STUDIES OF THE ROOTING OF CUTTINGS OF *HYDRANGEA MACROPHYLLA* L: DNA, PROTEIN AND ENZYME CHANGES

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FORWARD

An unusual format has been adopted for this thesis, which is divided into two main parts. The first part concerns the anatomical changes occurring during adventitious root development of *Hydrangea macrophylla* and associated protein and DNA changes. The second part deals with enzyme changes in root initials during adventitious root formation.

In addition to the general literature review, two manuscripts are presented in the format required by the Canadian Journal of Botany. A general discussion at the end of the thesis summarizes the findings of the entire investigation.

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ABSTRACT

Molnar, Joseph Michael, Ph.D., The University of Manitoba, October 1971. Studies of the rooting of cuttings of *Hydrangea macrophylla*: DNA, protein and enzyme changes. Major Professor: Lucien J. LaCroix, Department of Plant Science.

The anatomy of root initiation of *Hydrangea macrophylla* and associated changes in protein and DNA synthesis, and enzyme activities during root initiation, is described.

Observations showed that adventitious roots originated from preformed root initials in the phloem ray parenchyma cells. The roots appeared ten to twelve days after the cuttings were made. It was found that the total protein content of the root initials increased over 100% in the first four days, while there was no rapid increase in DNA content of the cells until the sixth day. The estimated mean content of total protein per cell stabilized on or before the sixth day from the time the cuttings were made. Thenceforth it appeared to come into equilibrium with the rate of cell division. While increased protein synthesis and doubling of DNA do not necessarily lead to cell division, in the tissue studied it appeared that this was the sequence of events.

Extensive changes occurred in enzyme activity. All enzymes investigated had increased activity in the tissue

responsible for root initiation. The first change observed was that of peroxidase in phloem and xylem ray cells, well before there was noticeable change in anatomy. Increased activity of cytochrome oxidase was detectable only in the root initials two days after the cuttings were made. Succinic dehydrogenase activity could be detected on the third day, and this enzyme exhibited increased activity not only in the root initial, but also in the phloem area surrounding the root initial.

Alpha-amylase was localized by the substrate film method. The highest amylase activity was observed in the epidermal tissues and vascular bundles. As the root primordia developed the enzyme activity shifted from the vascular bundles to the periphery of the bundles, especially to the phloem and xylem ray cells. This area was very high in starch content at the time the cuttings were made.

There was a positive correlation between the number of roots initiated and the starch content of the cuttings.

INTRODUCTION

Extensive research has been carried out on the rooting of cuttings. Much of this has been concentrated on the anatomy of root initiation and the environmental factors affecting rooting. In spite of the volume of literature on this subject, little information is available on some of the biochemical processes occurring in root initiation.

Microscopic histochemistry might be a useful tool in the identification and localization of some of the biochemical changes which occur before, or during adventitious root development. Knowledge of the actual site of action of a specific enzyme is essential to an understanding of the physiological role of an enzyme in the economy of the cell or organism.

The nucleus is the seat of genetic information, namely in its DNA component, and is primarily of importance in supplying the plan for development. Expression of the genetic plan, on the other hand, undoubtedly occurs principally in the cytoplasm, through control of the mechanism of protein synthesis. One of the main problems in developmental physiology is elucidation of the means by which control is achieved.

It is becoming evident that hormones do not act alone

in isolated systems, but in an interrelated manner in the plant as a whole. Hormonal growth control might thus involve an increase in the general protein producing machinery, rather than triggering the production of a particular protein. In some cases, possibly by promoting synthesis of messenger RNA molecules, hormones give rise to new synthesis of specific enzymes. The enzymes, in turn, control the biochemistry and thereby the physiology of the organism. It is therefore reasonable to presume that starch hydrolyzed by enzymes, provides the energy and substrate required for the execution of the metabolic processes of root initiation.

Most of the histochemical research has been concentrated on enzyme changes in the apical and root meristems. Little attention has been paid to the histochemical changes which occur during root initiation.

The purpose of this investigation is to present an anatomical description of root initiation of hydrangea cuttings, which has not been described before; and to obtain some insight into the protein and DNA synthesis, and some of the enzymatic changes which occur in the developing root primordia. The enzymes investigated were succinic dehydrogenase, cytochrome oxidase and peroxidase. Alpha-amylase was localized by the substrate film method in the cuttings. The effect of starch content on the rooting of cuttings was investigated.

LITERATURE REVIEW

Introduction

Adventitious root formation is a complex process in which many factors are involved (95). These include the action of auxin, the utilization of carbohydrates, the action of specific substances such as biotin and an internal co-factor specific to the organism. Rapidly growing buds actively promote root formation whereas dormant buds do not. Removal of the growing buds stops root formation almost completely. Adventitious root formation is due to the presence of specific substances formed in the actively growing shoot apex.

The aim of microscopic histochemistry is the localization and identification of substances and enzyme activity within cells and tissues. Histochemistry as such, was founded by the French botanist, Raspail in 1825. Raspail's difficulties in having his research published are indicative of the general position of microscopic histochemistry in his day. In 1830 Raspail published his major work at his own expense after it had been rejected by a committee consisting of a physiologist, a chemist and a botanist. The reason for the rejection, according to Raspail, was

that, "The physiologist was ignorant of chemistry, the chemist of microscopy and the botanist of both" (8).

Great advances have taken place in the field of histochemistry in the past thirty years. The number of procedures alone has increased many fold. Yet few of the procedures have been used by the plant scientist. There seem to be two primary reasons for this neglect of techniques by investigators in plant science (45). The first reason lies in the fact that most of the newer methods have been designed primarily for use with animal tissues (9,45), and secondarily, if at all, for use with plant tissues.

The second reason lies in the lack of appreciation for the application of histochemical techniques to the solution of botanical problems. Plants, and their parts, are extremely complex, both morphologically and physiologically. Histochemical procedures permit the recognition of this complexity and provide data which can be interpreted in terms of cells, tissues, and tissue systems. As plant scientists are becoming increasingly concerned about problems which require answers in their terms, histochemical methods must become increasingly important in research with plants (45).

Propagation

The art of propagating plants from cuttings is old and may have been started with man's initial interest in ornamental, medicinal, fruit and field crops. As a science, the propagation of plants from cuttings did not begin to develop until the eighteenth century (63). Probably the first paper describing the scientific approach to plant propagation was written by the French dendrologist, Duhamel du Monceau in 1758.

The rooting of stem cuttings is one of the widely used forms of asexual propagation. A knowledge of the internal structure of the stem is necessary in order to understand the origin of adventitious roots. The process of development of adventitious roots in stem cuttings can be divided into three stages: one, the initiation of groups of meristematic cells, the root initials; two, the differentiation of groups of these cell groups into recognizable root primordia; and three, the development and emergence of the new roots, including rupturing of other stem tissues and formation of vascular connections with the conducting tissue of the cutting (30,41).

In most plants, formation of adventitious roots takes place after the cuttings are made. The origin of most adventitious roots in stem cuttings is in groups of cells

which are capable of becoming meristematic. According to Esau, root initials tend to arise in the vicinity of differentiating vascular tissues. In young stems, roots originate from cells near the periphery of the vascular system, whereas in older stems, they initiate close to the vascular cambium (30).

In 1956, Stangler pointed out that few authors have adequately traced the developmental details of initiation and early division, or have published convincing photomicrographs as evidence (76).

Stangler reported that adventitious roots in chrysanthemum cuttings are first observed in the interfascicular region. Root initials in carnation cuttings arise in a layer of parenchyma cells inside a fiber sheath. The developing root tips upon reaching this band of fiber cells, are unable to push through it, and consequently turn downwards, emerging from the base of the cutting (76).

It was also reported that the origin of the adventitious roots of *Cucurbita pepo* and *Lycopersicon esculentum* is in the parenchyma cells of the phloem.

Delisle (27) reported that in white pine cuttings, root initials form in association with rays and leaf traces. In *Rosa delicata* adventitious roots originate near the cambium layers in immature, multiserate ray tissue of the secondary phloem (76). In certain plants they are

reported to be just outside and between the vascular bundles (61). For example, *Rosa* (18), *Salix* (19), and *Vaccinium* (10).

Girouard (37) reported that adventitious roots of English Ivy originate in the phloem ray parenchyma above the basal cut within a period of six to ten days. The actual emergence of the roots was at right angles to the main axis of the stem and the roots appeared ten to fourteen days after the cuttings were made.

Stangler reported that the time at which root initials develop after cuttings are placed in the propagating bed, varies. In chrysanthemum, the root initials were first observed microscopically after three days; after five days in carnation and seven days in rose. Visible roots emerged from the cuttings after ten days for chrysanthemum but three weeks were required for the carnation (76).

In some plants, adventitious root initials form during early stages of intact stem development, and already were present when the cuttings were taken (20,41). These are termed preformed root initials and generally lie dormant until the stems are made into cuttings and placed under environmental conditions favorable for further development and emergence of the primordia as adventitious roots. Such preformed root initials occur in a number of easily rooted

genera, such as willow (19).

McCahon in 1964 described some of the practical aspects of hydrangea propagation which deal only with the environmental conditions suitable for regeneration from cuttings (52). Laurie et al (50) also discuss the propagation of hydrangeas, mainly in terms of the environmental and cultural aspects, and the type of material suitable for cuttings.

Histochemistry

Nucleic acids, because of their role in chromosome structure and their function in protein synthesis, have assumed a place of eminence in the study of the cell (45). Both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) have been implicated in many important functions of the cell. Investigation of these has become important relative to cell development and function.

From the evidence already accumulated it would seem logical to investigate, on a quantitative level, changes in both DNA and RNA in plant tissue systems undergoing development. Interesting data on the processes of cell growth and differentiation would be expected from amylases of parts of developing plant embryos, flowers, stems, roots, and buds using the methods of quantitative histochemistry.

Yet, only a limited number of quantitative histochemical studies of the nucleic acids of plants, or plant parts, have been undertaken (36,45,56,64,73).

A study of apical dominance in *Tradescantia paludosa* revealed a conspicuous characteristic "zone of inhibition" within the apex of lateral buds of vegetative shoots (56). This spheroidal zone is located in the dome of the apex and includes both the single layered tunica and a group of cells in the underlying corpus. Mitoses were rare, if not totally absent in this zone. The interphase nuclei, particularly those of the corpus, are strikingly large, exhibit a faint Feulgen reaction, and apparently contain no condensed chromatin bodies.

Individual nuclei invariably contain the diploid telophase (2C) amount of DNA. These features distinguish the zone of inhibition from the subjacent portion of the apex where interphase nuclei appear much more condensed, and mitotic figures are found. This organization of the bud meristem is imposed by the auxin-dependent inhibiting mechanism. It disappeared on the release of inhibition brought about by decapitation of the shoot, but can be maintained in decapitated shoots by applying the growth regulator, alpha-naphthalene acetic acid to the stump of the cut stem. After release of inhibition by decapitation, one of the earliest manifestations of incipient

bud growth, is the doubling of the DNA content of nuclei in the zone of inhibition. This is followed by the onset of mitoses and cell division as the bud transforms into an actively growing shoot.

Setterfield (73) investigated the DNA content of individual nuclei within growing oat coleoptiles. In the ungerminated coleoptile (0 hour), essentially all of the nuclei were at the 2C level and this situation persisted during the first 12 hours of germination. Twenty-four hours after the start of germination about half of the nuclei reached the 4C level of DNA. Cell division also started after 24 hours. After 36 hours the rate of mitoses slightly exeeds that of DNA synthesis and there was a slow increase in the relative number of 2C nuclei. Cell division only continued until the coleoptile reached a length of about 10 mm. At this stage about two thirds of the nuclei were at the 2C level and only one third at the 4C level. However, although cell division ceases at this stage and growth continues slowly by cell elongation, it is clear that DNA synthesis is not blocked and that individual 2C nuclei continue to double their DNA until, at the cessation of growth (50-55 mm) the nuclei were predominantly 4C.

Dwivedi and Naylor (28) reported that in inhibited axillary buds of *Tradescantia paludosa*, DNA synthesis is

blocked at the 2C (G1) level in a group of cells which constitute a zone of "inhibition", in the bud apex. The apparent DNA/histone ratio of chromatin in these interphase cells was substantially higher than the standard value found in active terminal buds. The DNA/histone ratio of chromatin in the apex of axillary buds declines to the standard level when the cells undergo changes leading to mitosis, after the release of inhibition. In contrast to the histone level, the total protein content of chromatin in the zone of inhibition is not influenced by the change in the physiological state of the bud.

Rickson (64), demonstrated in *Paulownia tomentosa* that the cytological changes of the shoot apex are correlated with anatomical and histochemical differences. During the first days of germination and before leaf primordia are formed, there was an increase in cytoplasmic protein and RNA. Once the leaves were initiated the RNA and protein remained constant in the control mother cell zone, and further increase in protein and RNA was confined to the developing root primordia.

Van't Hof (92), measured the average mitotic cycle duration of root meristem cells of *Allium cepa, Lycopersicon esculentum* and *Tradescantia paludosa* by ^{3}H-thymidine incorporation. This study showed that the minimum mitotic cycle time increased with the increase in cellular DNA

content. The cycle duration for root meristem cells is the sum of the duration of DNA synthesis (S period) and a constant of 5.25 - 6.5 hours.

Van't Hof (93), showed that cells of pea root meristems cultured in the absence of a carbohydrate source are characterized by the absence of any proliferative activity. These cells accumulate in either the G1 or G2 stage of the mitotic cycle (stationary phase). Following the provision of sucrose G1 cells enter S (DNA synthesis) and G2 cells enter mitosis (transitional phase).Progression through the cycle resumed, but the meristem has not yet achieved complete asynchrony and hence steady-state kinetics. The term "proliferative phase" is used to describe completely asynchronous meristems.

