which quantitatively defined events of leaf growth and development can be related to specific qualitative aspects of the leaf's ontogeny. This study is the first detailed

analysis of tomato leaf ontogeny as well as the first description of a compound leaf which exhibits a basipetal sequence of leaflet initiation. Hopefully, this report will correct the misconception held by some physiologists that the tomato plant is a "twisted distortion of the ordinary rules of plant architecture" (see section 3.1). Besides enlarging upon Bedesem's (1958) summary of tomato leaf ontogeny, the present study corrects some of his interpretive errors and explores the relatively untouched developmental problem of minor vein ontogeny. Contrary to previous studies, I was able to derive a generalized sequence of stages for minor vein ontogeny (see Figure 3.17) based upon extensive studies of cleared and sectioned tomato leaf lamina tissues which were viewed paradermally as originally suggested by Foster (1952). Furthermore, this study of tomato leaf lamina and vein development has uncovered histological evidence to support the concept of "morphological fields" in terms of a close interplay between planes of mesophyll cell division and subsequent minor vein ontogeny. These events will probably be found to encompass both subtle changes in hormonal/nutritional fields and biophysically defined stresses on the individual cells of the growing organ.

If we hypothesize leaf form as the end result of the relative activity and duration of five interrelated meristems within the leaf primordium, then further research into leaf ontogeny must concentrate on the quantitative evaluation of

meristematic activity and must include a delineation of cell cycle populations within the different tissue regions. The use of single gene mutants which affect leaf form at different stages in the primordium's development may be used advantageously in this regard. At present, the literature contains exceptionally few attempts to examine this neglected, yet important, developmental problem.

Closely related to the leaf's developmental state is that leaf's ability to regenerate organs when severed or physiologically isolated from the remaining plant body. The present study of root regeneration from tomato leaf cuttings has validated this concept with subsequent use of the LPI for studies centering on the regulation of regeneration by GA_3. Contrary to an earlier suggestion put forth by Thorpe and his associates (Thorpe and Meier, 1974a; 1974b) that GA_3 inhibits starch accumulation and subsequent shoot primordium formation in tobacco callus cultures primarily by increasing the content of the starch hydrolyzing enzyme, α-amylase, the present study provides evidence which suggests that the initial GA_3 effect does not involve increased starch hydrolysis. Rather, GA_3 appears to inhibit localized starch synthesis in the incipient root primordia regions leading to a subsequent failure to produce primordia. Further work in this area should include an evaluation of the specific activities of the plastid-localized starch synthesizing enzymes as well as their synthesis and turnover as affected by GA_3. The role of starch synthesizing plastids in organ

regeneration has never been considered until now and should be explored at the biochemical level.

A second root regeneration system was developed which utilized 5 mm diameter tomato leaf discs cultured *in vitro*. When the leaf discs were cultured aseptically on defined nutrient media, root regeneration was totally dependent on a number of external factors such as organic and inorganic nutrients and hormones of the auxin class. Furthermore, root primordia could originate from two distinct types of tissues simultaneously, i.e. type I primordia from the phloem parenchyma of the primary and secondary veins, and type II primordia from the sheath parenchyma of the minor veins. However, the roots from both sites were histologically "normal" for induced root primordia. Histochemical studies at the light microscopy level established that the dedifferentiation → meristemoid sequence was accompanied by localized increases in nuclear and cytoplasmic RNA as well as increased nuclear and nucleolar volumes. Increased total and basic protein staining of the nucleolus and cytoplasm paralleled the increased RNA levels and probably signified the onset of increased ribosomal synthesis necessary for subsequent synthesis of proteins which was, in turn, necessary for organ formation.

Although crystalline protein microbodies developed in the organogenic and non-organogenic tomato leaf discs, they were interpreted as peroxisomes rather than protein storage bodies. Similar crystalline inclusions were observed in

tobacco callus cultures and were hypothesized to be involved in supplying substrates for developing shoot primordia in the callus cultures (Ross et al., 1973) although previous work (Matsushimo et al., 1969; Matsushimo, 1971) appears to have discounted this idea. Present evidence supports the concept of a simultaneous, yet essentially independent, development of peroxisomes associated with increased photorespiratory activity of the cultured tissues.

The use of the tomato leaf disc system has revealed multiple roles of GA_3 in the inhibition of rooting:

1) an early inhibitory effect (i.e. during the initial 48 hours of culture) which can be related to a GA_3-induced failure to rapidly accumulate starch within the leaf tissue;

2) a late inhibitory effect which can be related to a GA_3 mediated destruction of late forming primordia by a parenchymatization process which has its origin in an earlier GA_3 effect on growth stimulation of the adjacent cortical parenchyma.