In 1970, Webster and Van't Hof (96), reported that following provision of sucrose to starved, stationary phase pea root meristems, G1 cells enter DNA synthesis and G2 cells enter mitosis. Puromycin and cycloheximide completely prevent this initiation of progression through the cell cycle. Actinomycin D has no effect on the initial entry of G1 and G2 cells into S and mitosis, although later entry is prevented. The resistance of the cells to actinomycin D is lost slowly with time in medium without sucrose, suggesting that an RNA required for the resumption of

proliferative activity is being gradually lost. The effects of the inhibitors on transitional and proliferative phase meristem cells indicate that such dividing cells do indeed have sufficient of the requisite RNA for the 8 to 12 hour progression through the cycle, but that protein synthesis is required continuously. It is suggested that this RNA is the one lost slowly during starvation, allowing starved cells to reinitiate progression through the cycle in the presence of actinomycin D.

Steeves et al (79) reported that the vegetative shoot apex of *Helianthus annuus* contains a central zone in which the cell nuclei are relatively large and stain faintly in the Feulgen reaction. On the basis of autoradiography studies, it was concluded that during vegetative growth, DNA synthesis and mitosis are arrested in the central zone or reduced to an extremely slow rate. Microspectrophotometric measurement however, indicated that the central zone nuclei were not held at the 2C level. With the onset of flowering, cytological zonation disappears in the apex and incorporation of ^{3}H-thymidine is uniformly heavy throughout the region.

Alvarez in 1968 found in *Vanda (Orchidaceae)* an equal amount of DNA in all meristematic nuclei regardless of the developmental stage of the embryo, and this was taken as the 2C value (2). Most of the nuclei in the parenchyma

region fall into the discrete DNA classes 2C, 4C and 8C. The amount of DNA in the nuclei of the parenchyma region was shown to increase in direct proportion to the distance of the nucleus from the meristem.

Alvarez and Sagawa (3), noted that the large nuclei in the parenchyma region stained darker than those of the meristems, indicating an increase in the amount of DNA per nuclei during differentiation.

Bagchi et al (7) noted that the requirements of RNA and protein synthesis for the initiation of cell division in non-dividing systems, have also been demonstrated in animal cell types. Following explantation of amphibian lenses to culture medium there is an increase in RNA and protein synthesis in the epithelial cells, followed by consecutive bursts of DNA synthesis and mitosis. If these increases in RNA and protein synthesis are prevented by actinomycin D and puromycin, respectively, then subsequent DNA synthesis and mitosis are totally suppressed.

Jakob and Bovey (44) in their investigation on *Vicia faba* reported that primary roots of *Vicia faba* were pulse labelled during early stages of seed germination. DNA, RNA and protein synthesis were studied in homogenates and autoradiographs of 1 mm root tips. Selection of roots of uniform size reduced the great variation in the rates of DNA synthesis found in randomly sampled roots. Very low

levels of DNA and RNA synthesis were observed from 15 to 20 hours after the beginning of imbibition. A wave of DNA synthesis between 25 and 40 hours was followed by a wave of mitoses between 40 and 50 hours. It was shown that 96% of the cells which entered the first post-dormancy cell cycle were in G1 (i.e. before DNA synthesis). The curves describing RNA and DNA syntheses were parallel for the first 50 hours. Incomplete imbibition was excluded as a factor limiting the onset of synthesis of both nucleic acids, since imbibition by the 1mm root was completed by eight hours.

^{3}H-Leucine incorporation increased gradually from a very low level at 14.5 hours until 20 hours. Several sharp peaks of high precursor incorporation which precede the first wave of DNA synthesis were observed. Incorporation experiments with different specific activities of ^{3}H-Leucine indicated that these bursts of high incorporation represented changes in the level of protein synthesis, instead of abrupt changes in amino acid precursor pools only. The leucine precursor pool decreased in size from 20 to 30 hours. Protein synthesis between 30 and 50 hours paralleled DNA synthesis. The protein synthesis inhibitor cycloheximide was used to demonstrate that meristematic cells synthesized at least some of the protein essential for DNA synthesis throughout their S period.

Key (48) reported that not only RNA and protein synthesis were essential for auxin induced growth, but auxin causes an increased synthesis of both RNA and protein. In the actively growing plant form, it is controlled mainly by the relative amounts of the promoting hormones present. Apical dominance and the monopodial growth habit are governed by an interplay of hormones and by the states of various buds or shoots involved. Auxin produced by the apical bud is transported down the stem and prevents the enlargement of lateral buds by the production of ethylene in the buds.

Burg and Burg (17), suggested that ethylene, produced when auxin moves to the lower side of a horizontally positioned root, causes cellular swelling and the resultant geocurvature. However, such a mechanism must also take into account the fact that ethylene specifically impedes lateral auxin transport, and hence accumulation of the very hormone which stimulates its production. Possibly a dual feedback mechanism controls root geotropism, and ethylene not only modifies the action of auxin, but in addition, limits the amount moved laterally.

It is becoming increasingly evident that hormones do not act alone in isolated systems but in an interrelated manner in the plant as a whole. Thus, the proportions of various hormones present may vastly affect the growth

rate and subsequent differentiation patterns of the tissue in the complete organism. The presence of both promotive and inhibitory hormones permits a precise control of many developmental activities and in some cases, such as dormancy, on a stop to go basis (33). Hormonal growth control might thus involve an increase in the general protein producing machinery rather than triggering the production of a particular protein. Auxins, for example, appear to cause an increase in the amount of ribosomal RNA present in the tissue (48).

J. van Overbeek (59), described the hormonal control of plant growth through nucleic acid and protein synthesis. He states that auxins do more than produce elongation and cytokinins do more than promote cell division. Cytokinins cause elongation as well as cell proliferation. The functions of hormones overlap and plant hormones act in sequence. Cytokinins and gibberellins appear to dominate the early phase of development, auxin becoming dominant later. The site of action of hormones may be close to the gene. In some cases, possibly by promoting synthesis of messenger RNA molecules, hormones give rise to new synthesis of specific enzymes. The enzymes, in turn, control the biochemistry and thereby the physiology of the organism.

Auxins occur in minute quantities in growing tissue. The reason for this low concentration is that IAA is constantly being destroyed by indoleacetic acid oxidase (40). Indole 3-acetic acid oxidation is usually activated by monophenols and inhibited by orthodiphenols. Recognition of this fact has clarified the growth promoting activity of diphenols such as caffeic acid. Previously, they were thought to be auxins, now it is recognized that by inhibiting IAA oxidase, these compounds raise the level of native IAA considerably (59).

Even the two "inhibitory" plant hormones, ethylene and abscisic acid appear to exert their control through effects on nucleic acid metabolism, but they do this in different ways. Ethylene promotes nucleic acid and protein synthesis which leads to the synthesis of degradative enzymes, while abscisic acid appears to shut down the entire process (21). This enables an exact control on the protein formation machinery to be maintained according to the levels of promotive or inhibitory substances already present in the different tissues.

Ridge and Osborne (65) reported that ethylene may control the growth of plant cells by regulating hydroxylation of specific wall proteins. Their results were indicative of the parallel changes of hydroxyproline and peroxidases in the covalently bound cell wall proteins of pea shoots, and the several fold increases that follow exposure to ethylene. Although covalently bound peroxidases do not

function as proline hydroxylases, column fractionation and electrophoresis indicate that they separate with hydroxyproline-rich proteins, and are confined to the cell walls. They speculate, therefore, that ethylene increases cytoplasmic hydroxylation of proline, leading to enrichment in specific hydroxyproline-rich wall peroxidases.

Siegel and Galston stated that peroxidases have been traditionally viewed as toxic waste products of metabolism, but investigators of recent years indicate that they may be of great importance in the biochemical activities of the higher plants (74).

Peroxidase action is paralleled with various factors which control growth and development of activity in cells. While the exact biological function of this enzyme is not known (33), morphogenetic roles are suggested by its action in producing and inactivating auxin; in converting hydroxyphenolpropanes such as coniferyl alcohol to lignin-like materials and in oxidising such important metabolic compounds as reduced nicotinamide-adenine dinucleotide and its phosphate.

Peroxidase activity of plants is dependent upon genotype. Thus many genetic dwarfs have abnormally high peroxidase activity (59). When normal genotypes are stunted

by application of compounds which prevent gibberellic biosynthesis, the peroxidase activity rises markedly (34). From this it would appear that low gibberellin is conducive to high peroxidase and low growth role. It has been suggested that dwarfism is due to the abnormally high peroxidase which might act through auxin destruction.

Jensen (46) worked with *Vicia faba* and reported that the IAA-induced formation of peroxidase appears to be a possible factor of great importance in cellular differentiation, particularly of vascular tissue. It is significant that the cells respond very early in their development, often before there is any way of identifying them morphologically as different. That the induced peroxidase appears involved in lignin synthesis is of interest as it indicates a physiological role for the enzyme in a process that later manifests itself morphologically.

The observations on lignin formation help explain one of the most common morphological effects of IAA on roots, namely, the occurrence of vascularization and lignification closer to the root meristem. The sequence of events would appear to be that the cell in the presence of increased IAA forms a system which destroys IAA and part of this system is a peroxidase that is also active in the formation of lignin. Hence, if a peroxidase and eugenol type compound are present, lignin may be formed

in cells previously exposed to auxin.

Peroxidase activity was localized and measured in sections of the primary root of *Vicia faba* after treatment with IAA, to elucidate the physiological role of the induced peroxidase in the cell. The cells of the root cap, proepidermis, and vascular tissue, including the protophloem and protoxylem, show peroxidase activity. However, only the cells of the vascular tissue clearly show the induced formation of peroxidase. The maximum response to IAA shifts from the early provascular tissue at 10^{-8}M to the developing protoxylem at 10^{-7}M.

The cells that contain peroxidase activity are capable of producing a compound, when given eugenol and H_2O_2, which appears to be an intermediate in lignin biosynthesis. The vascular tissue that has high induced peroxidase is very active in lignin formation.

Van Fleet (87,88,89), reported the occurrence of vigorous peroxidase activity in the endodermis of roots of several species of plants; *Coleus*, *Zea mays*, and Sansevieria. He noted that peroxidase is detectable in all tissues but is most reactive in the basophilic cells of the histogens. Oxidation of applied phenols and aminophenols by peroxidase, produces quinones and quinonediimines that are absorbed by nucleic acids and other basophilic substances in the formative centers of the primordia.

Localization reactions for peroxidase occur in the axils of leaf primordia prior to bud formation, and on the surface of apical meristems in a spiral pattern, marking the points for future development of leaf primordia. Peroxidase is detectable in advance of or accompanying cell division, and declines after the division phase. Decline of peroxidase at the end of the division phase is related to the increase of phenols, naphthols and phenolases. Peroxidase declines in all tissues except the phloem. A continuous peroxidase system in the phloem connects primordia with adult tissue. The hypothesis is offered that the cellular units of the phloem peroxidase constitute a continuous system between primordia and adult tissue, and is functional in catalyzing the reduction of hydrogen acceptors essential to cell division and the initiation of primordia.

Vanden Born (86) demonstrated strong peroxidase activity in meristematic tissue, with a reduced intensity being produced in the differentiating and mature tissues.

Donald W. DeJong (24) noted that peroxidase localization is cytoplasmic in juvenile onion root tissues and associated with the walls in mature cells. The enzyme reaction is strongest in tissues which do not lignify, but is wholly absent in the lignifying xylem throughout all stages of development. One wall surface, the cortical-endodermal junction, exhibits a thermostable, cyanide insensitive peroxidase reaction. This "pseudoperoxidase" is located

at a site which stains most heavily for pectic material. Phenolic deposition occurs at the opposite wall surface in the endodermal cell. The reciprocal interaction observed between peroxidase enzyme and the plant growth hormone, indoleacetic acid, is not compatible with the concept that plant peroxidase is identical with indoleacetic acid-oxidase. Although auxin treatment changes the pattern of peroxidase localization in cell walls, the accumulation of hormone is greatest in hypodermal cells which are strongly positive for peroxidase. A mutual exclusion phenomenon observed for peroxidase and sulfhydryl proteins localized in the cells of young root tissues does not persist into the mature tissues, where these two reactions can occur at the same wall sites. His data do not support the theory that plant peroxidase is involved in cell wall synthesis. Its occurrence in conjunction with cell wall fractions may be incidental and only indirectly related to the natural redox function of the enzyme.

Hiatt in 1960 purified soluble succinic dehydrogenase from roots and leaves of *Phaseolus vulgaris* and found that in contrast with animal tissue it is not activated by phosphate ion (43).

Singer et al (12) reported that succinic dehydrogenase catalyzes the reversible oxidation of succinate to fumarate. In mitochondria and mitochondrial fragments, succinic

dehydrogenase is associated with a chain of enzymes and co-factors, which transfer electrons from succinate to oxygen by way of cytochrome C and cytochrome oxidase to the succinic oxidase system.

Avers (6) conducted histochemical tests to determine the activity and distribution of acid phosphatase, cytochrome oxidase, and succinic dehydrogenase in differentiating epidermal cells of timothy grass roots. The most intense activity for the first two enzymes was localized in the young trichoblasts during their early elongation phase. The hairless cells, which alternate with the trichoblasts in each cell row of the epidermis, lacked acid phosphatase activity and showed less cytochrome oxidase action. Both enzymes were most active in the trichoblasts in the growth zone, in which their rate of elongation was slower than that of their hairless sister cells. This correlation with previously observed growth rate differences was considered substantiation of the hypothesis that physiological differentiation occurred in this region of the root tip. Succinic dehydrogenase activity was localized in the meristem, but the enzyme was inactive in basal members of the pairs of sister cells which later mature into the two alternating types of epidermal cells. The apical cells, the members of the pairs which later produce root hairs, showed high

enzyme activity while the basal cell members showed no succinic dehydrogenase. This was the earliest sign of physiological differentiation, occurring in the meristem at the time of cytokinesis or shortly afterwards.

Mia and Pathak (55) demonstrated that the apical meristem and procambial strands of *Rauwolfia* give a strong positive reaction for cytochrome oxidase, succinic dehydrogenase and total protein. The pith cells reacted positively for peroxidase.

Ekes (29) indicated that succinic dehydrogenase (SDH) activity was demonstrated in unfixed root segments from *Lupinus luteus* at the ultrastructural level. The reaction product was found to be localized in the mitochondria and to a lesser extent on the membranes of plastids. Different mitochondria of the same cell often showed different intensity of the staining reaction. Different cells of the same tissue exhibited varying degrees of enzyme activity. An increase was found in the number of cells exhibiting the SDH reaction as well as in the intensity of the reaction itself, from the meristematic zone of the root to the more differentiated regions.

Bonner (12) stated that it is known that alpha-amylase attacks both amylose and amylopectin randomly throughout the molecule. However, the details of the action of alpha-

amylase are not completely known, and there are differences in enzymatic properties of each individual alpha-amylase. The biological function of alpha and beta amylase in tissue, other than storage tissue, remains obscure. There is not sufficient information available to assure that the role of beta amylase in hydrolysis of starch molecules in plant cells is to supply low molecular sugars for metabolic processes.

Simpson and Naylor (75) reported that initiation of germination in excised embryos requires an exogenous energy source. Normally this is obtained from the endosperm. In dormant seeds the hydrolysis of starch is blocked despite the fact that dormant and non-dormant seeds contain similar amounts of alpha and beta amylase. Alone, or in combination, the amylases are unable to break down raw endosperm starch granules to simple sugars in vitro. Exogenous maltose in combination with alpha-amylase hydrolyzes raw starch to glucose. Exogenous maltase also eliminates the requirement for exogenous sugar.

Examination of the maltase content of imbibed dormant and non-dormant seeds showed a marked increase in non-dormant seeds during the first 40 hours. This did not occur in dormant seeds unless they were treated with gibberellic acid. The results lead to the conclusion that an important effect of gibberellic acid is to induce the synthesis of maltose, or in someway activate the preformed enzyme.

Chandra (33) also reported that in germinating barley grains, the addition of gibberellin increases the hydrolysis of starch in the endosperm. In isolated aleurone layers (22), the addition of gibberellic acid caused the production of alpha-amylase after an initial lag period, and the most enzyme was secreted from the aleurone layer into the medium. Production of alpha-amylase was prevented by inhibitors of oxidative phosphorylation and protein synthesis.

Gates and Simpson (35) conducted a survey for alpha-amylase activity in seventy-nine species of plants, selected from thirty families. The results indicated that the enzyme was present in the leaves of every species. Starch was also present in the leaves of most species. Their results indicated an association between starch and alpha-amylase activity in leaves of plants.

The fact that alpha-amylase seemed to be present in the leaves of each of the species examined, raised an interesting question about the function of this enzyme. Alpha-amylase is known to hydrolyze starch (1,75,84,85). It is known also to assist transglucoside reactions, possibly to produce the primers needed for starch synthesis. Where starch levels are low in leaves, such as in the monocotyledonae, alpha-amylase may function to

prevent accumulation of starch (1). In those species producing a lot of starch, its function may be to speed mobilization of carbohydrate reserve.

SECTION I.

STUDIES OF THE ROOTING OF CUTTINGS OF *HYDRANGEA MACROPHYLLA*: DNA AND PROTEIN CHANGES

ABSTRACT

The anatomy of root initiation of *Hydrangea macrophylla* and associated changes in protein and DNA synthesis during adventitious root formation is described. Observations showed that adventitious roots originated from preformed root initials in the phloem ray parenchyma cells. The roots appeared ten to twelve days after the cuttings were made.

The total protein content of the root initials increased over 100% in the first four days, while there was no rapid increase in DNA content of the cells until the sixth day from the time the cuttings were made, and thenceforth they appeared to come into equilibrium with the rate of cell division.

INTRODUCTION

Extensive research has been carried out on the rooting of cuttings. Much of this has been concentrated on the anatomy of root initiation (4,10) and on the environmental factors which affect rooting (3,11). The effect of growth substances on rooting is another area of research which has been prominent (11,12), especially since Went demonstrated that auxins stimulate adventitious root formation

in stem cuttings (20). Rooting co-factors have also received a great deal of attention (12,14). Limited research, however, has been carried out on the biochemical changes associated with root initiation. Reference to only one investigation was found wherein DNA content was studied in relation to rooting, and that was based on cauliflower leaf cuttings as the experimental material (11).

The nucleus is the seat of genetic information, namely in its DNA component, and is primarily of importance in supplying the plan for development. Expression of the genetic plan, on the other hand, undoubtedly occurs principally in the cytoplasm through control of the mechanism of protein synthesis (2). One of the main problems in developmental physiology is elucidation of the means by which control is achieved.

The purpose of this investigation is to present anatomical description of root initiation of hydrangea cuttings which has not been described before, and to study the DNA and protein synthesis in the developing root primordia. It is hoped that the basic information obtained might lead to further research on the developmental physiology of the phenomenon of rooting.

MATERIALS AND METHODS

Stem cuttings of *Hydrangea macrophylla 'Kuhnert'* cultivar were obtained from one-year-old stock plants grown in the greenhouse. The cuttings were treated with a talc-hormone mixture containing 0.1% 3-indolylbutyric acid, and were rooted under intermittent mist in perlite maintained at 75°F.

A. Anatomical studies

Cuttings were lifted from the propagation bench at daily intervals until the roots emerged through the cortex and the epidermis, at which time 2cm pieces were cut from the basal end and fixed in FAA (50ml 95% ethanol, 5ml glacial acetic acid, 10ml 37-40% formalin and 35ml water). The samples were stored in FAA until the sections were made (15). The fixed stem pieces were then sectioned with a Hooker Plant Microtome (Labline Instruments, Inc.,) without embedding. THe sections were 24-30 microns thick, stained according to the safranin-fast green procedure (15), dehydrated and mounted on slides in Canada balsam.

B. DNA measurements

Cuttings were collected at daily intervals from the propagation bench and fixed in 4% formalin in .067M phosphate buffer of pH 7 (16). The fixed stem pieces were then sectioned with a Hooker Plant Microtome without embedding.

The sectioned material was hydrolyzed in IN HCl at 60° for ten minutes. They were then stained with Feulgen for one hour in the dark at room temperature, washed in two changes of fresh SO_2 water (5ml IN HCl and 5ml 10% $Na_2S_2O_2$ made up to 100ml with distilled water) for ten minutes each, and rinsed thoroughly in distilled water.

Sections from the cuttings collected during the first three days were 16 microns thick. These were dehydrated and mounted in immersion oil (refractive index 1.5150). From the fourth day on, sections 60 microns thick containing developmental roots were treated with 4% pectinase for five minutes and rinsed in distilled water (21). The root primordia were dissected from the sections, squashed the same way as root tips, dehydrated and mounted in oil.

Extinction values of nuclei at 550 nm were determined by using a type GN2 integrating microdensitometer (Barr and Stroud Limited, Glasgow, Scotland). This instrument incorporates a scanning device that minimizes distributional error. Extinction is integrated as the scanning progresses, so that a direct measurement of total extinction is provided in arbitrary units for a single nucleus (7). Nuclei were chosen at random. A mean of extinction readings for half anaphase figures was assigned the 2C value and all other extinction readings were relative to this figure. Each determination was the mean of three readings. All

measurements were made with a 45 X objective.

The nuclear DNA content of the ray parenchyma cells was measured at the time the cuttings were made, and again after two, three, four and six days. Subsequent determinations were made on nuclei of meristematic cells of roots 0-2,5,10,15, and 25mm long.

C. Total proteins

The material was fixed with Carnoy's fixative (3:1 ethanol:acetic acid). The ninhydrin-alloxan test was carried out for detecting total proteins by the method of Yasuma and Tchikawa (13). The fixed stem pieces were then sectioned with a Hooker Plant Microtome without embedding. The sections were cut 16 microns thick, and incubated at 37°C for 20 hours in 0.5% ninhydrin solution in absolute ethanol. After rinsing in two changes of absolute alcohol and distilled water, they were then treated with Schiff's reagent for 30 minutes.

Following staining, the sections were washed with two changes of distilled water, two minutes in 2% sodium bisulfite, and for ten minutes in running tap water, dehydrated and mounted in immersion oil. Controls were incubated in ethanol without ninhydrin.

From the sections, circular fields 3,523 square microns in area were selected, each containing a root initial or primordium. Total extinction at 550 nm for each field was

determined by the microdensitometer as a measure of total protein. Readings were made on samples collected at two day intervals from the time the cuttings were taken until the adventitious roots emerged through the cortex and the epidermis.

The total cell number was determined by counting the number of cells in one plane of the circular field with a diameter of 67 microns, and multiplying by the section thickness of 16 microns. The number of cell layers calculated, was based on mean cell diameter and assuming isodiametric cell shape. This procedure was repeated five times and the mean cell number was recorded.

RESULTS

A. Anatomical studies

The adventitious roots on stem cuttings arise endogenously from a group of meristematic cells between the vascular bundles (root initials) just outside the interfascicular cambium. They appear to have large nuclei and dense cytoplasm (Figs 1,2).

These cells first divide anticlinally to form an organized group of cells (Figs. 3,4), and these differentiate into a recognizable structure, the root primordium. Cell division continues and the vascular system develops in the new primordium and becomes connected to the adjacent

vascular bundles. The root tip continues to grow outwards through the cortex and in approximately ten days, emerges from the epidermis of the stem. The adventitious roots in all the observations arose from the root initials between the vascular bundles (Figs. 2,3,4,5).

Thus it would appear that in hydrangea adventitious root initials form during early stages of intact stem development, and are already present at the time the cuttings are made (Fig.2). Preformed root initials generally lie dormant until the stems are cut and placed under environmental conditions favorable for further development and emergence of the root primordium (6). However, with intact plants, the preformed root initials may differentiate into root primordia and develop until the root breaks through the epidermis of the stem (Fig. 7). This occurred mostly in hydrangea stock plants which were pruned severely several times. Further growth of these roots occurred if they were either air-layered or made into cuttings.

Figures 1 to 6 demonstrate the anatomical changes with occurred in the hydrangea stem during adventitious root development, at different times after the cuttings were made. Figure 7 demonstrates intact hydrangea stems.

Legend: E, endodermis; P, phloem; Pr, phloem ray; X, xylem.

Figure 1 — Transection at the time the cuttings were made. Section was stained with Feulgen.
X 300.

Figure 2 — Transection at the time the cuttings were made. Stained with safranin-fast green.
X 1000.

Figure 3 — Transection after three days. Note the division of the root initials (Arrow).
X 1000.

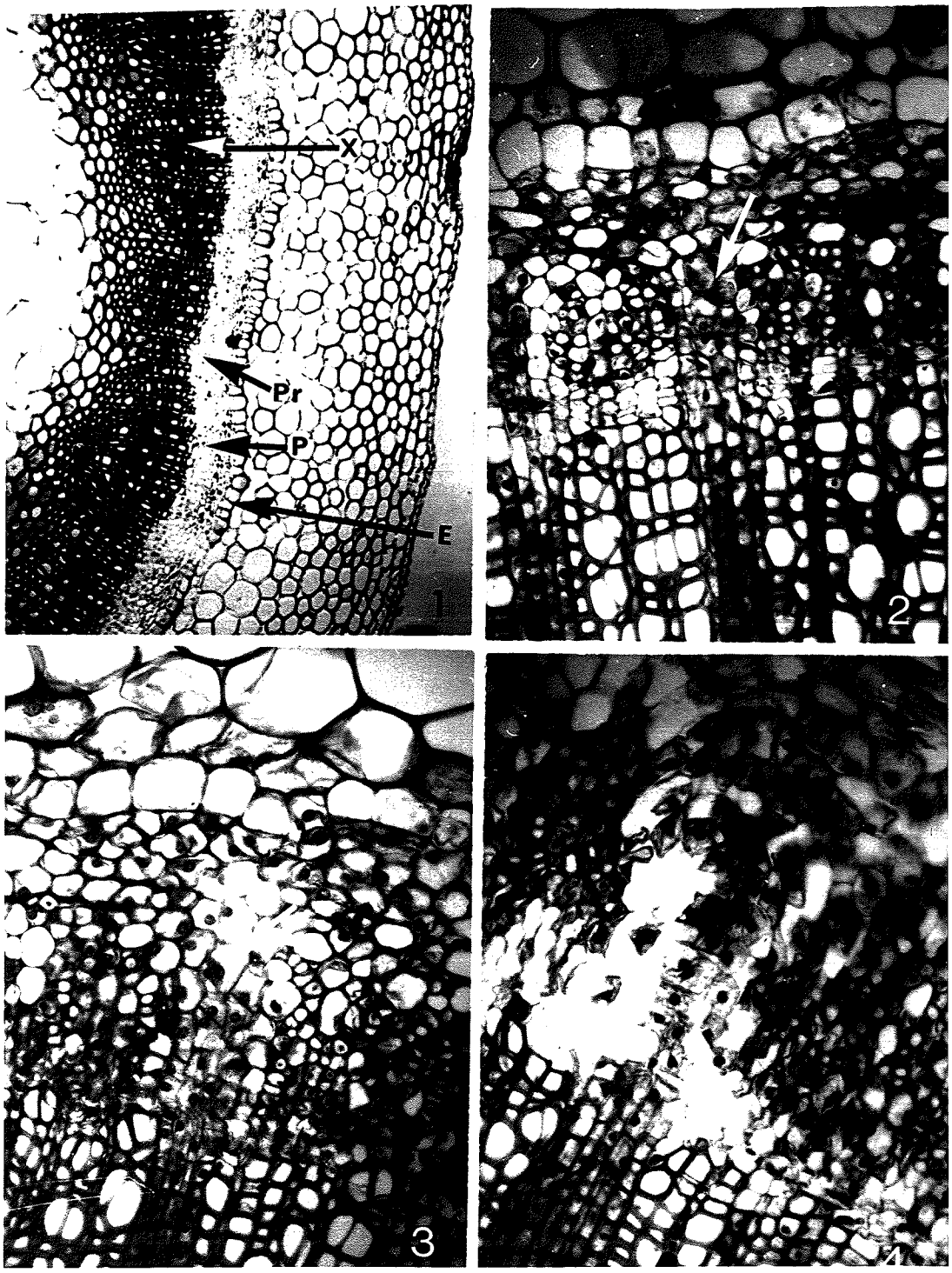
Figure 4 — Transection after five days. Stained with safranin-fast green.
X 1000.