3) an inhibition of meristemoid formation from sheath parenchyma derivatives surrounding the minor veins. This inhibition would insure an absence of type II primordia formation.

Further work with the leaf disc culture system has revealed an apparent GA_3 stimulation of rooting in continuous darkness which can be related to the specific auxin precursors, TPP, TNH_2 and ILA. Attempts to study the possible GA_3 effects on endogenous IAA synthesis through the

continuous feeding of TNH_2-2-^{14}C over a 24 hour period revealed no significant effects on label incorporation into IAA although IAA synthesis was detected within 6 hours after the initiation of the experiment. As pointed out in section 7.4, the present experiment cannot be considered as definite proof for a lack of GA_3 involvement in the synthesis of IAA from TNH_2 as no consideration was given to such aspects as uptake rates, intracellular compartmentation of indole compounds and turnover of metabolites. Further quantitative studies are needed in these areas.

The observation that ILA is a highly active indole derivative in the induction of rooting in tomato leaf discs cultured in vitro and can be further increased in activity by GA_3 is quite surprising and without precedent in the literature. As noted in Chapter 7, ILA has been reported only in tomato although its absence in other species may simply be due to lack of detection. Whether GA_3 prevents its breakdown leading to increased ILA (and possibly IPyA) levels or enhances the synthesis of a highly active (and presently unknown) indole auxin from ILA is unknown. The literature has consistently pointed out the possibility of other naturally occurring indole auxins such as IPA and IBA. Furthermore, Wightman (1973) has recently uncovered a separate endogenous auxin pathway which converts phenylalanine to the weak growth promoting substance, phenylacetic acid.

It is apparent that this area of hormonal physiology is ripe for detailed metabolic studies on the origins and

fates of endogenous indole auxins. The relevance of these additional, and as yet uncharacterized, auxin pathways to the plant's growth and development remain to be uncovered.

APPENDIX

STARCH STAINING TECHNIQUE

1. Introduction

During a histochemical examination of adventitious root initiation from tomato leaf discs cultured in vitro (see Chapter 5), attempts to stain starch grains within the fixed tissue sections using IKI solutions (Jensen, 1962) were unsuccessful although starch was readily demonstrated in fresh tissue preparations. The alternative method available to the plant histologist (i.e. the PAS method; Jensen, 1962) utilizes a very light sensitive and labile stain and is quite time consuming, with numerous steps in the procedure. Fortunately, histochemists have developed a number of techniques for staining glycogen, a macromolecule similar in composition to the amylopectin component of starch although differing in the number of glucose residues found in the side branches. Of the methods available, the demonstration of glycogen by means of silver complex formation is relatively specific and gives a dark metallic deposit which is readily photographed (Pearse, 1968). The silver methods are, in general, similar to the PAS method in the sense that they employ acid hydrolysis and oxidation

of the glucose residues to reveal aldehyde groups in the polysaccharide chain prior to their combination with silver. However, the available methods are still lengthy. Furthermore, no attempts have been made to adapt these silver methods to plant materials. Consequently, the following procedure (i.e. the Periodic acid-ammonical silver or PAAS method) for starch staining by means of a silver complex was developed as a simple alternative to the two conventional histochemical procedures for the characterization of starch.

2. Materials and Methods

Due to the large deposits of starch found in the root cap cells, adventitious roots induced on defined culture media from tomato leaf discs (see section 5.2) were subsequently fixed in cold 10% formalin in .1 M phosphate buffer (pH 7.1) for 1 - 1 1/2 hours. Subsequent dehydration, embedding and longitudinal sectioning of the root tips at 5 μ were carried out using the standard microtechniques of Jensen (1962). Following dewaxing, hydration and brief washing in tap water, the staining procedure was carried out as follows:

1. Immerse slides in 5% aqueous periodic acid for 60 minutes at 60°C. Variations of this treatment and their consequences for starch staining are listed in table 1. Control sections were untreated.