Figure 5 — Transection after seven days showing three root primordia all originating from the initials between the phloem.
X 200.

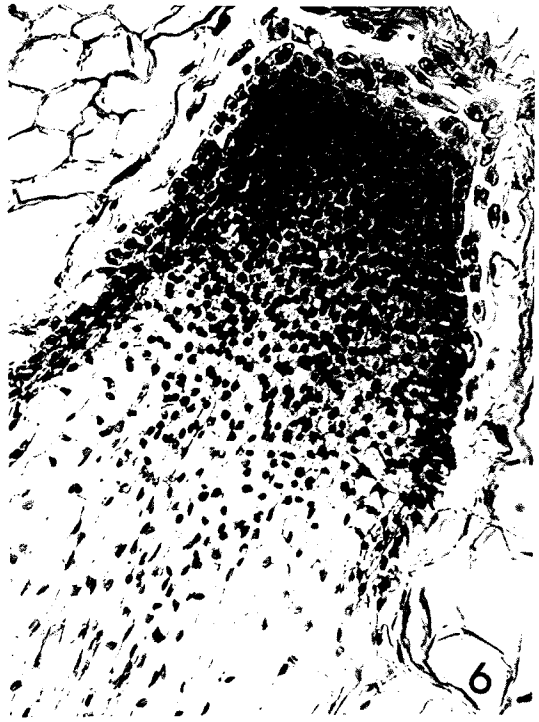
Figure 6 — Longitudinal section of adventitious root after ten days. Stained with Feulgen.
X 800.

Figure 7 — Photograph of intact hydrangea stems. Arrows indicate the adventitious root development.
Actual size.

Figures 1 to 4.



Figures 5 to 7.



B. DNA synthesis

Figure 8 shows the DNA content of nuclei of meristematic cells involved in the different stages of root formation. Changes in DNA content were not very noticeable in the first four days of rooting (Figs. 8a,b,c,d). The majority of the nuclei remained at the 2C level with slight increase in synthesis and only a few being at the 4C level.

The first major change in DNA content was observed in the six day old primordium (Fig. 8e). Of the measured nuclei observed, 45% were at the 2C level; 25% at the 4C level and 30% were in synthesis.

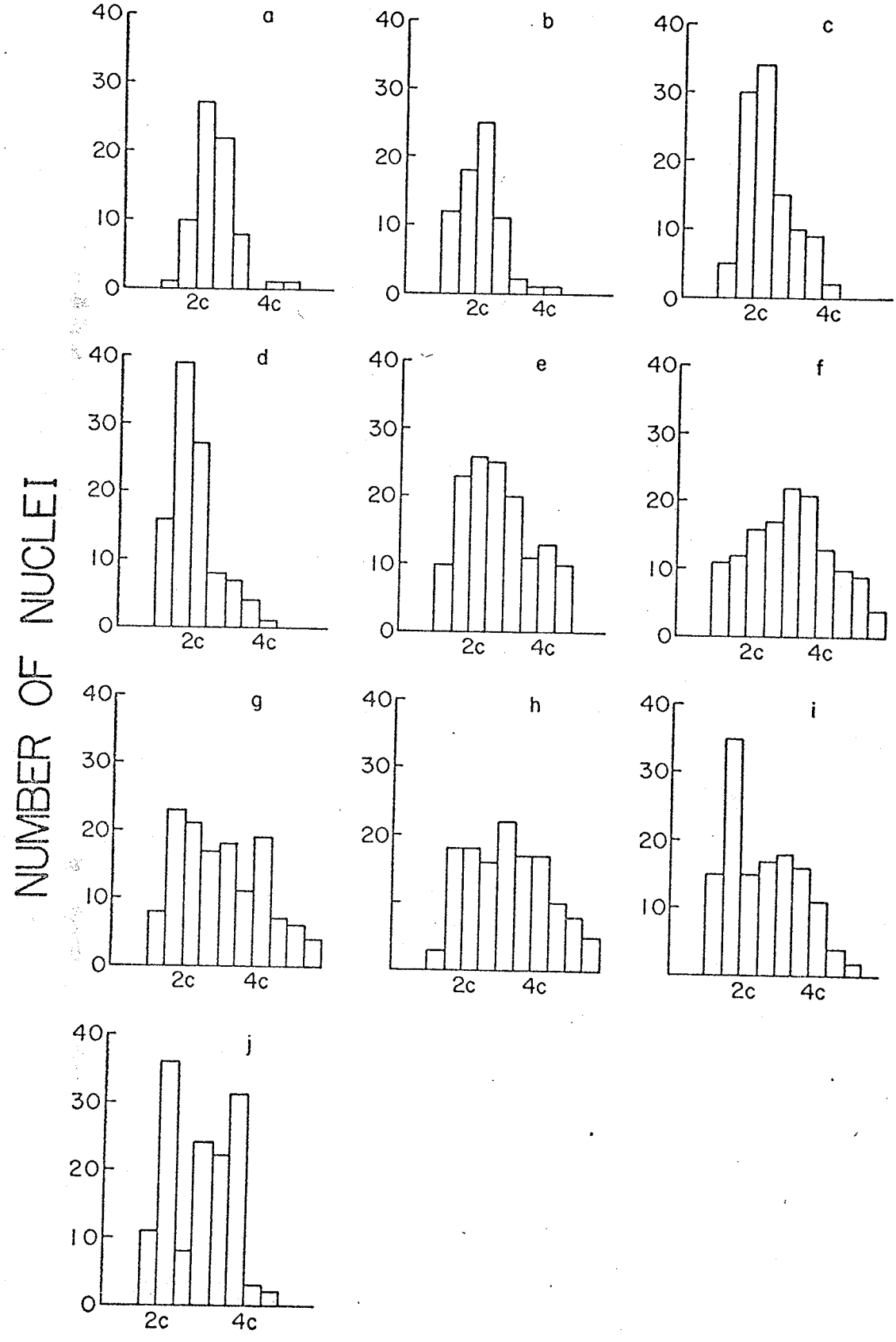
DNA values of nuclei of root tip cells of roots varying from 0-2mm to 25 mm in size are shown in figures 8f to 8j. The shortest roots were the most active in DNA synthesis (Fig. 8f). As the root length increased there was a general trend towards reduction in the number of cells between the 2C and 4C values. This trend resulted in the bimodal distribution finally observed in root tips of roots 25mm long (Fig. 8j). Despite bimodulity, more cells appeared to be at the 2C level than at the 4C level; that is, more cells were in G1 than in G2 (Figs. 8g,i,j).

A progressive increase in the relative length of the phases G1 and G2 with respect to S would transform such a unimodal distribution to a bimodal distribution.

This further implies that the division rate was declining in the root tips as the rootlets elongated. A considerable number of root tip cells had greater values than 4C. The extent of this tail declined as the rootlets elongated. Such cells might represent a sub-population beginning a new cycle of synthesis without intervening divisions, which results in endopolyploidy.

As the initiation of the new series of DNA replication by root tip cells at the 2C level falls with increasing root length, then the rate at which 4C cells begin replication up to 8C might also be expected to fall. In this manner, the tail of cells with DNA content greater than 4C would also decline.

Figure 8.


a
b
c
d
e
f
g
h
i
j
40
30
20
10
0
2c
4c
NUMBER OF NUCLEI
DNA CONTENT (ARBITRARY UNITS)

C. Total protein

Total protein analysis was carried out for developing root initials and meristematic area of developing roots. The results of this analysis based on the total protein per unit volume of tissue are summarized in Table 1. A 58% increase occurred from the time the cuttings were taken until the second day. From two to four days there was a further increase of 32% and in the following two days there was a 21% increase.

On the basis of estimated cell number, the protein content per cell increased in a similar manner in the first four days, but decreased substantially between the fourth and sixth day. Between the sixth and tenth day, the protein content per cell remained relatively constant. This stage coincides with the increase in DNA synthesis and also an increase in cell division.

TABLE I

Total protein content of root initials and root primordium in various stages of development.

No.of days	Vol.Measured in cubic microns	Total protein per volume arbitrary units	Estimated cell number per volume(1)	Estimated total protein per cell
0	56,368	8.92a	28.80	.3097
2	56,368	14.06b	29.83	.4713
4	56,368	18.60c	33.61	.5534
6	56,368	22.50d	43.05	.5226
8	56,368	25.12e**	50.82	.4992
10	56.368	24.88e	50.69	.4908

** Figures followed by the same letter are not significantly different from each other at P 1% (lsd=1.02).

1 Cells were counted in each stage on four slides in the root initials region and subsequently in the root primordium.

DISCUSSION

Adventitious root formation consists of three basic processes: cell division, cell enlargement and cell differentiation (9). DNA synthesis is a prerequisite, and protein synthesis is a common occurrence prior to cell division. Therefore it seemed appropriate to investigate the changes occurring in the total protein and DNA content in the developing adventitious roots of hydrangea cuttings.

In most plants, after cuttings are made, adventitious root formation evolves from a group of potentially meristematic cells of diverse tissue origin. In hydrangea they originate from pre-existing initials in the phloem ray parenchyma. Roots originating from this same tissue were reported for English Ivy (10), and also for *Rosa*, *Salix*, *Taxus* and *Vaccinium* (4,5,11). In other species, roots may arise from different tissues, for example, in chrysanthemum from the interfascicular cambium (11), and in carnation from a layer of parenchyma cells inside the fiber sheet.

In hydrangea cuttings the phloem ray parenchyma cells actually appear as preformed root initials which are capable of further development while the stem is still attached to the stock plant. This was observed only in plants grown under stress, for example, on newly generated

shoots of stock plants after pruning of the main shoot (Fig. 7). This adventitious root development may have been triggered by some change in the plant's hormone or nutritional balance as a result of the severe pruning.

It appeared from this study that within two days after cuttings were made, a major increase in the cellular protein occurred in the root initial area. On the other hand, the first division of the root initials from the cuttings could not be observed microscopically until three to four days after the cuttings were made. While the DNA content of the cells does show some increase by the third day, it does not begin to increase rapidly until the sixth day (Fig. 8c). By comparing the estimated mean total protein content per cell with the DNA content per cell (Table I and Fig. 8 respectively), it is clear that much protein synthesis precedes the large scale replication of nuclear DNA. The total protein per cell stabilized on or before the sixth day from when the cuttings were made, and therefore appeared to come into equilibrium with the rate of cell division. While increased protein synthesis and doubling of DNA do not necessarily lead to cell division, in the tissue studied, it appeared that this was the sequence of events. This finding supports Webster and Van't Hof's observations with *Pisum sativum* (19), in which they found that increased protein synthesis

is required for both the initiation of DNA synthesis by GI cells, and entrance into mitosis of G2 cells.

The requirements for RNA and protein synthesis for the initiation of cell division in non-dividing systems has also been investigated in animal cell types (1). Following explantation of amphibian lenses to culture medium, there is an increase in RNA and protein synthesis in the epithelial cells, followed by consecutive bursts of DNA synthesis and mitosis. If these increases in RNA and protein synthesis are prevented by actinomycin D and puromycin, respectively, subsequent DNA synthesis and mitosis is totally suppressed.

It therefore seems likely that the protein synthesis observed was a consequence of the synthesis of one or more species of RNA. Further, it is possible that the synthesis of DNA is conditional upon the presence of one or several fractions of the newly synthesized protein. This theory is supported by other authors. McLeish (18) suggested that the DNA duplication in mitosis is accompanied by the nuclear protein duplication. If mitosis is to proceed in a normal manner there may be a requirement for exact duplication, not only for the DNA complement but also for a specific protein moiety to be synthesized by the nuclear RNA. Schrader (17) discussed the interrelation of protein and DNA synthesis in animal tissues. He

suggests that DNA and protein synthesis in the same cell are independent processes in the sense that protein synthesis may occur without simultaneous DNA synthesis.

In 1965, Galle reported finding an increase in the RNA content of the basal tissues of cauliflower leaf cuttings, during the period when the root primordia were forming, but he did not find any increase in the amount of DNA (11).

It appears that in the root initial of hydrangea, the DNA content remains constant, since most cells were observed at the 2C level, which would indicate that the process leading to DNA replication is blocked. This is supported in previous reports (22) that prior to nuclear division diploid nuclei contain a basic amount of DNA, designated in relative terms as 2C. In many specialized cells where division is inhibited the reversible control mechanism appears to be the absence of DNA synthesis (8). This fact is demonstrated when a mature cell gives up its functional specialization and again enters the reduplication cycle. In many plants this activation of individual functions can be induced by isolation of specialized cells, thus interrupting physiological correlations which have evolved during cell differentiation. Activation of nuclear metabolism in isolated cells has been reported to precede the first nuclear and cell divisions (22). Probably the processes

which are blocked during differentiation are activated first, and in the case of hydrangea root initiation, may be protein synthesis.

In hydrangea cuttings, the root originates from a specialized group of cells within the phloem ray parenchyma. Under normal growing conditions, they do not divide. Perhaps one or more conditions of their normal cellular environment provides such a block. If the stem is cut off and put into a rooting medium, or when the plant is grown under unfavorable conditions, they resume nuclear metabolism and cell division.

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LITERATURE CITED

1. Bagchi, M., Rothstein, H. and Stein, G.S. 1968. Synthesis of macromolecules in epithelial cells of the cultured amphybian lens. II. Effects of puromycin and actinomycin D on protein synthesis. Exp. Cell Res., 50: 554-559.

2. Bonner, J. 1959. Protein synthesis and the control of plant processes. Amer. J. Bot., 46: 58-62.

3. Brandon, R. 1939. Seasonal variations in the starch content of the genus *Rosa* and their relation to propagation by stem cuttings. J. Pom. Hort. Sci., 17: 233-253.

4. Carlson, M.C. 1933. Comparative anatomical studies of Dorothy Perkins and American Pillar Roses. I. Anatomy of canes. II. Origin and development of adventitious roots in cuttings. Contribution from Boyce Thompson Institute for Plant Research, Inc., 5: 313-329.

5. Carlson, M.C. 1938. The formation of nodal adventitious roots in *Salix cordata*. Amer. J. Bot., 25: 721-725.

6. Carpenter, J.B. 1961. Occurrence and inheritance of preformed root primordia in stems of citron *(Citrus medica* L.).Proc. Amer. Soc. Hort. Sci., 77: 211-218.

7. Deeley, E.M. 1954. An integrating microdensitometer for biological cells. J. Sci. Inst., 32: 263-267.

8. Dwivedi, R.S. and Naylor, J.M. 1968. Influence of apical dominance on the nuclear proteins in cells of the lateral bud meristem in *Tradescantia paludosa*. Can. J. Bot., 46: 289-298.

9. Esau, K. 1960. Anatomy of seed plants. John Wiley and Sons, Inc., New York.

10. Girouard, R.M. 1967. Initiation and development of adventitious roots in stem cuttings of *Hedera helix*. Anatomical studies of the juvenile growth phase. Can. J. Bot., 45: 1877-1882.

11. Hartmann, H.T. and Kester, D.E. 1968. Plant Propagation. Principles and Practices. Second Edition. Prentice-Hall, Inc., New Jersey.

12. Hess, C.E. 1967. Root initiation and juvenility - a possible implication of terpene compounds. Proc. XVII Int. Hort. Cong., 3: 443-451.

13. Jensen, W.A. 1962. Botanical Histochemistry. W.H. Freeman and Company, San Francisco.

14. Kawase, M. 1967. Effect of centrifugation on rooting of softwood cuttings. Proc. XVII Int. Hort. Cong., 3: 453-467.

15. Sass, J.E. 1968. "Botanical Microtechnique". Third Edition. Iowa: The Iowa State University Press.

16. Saurer, W. 1969. Effect of fixation on histochemical determination of deoxyribonucleic acid in plant material. I. Feulgen spectrophotometric and fluorometric measurements. Mikroskopie, 24: 284-290.

17. Schrader, F. and Leuchtenberger, C. 1950. A cytochemical analysis of the functional interrelations of various cell structures in *Arvelius albopunctatus* (De Geer). Exp. Cell Res., 1: 421-452.

18. Symposia of the Society for Experimental Biology. Number XIII. Utilization of nitrogen and its compounds by plants. Cambridge: 1959. University Press, Cambridge, England.

19. Webster, P.L. and Van't Hof, J. 1970. DNA synthesis and mitosis in meristems: Requirements for RNA and protein synthesis. Amer. J. Bot., 57: 130-139.

20. Went, F.W. 1934. A test method for rhyzocaline, the root forming substance. Proc. Kon. Ned. Akad. Wet., 37: 445-455.

21. Wied, G.L. 1966. Introduction to Quantitative Cytochemistry. Academic Press, New York.

22. Wied, G.L., and Barr, G.F. 1970. Introduction to Quantitative Cytochemistry - II. Academic Press, New York.

SECTION II.

STUDIES OF THE ROOTING OF CUTTINGS OF *HYDRANGEA MACROPHYLLA*: ENZYME CHANGES

ABSTRACT

Enzyme changes in root initials of *Hydrangea macrophylla* during adventitious root formation are described. Extensive changes in enzyme activity were demonstrated by histochemical staining and all enzymes investigated showed increased activity in the tissue responsible for root initiation.

The earliest change observed was that of peroxidase in the phloem and xylem ray cells. This was followed by cytochrome oxidase and succinic dehydrogenase. Alpha-amylase was localized by the substrate film method. The highest amylase activity was demonstrated in the epidermal tissues and vascular bundles. As the root primordia developed, enzyme activity shifted from the vascular bundles to the periphery of the bundles. A positive correlation was found between the starch content and root number of cuttings.

INTRODUCTION

Some of the anatomical and histochemical changes which occur during root initiation of hydrangea stem cuttings were described earlier (12). It was reported that DNA synthesis was preceded by protein synthesis and cell division in the preformed root initials.

Extensive research has been carried out in histochemistry

and much of this has been concentrated on enzyme changes in the apical and root meristems (4,5,10,11,17,19). Van Fleet demonstrated peroxidase activity in differentiating vascular plants - *Peperomia, Smilax, Allium* (17). Histochemical characteristics of epidermis and hypodermis have been demonstrated in *Allium sativum, Asparagus, Pelargonium* (18). Little attention has been paid to histochemical changes occurring during root initiation in cuttings.

The purpose of this investigation was to obtain some insight into enzymatic changes which may occur during adventitious root initiation and development of hydrangea cuttings. The enzymes investigated were succinic dehydrogenase, cytochrome oxidase and peroxidase. Alpha-amylase was localized by the substrate film method. This has been demonstrated in animal tissues by Tremblay (15,16). The gel-diffusion method was used to demonstrate alpha-amylase activity by other authors (3,13,14).

The effect of starch content on the rooting of cuttings was investigated.

MATERIALS AND METHODS

Stem cuttings of *Hydrangea macrophylla 'Kuhnert'* cultivar were obtained from one year old stock plants grown in the greenhouse. The cuttings were rooted under intermittent mist in perlite maintained at 75°F. Cuttings were lifted from the

propagation bench at daily intervals until the roots emerged through the cortex and epidermis.

In all enzyme and starch determinations, fresh, unfixed tissues were used. The stem pieces were sectioned with a Hooker Plant Microtome (Labline Instruments, Inc.,) without embedding.

A. Succinic dehydrogenase

Fresh stem pieces were sectioned 50 microns thick into 0.05M phosphate buffer pH 7. They were immediately transferred into the incubation medium consisting of; 5ml .067M sodium phosphate buffer pH 7; 5ml 0.02M sodium succinate; 5ml 0.1% neotetrozolium chloride and 5ml of distilled water (1). The mixture was boiled and cooled before use. Sections were incubated in the above medium for two hours at 37°C. Controls were either heat killed before incubation or incubated in the above medium without sodium succinate. The sections were mounted in glycerol after staining.

Sections were prepared at the time the cuttings were made, and at daily intervals up to ten days. Enzyme reactions in the tissues were recorded photographically and by microdensitometric measurements. The absorption of the reaction product of the succinic dehydrogenase in the tissues was determined at 550 nm with a type GN2 integrating microdensitometer (Barr and Stroud Limited, Glasgow, Scotland).

B. Cytochrome oxidase

Fresh stem pieces were sectioned 50 microns thick into 0.05M phosphate buffer pH 7.5. They were transferred immediately to the reaction mixture containing 25ml of 0.05M phosphate buffer at pH 7.5; 1ml of 1% alpha naphthol solution in 40% ethanol, and 1ml of a 1% solution of dimethylparaphenylenediamine hydrochloride. The sections were incubated in the above medium for 15 minutes at room temperature. They were then rinsed in distilled water and mounted in glycerol (9). Controls were either heat treated or killed in alcohol and incubated in a complete reaction mixture containing 5ml of 0.005M sodium azide.

The color formation of the enzyme reaction product of the tissues was recorded on photographs and by the integrating microdensitometer at 550 nm in similar stages as described for succinic dehydrogenase.

C. Peroxidase

Fresh stem pieces were sectioned 50 microns thick into 0.05M phosphate buffer at pH 7. They were then transferred immediately into the reaction mixture containing either 5ml of saturated benzidine hydrochloride and 5ml of 1% hydrogen peroxide and 1 ml 5% ammonium chloride (17); or 5ml of 1% 8-aminoquinoline in 95% ethanol and 5 ml of 1% hydrogen peroxide (4). Controls were heat killed by boiling in water for five minutes. After staining, sections were mounted in

glycerol.

The color formation of the enzyme reaction of the tissues was recorded on photographs and by the integrating microdensitometer at 580 nm in similar stages as described for succinic dehydrogenase.

D. Amylase

Localization of amylase activity was determined by a modification of the methods used by Briggs (3) and Tremblay (15,16). A 3% suspension of soluble starch containing 1% bacto-agar was heated to 100°C in a water bath for 15 minutes, and while still hot, filtered through a Millipore filter. (Cat.No. PHWP01300). Ten to twelve drops of this preparation were deposited on a glass slide and spread over the surface of the slide. The slide was placed vertically on a piece of filter paper and the excess suspension was allowed to drain.

The film was allowed to dry at room temperature and fixed overnight in methanol: acetic acid: water: (5:1:5: v/v). Finally, the film was washed in three baths of distilled water, for five minutes each, and dried at room temperature. Before exposure, the film was kept for two hours at room temperature in a moist chamber consisting of a petri dish containing a wet filter paper (16).

Fresh stem pieces were sectioned 40 microns thick into 0.05M calcium acetate buffer, pH 5.3. The sections were

lifted from the buffer solution with a camel hair brush and drained from the excess moisture by placing the tip of the brush on a blotting paper. They were then carefully placed on the humidified starch agar film and covered with a cover-slip to prevent desiccation.

Incubation was carried out at room temperature in the moist chamber for 30 to 45 minutes. After incubation, the tissue sections were flushed off the film with a stream of water. The film was then immersed in the methanol acetic acid fixative for 15 minutes and stained by the periodic acid Schiff (PAS) reaction (9). The fixed starch agar films were oxidized in 1% periodic acid ($HIO_4 \cdot 2H_2O$) for 10 minutes and washed for five minutes in running tap water, stained for 10 minutes in Schiff's reagent (9), washed directly in three successive baths of M/20 $NaHSO_3$ for two minutes each and 10 minutes in running tap water. They were allowed to dry at room temperature and observed under the microscope. Heat killed sections were also used as controls.

Specificity of the reaction was verified by controls treated with alpha-amylase and by the exposure of films to sections of triticale grains (a new species synthesized from a wheat-rye cross), sectioned after overnight soaking.

E. Starch content of cuttings and root formation

Prior to the time the cuttings were made, ten hydrangea stock plants were kept in a dark growth chamber for 72 hours. Thirty cuttings each were taken from these plants and stock

plants under normal light regime in the greenhouse. Each cutting was individually labelled and a 1 cm. piece cut from the ends for starch determination.

Four sections 60 microns thick were cut from each stem piece, placed on the end of a glass cuvette and the thickness determined by means of an ocular micrometer. The sections were stained for starch by the method of Gates and Simpson (8). The sections were killed in boiling water for thirty seconds, decolorized in cold methanol and stained with dilute iodine solution (0.44 g. iodine and 22.088 g. KI dissolved in distilled water and made up to 500 ml with distilled water (14). The sections were then mounted in glycerol.

The absorption of the iodine stained starch was determined with four root initial areas in each section with the microdensitometer at a wavelength of 550 nm. When the cuttings were rooted, the roots were counted on each cutting. A correlation analysis of initial starch content of cuttings versus number of roots formed, was carried out.

RESULTS

A. Succinic dehydrogenase

Succinic dehydrogenase reduces neotetrazolium chloride to a bright purple, insoluble formosan. This enzyme appeared to have very low activity in the hydrangea stem cuttings before rooting. The highest activity was detected in the epidermis, followed by the vascular bundles, and no activity was notice-

able in the cortex and pith tissue (Table I).

Two to three days after the cuttings were made, the enzyme activity started to increase noticeably in the region of the phloem and phloem rays (Figs. 1,2,3). The microdensitometric readings also indicated a general increase in enzyme activity in the cuttings with about an eight fold increase in the root primordia region.

The enzyme activity appears to be associated with some densely stained particles in the cell (Fig. 4). The embryonic tissue of the root apex and procambial strands stained very deeply as a result of enzyme activity, while the root cap of the adventitious roots showed less activity. The heat treated controls did not show any enzyme activity.

B. Cytochrome oxidase

The site of the enzyme activity was indicated by the formation of the indophenol blue granules in the tissues. Cytochrome oxidase was found to be most active in the epidermis, xylem and phloem rays at the time the cuttings were taken, with less activity in the cortex and phloem (Table II, Fig. 5). When the cuttings were taken from fairly lignified stems the endodermis gave a very strong enzyme reaction. The activity was approximately the same as that of the epidermis (Fig. 6). This was probably due to lignification of the cell walls of the endodermis. Lignified tissues exhibit

strong cytochrome oxidase reaction (20).

The first increase in enzyme activity could be noticed microscopically two days after the cuttings were taken (Fig. 6),and reactions further intensified with the development of the root primordia to about five fold compared to the root initials (Table II). The root apex and procambial strands stained very deeply as the result of enzyme activity (Figs. 7,8). Indophenol blue did not form in the control sections.

C. Peroxidase

When benzidine was used as the substrate, the enzyme activity was indicated by a dark blue coloration, but this reaction was very unstable as reported by other authors (4,17). Benzidine, in the presence of H_2O_2 and peroxidase enzyme forms benzidine blue, which breaks down to benzidine brown (diimine) which made it difficult to record the results on photographs and take microdensitometric measurements. Ammonium chloride is reported to stabilize the reaction, and stop it in the benzidine blue stage, but it was found to be unsatisfactory in these experiments.

The 8-aminoquinoline reaction occurs more slowly and furnishes results which yield a better contrast between strong and weak peroxidase containing tissues. The reaction product is stable and does not diffuse as readily as does the quinone-diimine of benzidine. The observations and measurements were

based on the 8-aminoquinoline reaction.