2. Wash slides in 3 to 4 changes of distilled water. In order to test the hypothesis that the periodic oxidation step was in fact producing aldehyde groups necessary for

subsequent silver staining, duplicate slides were blocked at this step by one of four different procedures (see Pearse, 1968):

i) Methylation a) mild; acidified (HCl) methanol for 24 hours at 37°C. This step will block carboxyl groups (among others); b) drastic; acidified methanol for 24 hours at 60°C. This step abolishes the PAS staining reaction. Sections are then rinsed briefly in several changes of distilled water.

ii) Bisulphite blocking for aldehydes; 10 ml ethanol are added to 40 ml of 50% aqueous sodium bisulphite. Sections are treated for 3 hours at room temperature and then rinsed briefly in several changes of distilled water. According to Pearse (1968), sodium bisulphite forms addition compounds with aldehydes and ketones.

iii) Amine-aldehyde condensation; 90 ml acetic acid are added to 10 ml aniline. Sections are treated for 30 minutes at room temperature and then rinsed briefly in several changes of distilled water.

iv) Dimedone blocking for aldehydes; saturated solution of dimedone (5:5 dimethyl-cyclo-hexane-1,3-dione) in 5% acetic acid. Sections are treated for 3 hours at 60°C and then rinsed briefly in several changes of distilled water. According to Pearse (1968), this reagent forms condensation products with glacial acetic acid solutions of aldehydes. The reaction is believed to be specific for aldehydes and is capable of abolishing the PAS reaction.

3. The slides were subsequently stained in freshly prepared ammoniacal silver for 60 seconds at room temperature with agitation. To prepare the stain, add 10% aqueous silver nitrate dropwise to 4 ml of concentrated ammonium hydroxide until a persistant turbidity occurs (approximately 10:1 v/v).

4. The slides were washed briefly in five changes of distilled water.

5. Dehydrated and mounted in Permount.

3. Results

Periodic acid oxidation of insoluble polysaccharides for 60 minutes at 60°C allowed excellent staining of starch granules by the ammoniacal silver method (Table 1). Starch grains stain a deep yellow to yellowish brown and are readily photographed by any of the commercially available colour films. The stained preparations have not faded after four years of storage at room temperature. When lower temperatures of periodic acid treatment were used, comparable staining was achieved only after 24 hours treatment in periodic acid. No appreciable staining of primary cell walls was observed in any of the treatments and no staining occurred without the periodic acid treatment. While methylation, dimedone and aniline/acetic acid were able to abolish the subsequent staining reaction, the bisulphite blocking for aldehydes had no apparent effect. The reason for the failure of the bisulphite treatment to block the PAAS staining is unknown. The bisulphite forms addition

Table 1. Effects of periodic acid treatment duration, post-formalization and selective blocking procedures on starch grain staining.

Periodic acid duration	Temperature	Post-periodic acid blocking procedure	Post-formalinization	Staining results*	
				Starch grains	Cytoplasm, nucleus, cell walls, etc.
0 min.	---	---	---	0	0
10 min.	RT (25°C)	---	---	0	0
60 min.	RT	---	---	+	0
24 hrs.	RT	---	---	++	0
60 min.	60°C	---	---	++	0
60 min.	60°C	---	2 min. RT	++	++
60 min.	60°C	mild methylation	---	0	0
60 min.	60°C	drastic methylation	---	0	0
60 min.	60°C	bisulphite	---	++	0
60 min.	60°C	aniline-acetic acid	---	0	0
60 min.	60°C	dimedone	---	0	0

* 0 - no staining; + - faint staining; ++ - intense staining

compounds with aldehydes and ketones which can, in turn, be broken down in dilute alkali into aldehydes or ketones (Pearse, 1968). Since the staining solution is alkaline, this blocking product could conceivably be broken down rapidly enough to allow aldehyde-silver ion interactions. Another possible explanation - too short a blocking time - should also be mentioned.

Post-formalin treatment allowed a wider variety of cell constituents to be stained (e.g. cytoplasm, Casparian strip, vessel elements) without affecting the starch staining.

4. Discussion

The use of ammoniacal silver solution for staining polysaccharides in animal cells depends on prior acid hydrolysis and oxidation of the glucose residues to produce aldehyde groups *in situ* which reduce the diamminesilver (I) ion to metallic silver (Pearse, 1968). On the basis of the present results a similar mechanism appears to operate in the procedure outlined in this paper.

The reasons for the lack of appreciable cell wall staining are obscure. While a periodic acid oxidation of the primary cell wall material would be expected, the subsequent yellow colouration produced by silver deposition may be too faint to be distinguished from light refracted through the wall. The starch grain must present a very favourable site for colloidal silver deposition by virtue of the high density

of periodic acid-produced aldehyde groups. Consequently, this method would be suitable for defining starch grains in fixed plant material in cases where the traditional iodine-potassium iodide method is undesired or inadequate and the PAS method too cumbersome. The described TAAS method for starch grains is rapid, simple and gives permanent reproducible staining of good contrast with no evidence of starch grain degradation.

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