Peroxidase was active in all tissues at the time the cuttings were taken, with somewhat lower activity in the cortex and the epidermis. The vascular bundles gave the strongest reaction, followed by the phloem ray cells and the epidermis (Fig. 9). The first change in enzyme action was noticed two days after the cuttings were made, where the whole phloem and xylem ray area gave a very strong peroxidase reaction (Fig. 10). It was so dark that it could not be measured with the microdensitometer. With the development of the root primordia, the enzyme activity decreased somewhat. The four to five day old primordia had twice as much activity as the root initials at the time the cuttings were made (Table III). There was also an increase in enzyme activity in other tissues, especially in the xylem and the epidermis. The periphery of the root primordia had such a high peroxidase activity that it was impossible to measure it microdensitometrically (Figs. 11,12). The results of the measurements are summarized in Table III. The control sections did not exhibit any enzyme activity.

TABLE I

Succinic dehydrogenase activity in stem cuttings of *Hydrangea macrophylla* before rooting and during the development of the root primordia.

Tissue	Before rooting (Arbitrary Units)	5-6 day old roots (Arbitrary Units)	Differences
Epidermis	18.8	21.8	3.0 xx
Cortex	2.6	3.8	1.2 ns
Phloem	9.4	29.2	19.8 xx
Root initials or primordia	7.4	68.2	60.8 xx
Xylem	13.2	22.6	9.4 xx
Pith	2.4	3.8	1.4 ns

xx Differences are significant at the 1% level according to the 't' test (t=2.18).

Figures 1 to 4 demonstrate localization of succinic dehydrogenase in hydrangea stem sections at different times after the cuttings were made.

Legend: P, phloem; Pr, phloem ray; X, xylem; R, root initial.

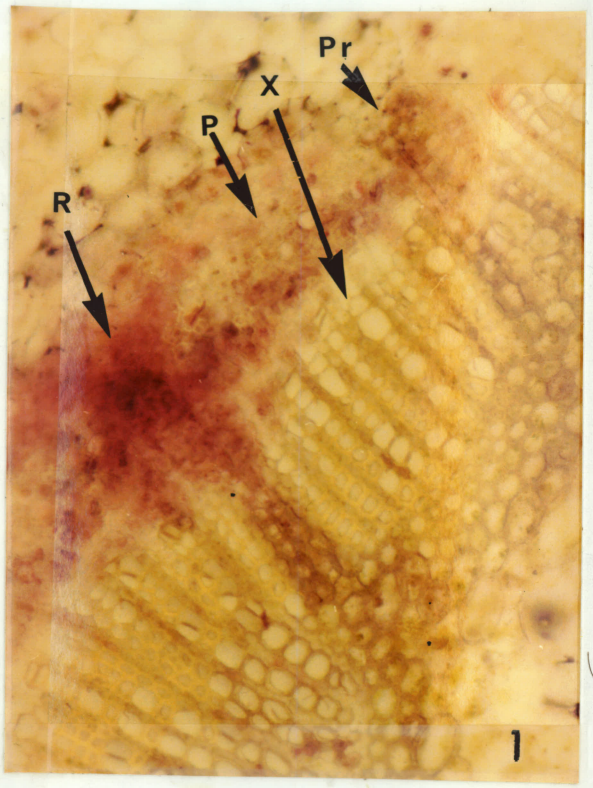
Figure 1 — Transection after four days. X 400.

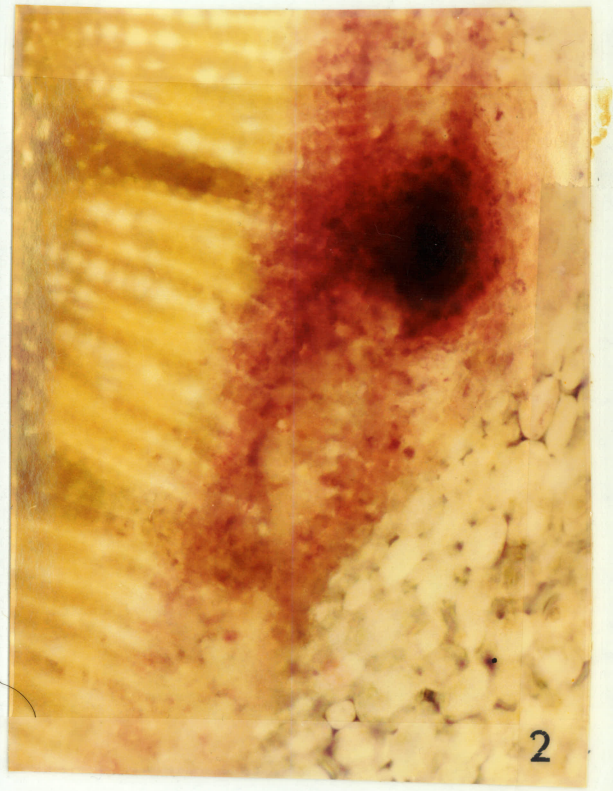
Figure 2 — Transection after five days. X 400.

Figure 3 — Transection after seven days showing three root primordia. X 200.

Figure 4 — Longitudinal section of adventitious root after ten days. X 800.

Figures 1 to 4.





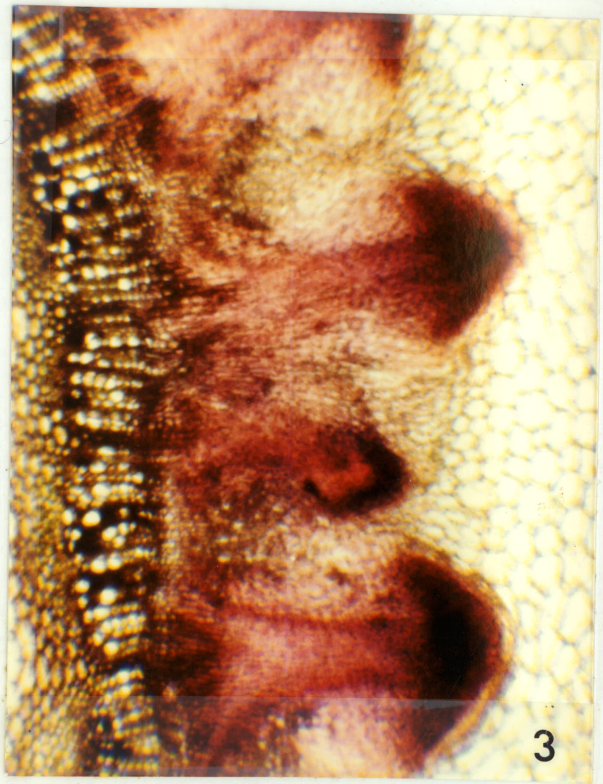


TABLE II

Cytochrome oxidase activity in stem cuttings of *Hydrangea macrophylla* before rooting and during the development of the root primordia.

Tissue	Before rooting (Arbitrary Units)	5-6 day old roots (Arbitrary Units)	Differences
Epidermis	42.0	39.0	3.0 xx
Cortex	7.6	12.6	5.0 xx
Phloem	7.0	9.2	2.2 xx
Root initials or primordia	14.8	71.8	57.0 xx
Xylem	32.6	34.8	2.2 xx
Pith	4.0	5.6	1.6 ns

xx Differences are significant at the 1% level according to the 't' test (t=2.1003).

Figures 5 to 8 demonstrate localization of cytochrome oxidase in hydrangea stem sections at different times after the cuttings were made.

Legend: E, endodermis; P, phloem; Pr, phloem ray; X, xylem; R, root initial.

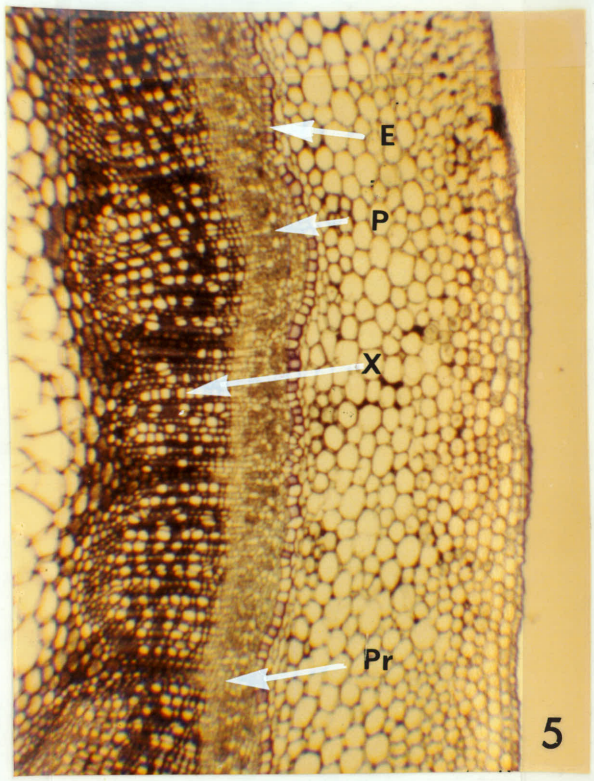
Figure 5 — Transection at the time the cuttings were made. X 300.

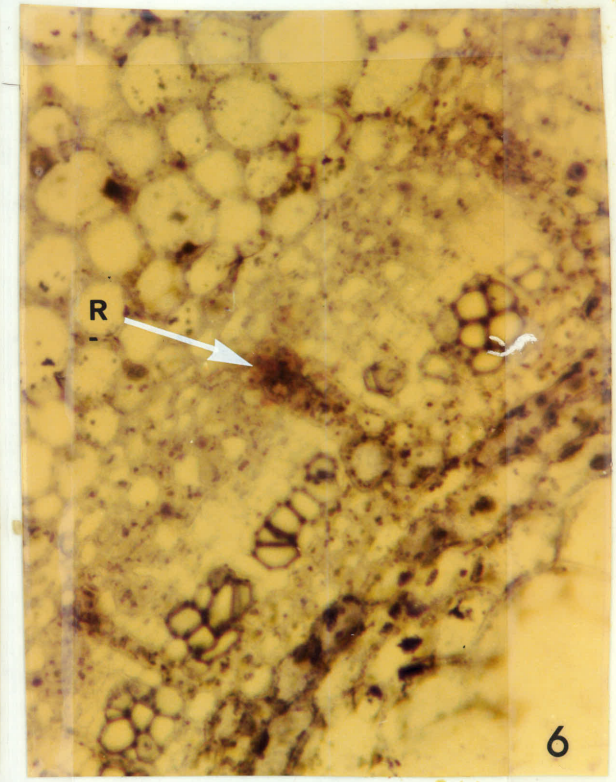
Figure 6 — Transection after two days X 750.

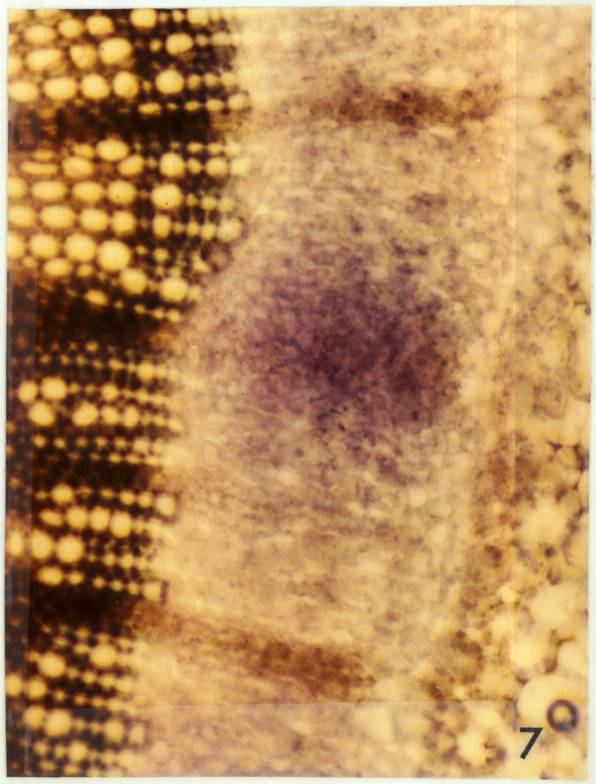
Figure 7 — Transection after four days. X 400.

Figure 8 — Transection after seven days showing root primordium. X 400.

Figures 5 to 8.







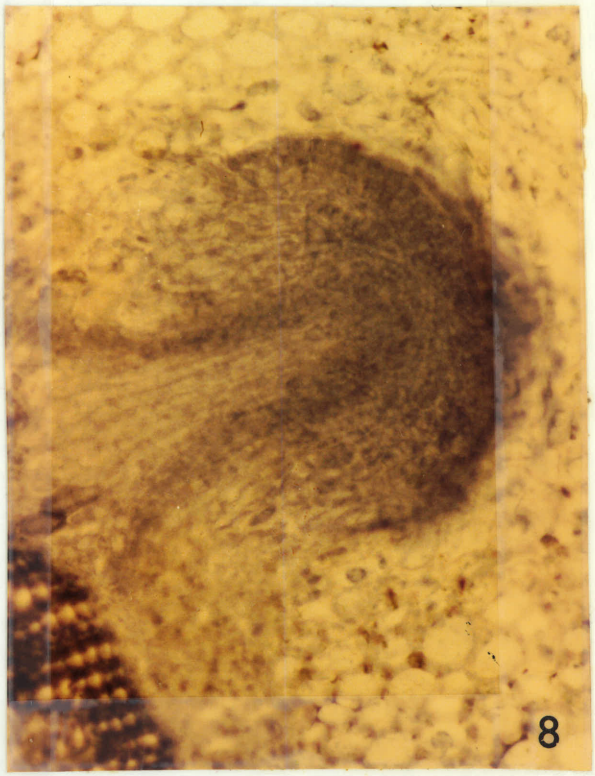


TABLE III

Peroxidase activity in stem cuttings of *Hydrangea macrophylla* before rooting and during the development of root primordia.

Tissue	Before rooting (Arbitrary Units)	5-6 day old roots (Arbitrary Units)	Differences
Epidermis	14.2	18.2	4.0 xx
Cortex	4.8	5.8	1.0 ns
Phloem	23.4	27.8	4.4 xx
Root initials or primordia	24.4	49.4	25.0 xx
Xylem	22.4	38.0	16.0 xx
Pith	3.8	5.0	1.2 ns

xx Differences are significant at the 1% level according to the 't' test (t=2.0398).

Figures 9 to 12 demonstrate localization of peroxidase in hydrangea stem sections at different times after the cuttings were made.

Legend: P, phloem, Pr, phloem ray; X, xylem.

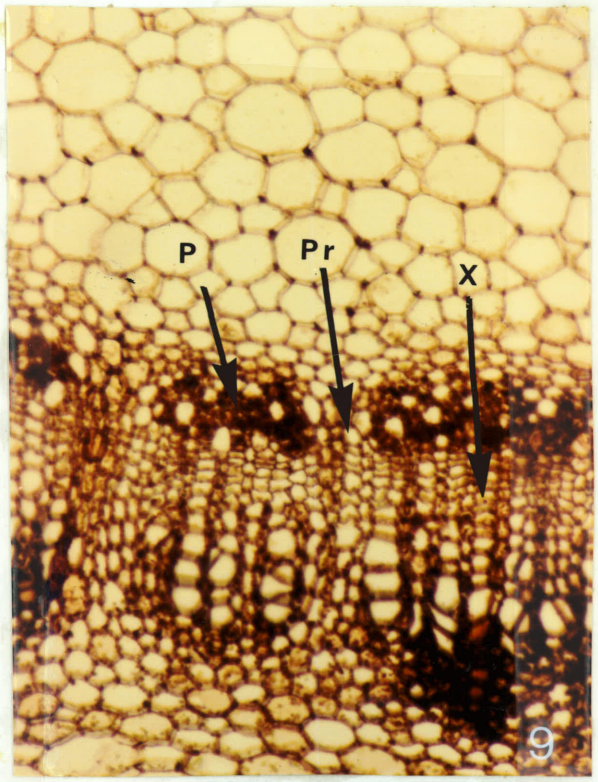
Figure 9 — Transection at the time the cuttings were made. X 600.

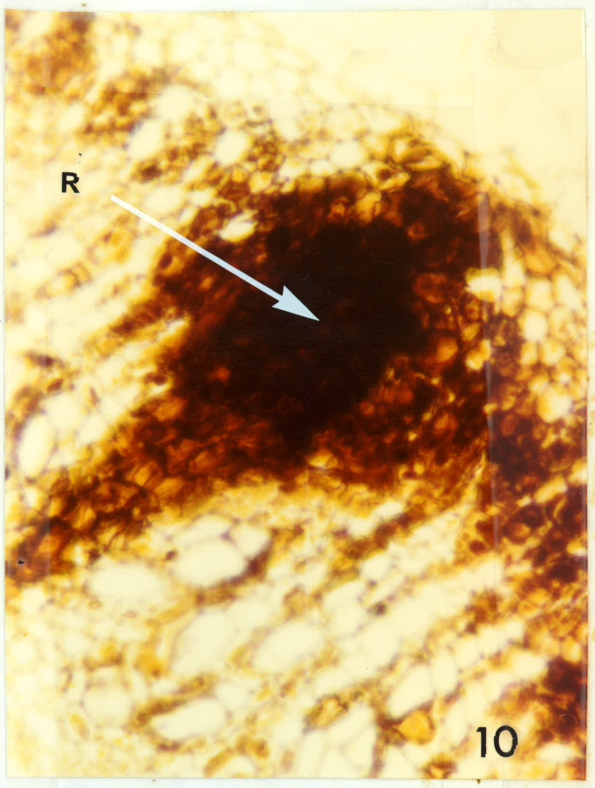
Figure 10 — Transection after two days. X 1000.

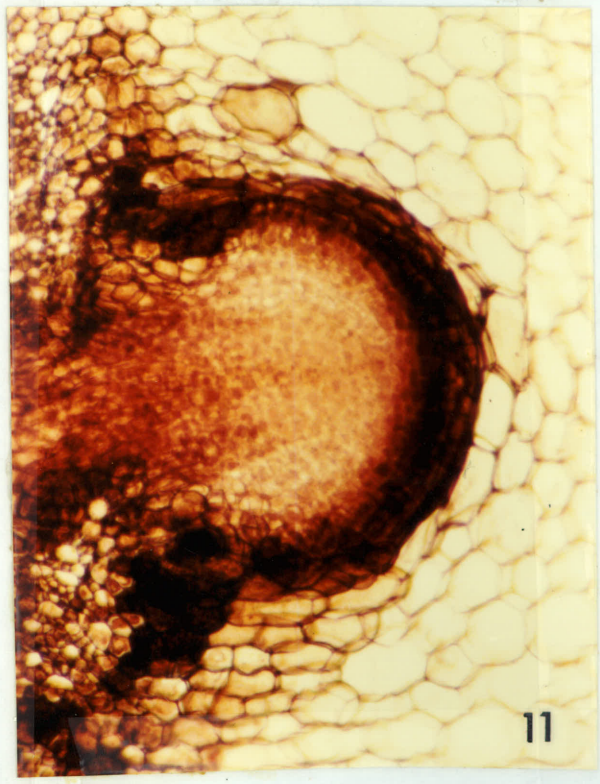
Figure 11 — Transection after seven days showing root primordia. X 400.

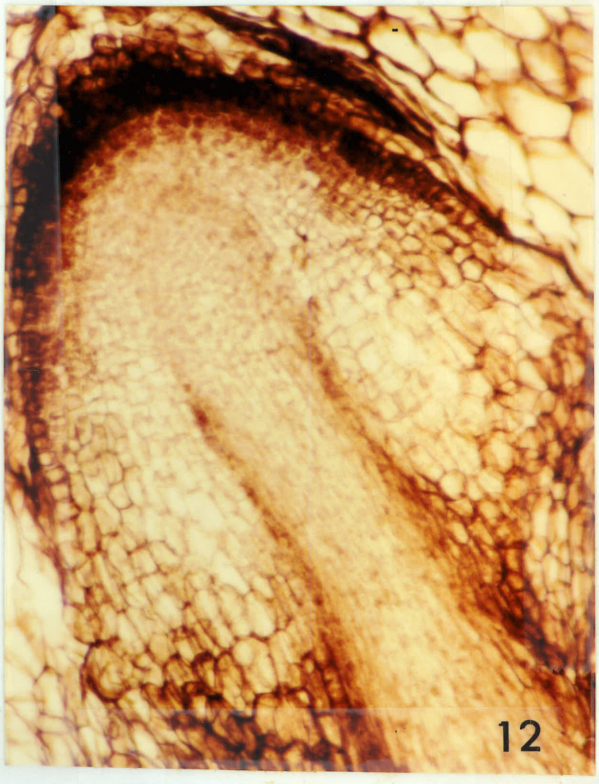
Figure 12 — Longitudinal section of adventitious root after ten days. X 800.

Figures 9 to 12.









D. Amylase

Starch films exposed to fresh tissue sections of hydrangea cuttings indicated strong amylase activity, as revealed by the unstained hydrolyzed area. Figure 13 illustrates a slide stained by the PAS reaction. Amylase or another starch hydrolyzing enzyme was active in the epidermis and in the vascular bundles, especially in the xylem area below the interfascicular cambium (Fig. 14). There appeared to be somewhat lesser activity in the phloem ray parenchyma and also in the cortex, just outside the endodermis and the exterior of the pith (Fig. 15). When sections from three day old cuttings were exposed to the starch film, there appeared to be a shift in enzyme activity to the periphery of the vascular bundles, especially to the phloem and xylem rays (Fig. 16). This area corresponds to the area where the starch accumulated in the cuttings (Fig. 18).

When sections with developing root primordia were exposed to the starch film, the film lost its stainability in the vascular bundle area behind the developing root primordia (Fig. 17). The root primordia appear to lack any amylase or other starch hydrolyzing enzyme activity.

Starch films exposed to heat killed sections did not lose their stainability with the PAS reaction. Where starch films were exposed to a 0.1% alpha-amylase solution they lost their stainability with the PAS reaction.

Unstained hydrolyzed areas of the films exposed to kernels of triticale after overnight soaking, corresponded with that of the aleurone layer in the sections known to have high amylase activity.

E. Effect of starch content on the rooting of cuttings

In the first experiment (Table IV) cuttings from the dark treated plants had an average starch content of 13.39 arbitrary units; 12.51 roots per cutting, and rooted only 88% compared to the cuttings collected from plants grown under normal growing conditions. These had an average starch content of 27.13, average root number of 72.43 and 100% rooting. There was a positive correlation of + 0.675 between the starch content and root number. The dark treated cuttings did not show such a relationship in this experiment.

In the second experiment, (Table V) cuttings from the dark treated plants had an average of 8.55 starch content; 25.33 roots per cutting, and only 85% of the cuttings rooted. In spite of the lower rooting percentage and fewer roots per cutting, a positive correlation of + 0.658 between starch content and root number per cutting was found. Cuttings from plants grown in greenhouse under normal light regime had much higher starch content and had a positive correlation of + 0.771 between starch content and root number. As illustrated in Figure 18, the starch accumulated in the endodermis, phloem and xylem rays and also in the pith. Figure 19 illustrates a section with developing root primordia. It is

apparent that the starch has been hydrolyzed from the phloem and xylem rays and the pith in proximity to the adventitious roots, and also partially from the endodermis in the vicinity of the root primordia.

Figures 13 to 17 demonstrate alpha-amylase activity in hydrangea stem sections. Amylase activity is revealed by the unstained hydrolyzed area.

Legend: E, epidermis; Vb, vascular bundles; R, ray cells; Rp, root primordia.

Figure 13 — Starch film after exposure to sections for 45 minutes. Actual size.

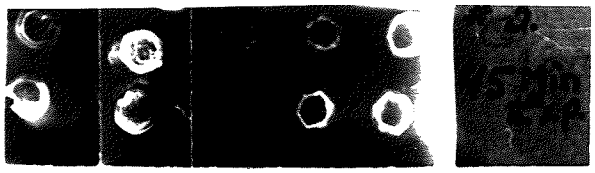
Figure 14 — Starch film exposed to a section at the time the cuttings were made. Note enzyme activity in the epidermis and vascular bundles. X 200.

Figure 15 — Starch film exposed to a section at the time the cuttings were made. There is lower enzyme activity in the ray cells. X 300.

Figure 16 — Starch film exposed to a section two days after the cuttings were made. Enzyme activity appears higher in periphery of vascular bundles. X 400.

Figure 17 — Starch film exposed to a section containing two root primordia. There is no enzyme activity indicated in the root primordia. X 400.

Figures 13 to 17.



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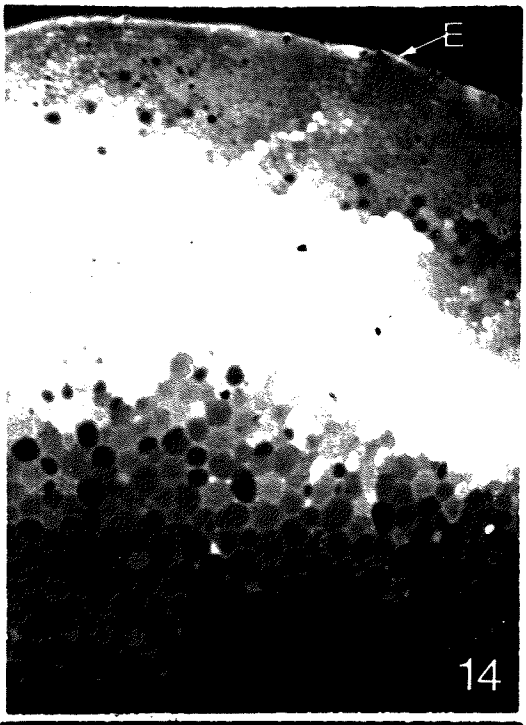
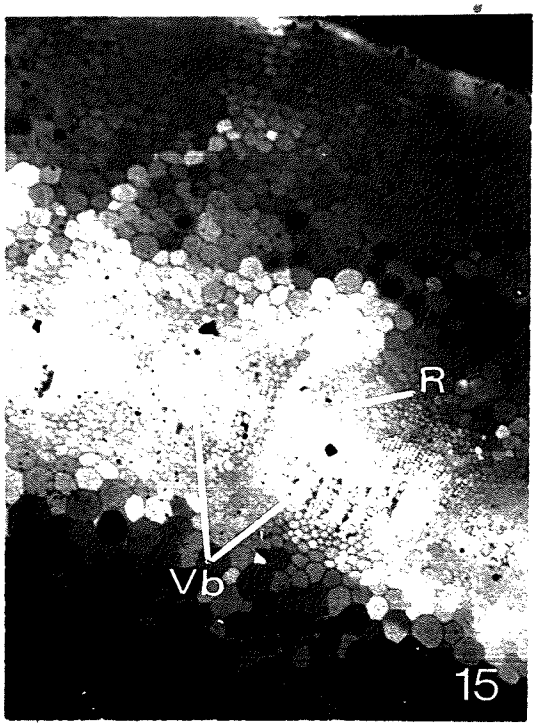
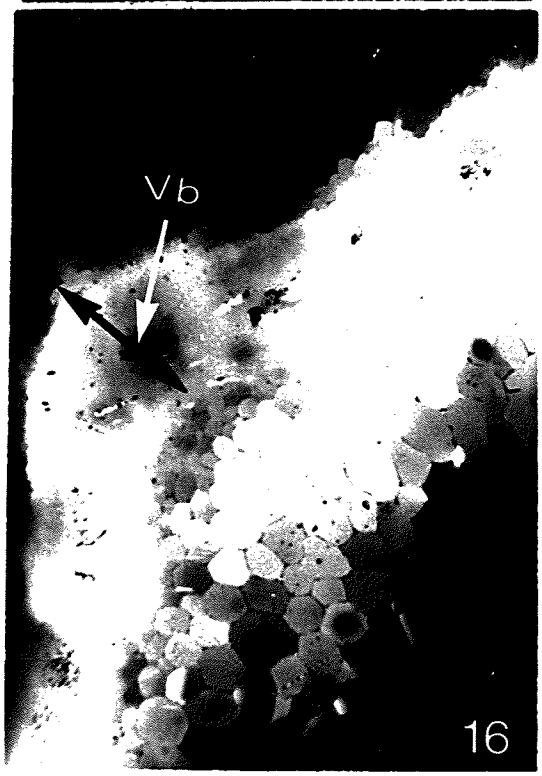
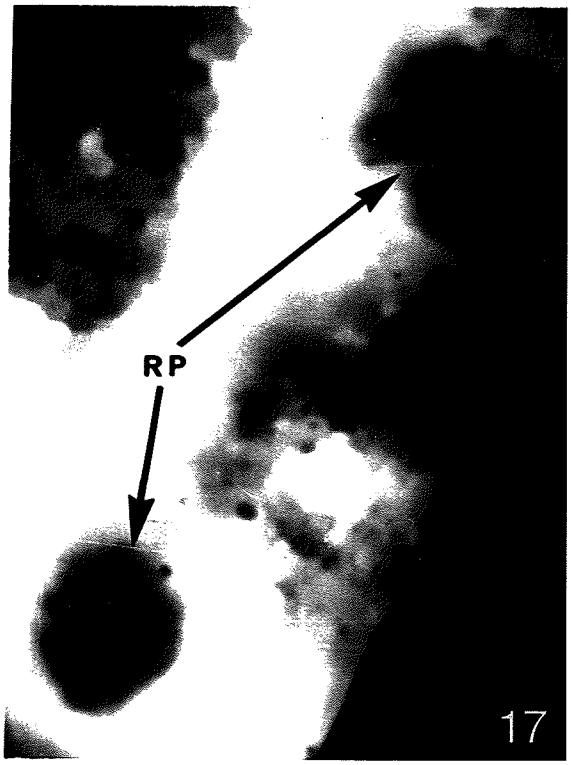
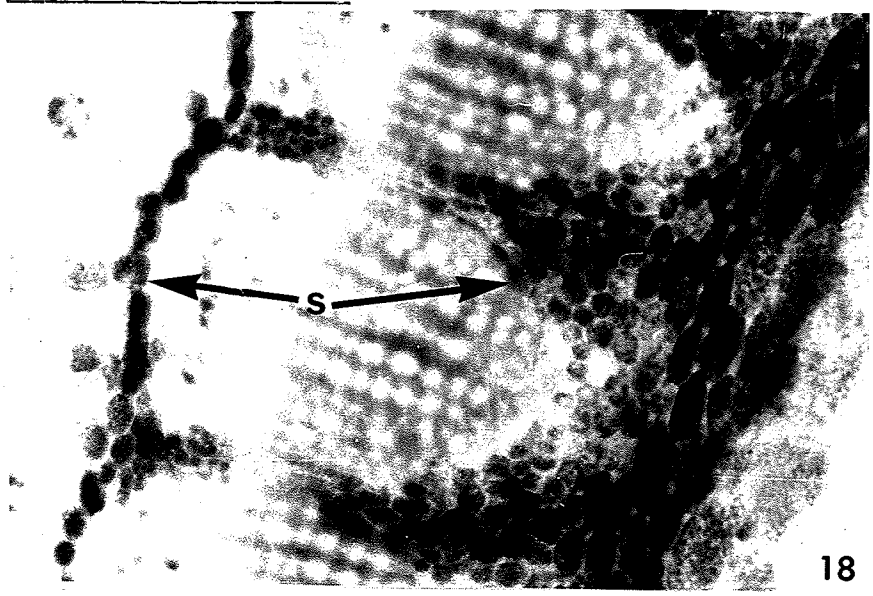


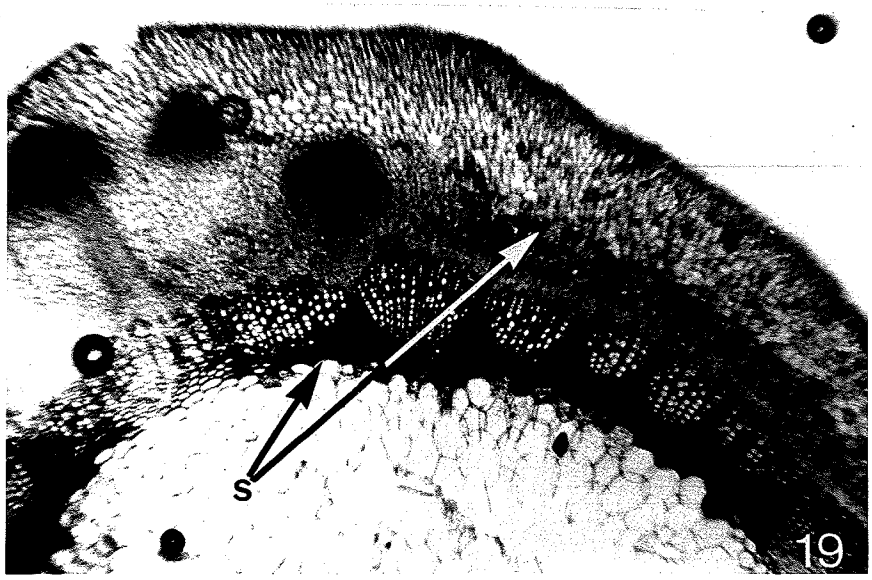






Figures 18 and 19.





Figures 18 and 19 demonstrate starch content.

Figure 18 — Transection at the time the cuttings were made showing starch accumulation in the endodermis, phloem and xylem ray and pith. X 600.

Figure 19 — Transection showing the lack of starch behind the root primordia (S, starch). X 400.

TABLE IV

The effect of starch content on the root initiation of *Hydrangea macrophylla* cuttings

EXPERIMENT I

DARK TREATED THREE DAYS			NO DARK TREATMENT		
Starch content (Arb.Units) Classes	Frequency No.	Av. root No./cutting in class	Starch content (Arb.Units) Classes	Frequency No.	Av.root No./cutting in class
7-8	2	1.33	21-22	2	40.50
9-10	4	16.00	23-24	6	54.57
11-12	3	14.00	25-26	5	65.00
12-13	4	18.00	27-28	4	83.75
14-15	9	9.00	29-30	2	109.00
16-17	4	23.00	31-32	5	68.00
18-19	3	11.00	33-34	4	106.00
20-21	1	10.50	35-36	2	127.00
Average starch content/cutting = 13.39			Average starch content/cutting=27.13		
Average root number/cutting = 12.51			Average root number/cutting = 72.43		
Percent rooting = 88%			Percent rooting = 100%		
$r = + 0.134$			$r = + 0.675$		

TABLE V

The effect of starch content on the root initiation of *Hydrangea macrophylla* cuttings

EXPERIMENT II

DARK TREATED THREE DAYS			NO DARK TREATMENT		
Starch content (Arb.Units) Classes	Frequency No.	Av. root No./cutting in class	Starch content (Arb.Units) Classes	Frequency No.	Av. root No./cutting in class
4	3	2.00	13-14	2	19.00
5	3	9.33	15-16	4	29.75
6	3	7.33	17-18	3	64.00
7	5	24.00	19-20	4	44.50
8	2	33.00	21-22	5	92.40
9	4	36.75	23-24	6	98.00
10	3	26.33	25-26	4	115.50
11	5	28.40	27-28	3	112.00
12	5	34.40	29-30	2	122.33
Average starch content/cutting = 8.55			Average starch content/cutting = 22.88		
Average root number/cutting = 25.33			Average root number/cutting = 83.36		
Percent rooting = 85%			Percent rooting = 97%		
$r = +0.658$			$r = +0.771$		

DISCUSSION

Some of the anatomical and histochemical changes that occurred during root initiation of hydrangea stem cuttings have been described earlier (12). It was reported that protein synthesis preceded DNA synthesis and cell division in the preformed adventitious root initials. It is clear from the results presented above, that extensive changes also occur in enzyme activities. In the normal course of development of the root primordia, all the enzymes investigated had increased activity in the tissue responsible for root initiation. The first change was noted in peroxidase activity within phloem and xylem ray cells, well before any noticeable change in anatomy (Fig. 10). Similar localization was reported in developing roots of *Vicia* by Jensen (10). He suggested that peroxidase could be important in the cellular differentiation, particularly in vascular tissue. Van Fleet (17) also detected peroxidase activity both before cell division and accompanying cell division in pro-epidermis, proendodermis, protophloem and primordial centres of origin of buds and roots.

In the present study, it was found that peroxidase activity was highest in cells of the phloem and xylem rays two days after the cuttings were made. This high enzyme activity decreased considerably as the tissues developed with highest activity remaining in the root cap and epidermal tissues

(Figs. 11,12). Microdensitometric measurements indicated that there was a general increase in peroxidase activity from zero to five days. The largest increase was found to be in the root primordia, while other tissues also showed an increase in peroxidase activity. Peroxidase has been reported to play a role in lignin synthesis, converting hydroxyphenylpropanes such as coniferyl alcohol to lignin-like material (7). The hydrangea stem cuttings used in this experiment were still undergoing lignification. This might explain the general increase in peroxidase activity in the entire section.

Cytochrome oxidase was found to increase in activity and could be detected in the root initials two days after the cuttings were made (Fig. 6). This activity was further intensified with the development of root primordia. The meristematic regions of the root apex and the procambial strands stained very deeply as a result of enzyme activity (Figs. 7,8).

Cytochrome oxidase appeared to be active in the entire hydrangea stem section, with lesser activity in the cortex and pith area (Table II). Avers (1) showed that all growing epidermal cells also have high cytochrome oxidase activity, but that some activity of cytochrome oxidase could be detected in all tissues of the section.

The Nadi reaction is not specific for cytochromes. It also

stains peroxidase and polyphenol oxidase. This may explain the high cytochrome oxidase in lignified tissues.

The other respiratory enzyme investigated was succinic dehydrogenase. Increased activity of this enzyme could be observed in the root initial area only, about three days after the cuttings were taken. Activity of this enzyme in the stem sections appeared to be very low when the cuttings were made. The highest activity was observed in the epidermis. This enzyme has been demonstrated in plants by other authors (1,6,11). They found it to be most active in the meristematic areas of the shoot apex and in the procambial strands. Pith and cortex tissue showed very little activity of this enzyme. Avers (1) also detected activity in meristematic cells of roots and root caps. DeJong (5) reported strong succinic dehydrogenase activity in the epidermis of onion root.

It is interesting to note that both succinic dehydrogenase and cytochrome oxidase are found in mitochondria and associated with a chain of enzymes and co-factors. Succinic dehydrogenase transfers electrons from succinate to oxygen by way of cytochrome C and cytochrome oxidase (2). One might have expected both enzymes to be detected in the same tissue at the same time. Activity of cytochrome oxidase was detectable in the root initials about two days after the cuttings were made, while succinic dehydrogenase activity could be detected only on the third day. There were

differences in the location of the increased enzyme activity. For example, while cytochrome oxidase was confined to the root initials, the succinic dehydrogenase enzyme exhibited an increased activity not only in the root initial, but also in the phloem area surrounding the root initial. One might consider that initially, higher levels of some inhibitor could mask the immediate activity of succinic dehydrogenase and not that of cytochrome oxidase. It is known, for instance, that oxalacetate is a powerful inhibitor of succinic dehydrogenase. Even in extremely well washed mitochondria there are traces of endogenous substrate which allow a slow rate of respiration, and this results in the accumulation of oxalacetic acid (2).

The other possibility is that peroxidase and perhaps catalase, were masking the expression, since both peroxidase and catalase inhibit staining reaction. Diophorase (part of the electron transport system from NAD(P)H to cytochromes) diverts H· from the NAD(P)H to the tetrazolium system, which also results in inhibition of staining. This may be the reason why the enzyme in lower concentration, could not be detected in early stages of root development (22).

Activity of amylase was the last to be investigated. The method used for localization of this activity was a modification of that used by Tremblay for animal tissues (15,16). Satisfactory localization of starch hydrolytic

activity is now possible within sections of plant material.

Activities of the enzyme or enzymes localized in hydrangea cuttings appeared essentially similar both to that of 0.1% alpha-amylase and to the activity of starch hydrolysis of the aleurone layer of sections of soaked triticale kernels. No definite conclusion is possible, but it seems likely that most of the activity detected and localized was of alpha-amylase.

Starch hydrolyzing enzymes in the tissue sections were most active in the area of the vascular bundles, and in the epidermis at the time the cuttings were made. Subsequently, localization of enzyme activity shifted from the vascular bundles to the periphery of the bundles, especially to the phloem and xylem ray cells (Fig. 16). This area was very high in starch content at the time when the cuttings were made (Fig. 8). It is reasonable that this hydrolyzed starch provides the energy and substrate required for the execution of the metabolic processes of root initiation. This supposition appears to be correct since there was a positive correlation between the number of roots initiated and the starch content of the cuttings. When sections of five to six day old cuttings with root primordia were placed on the starch film, very high enzyme activity was revealed behind and around the primordia, but not in the primordia themselves. This correlates with a lack of starch in

the primordia (Fig. 19).

The high amylase activity internal to the new root initials (Fig. 7), was reflected in the disappearance of starch from rays behind developing root initials in comparable areas of iodine stained sections (Fig. 9).

Thus starch disappears from the endodermis, phloem and xylem rays and pith in close proximity to the developing root primordia. This suggests that starch plays an important role in adventitious root development. This observation is supported by Webster and Van't Hof's report (21). They cultured meristems of pea root which failed to proliferate in the absence of a carbohydrate source. The provision of sucrose increased rates of RNA and protein synthesis which they concluded were prerequisites for cell division and DNA synthesis.

It appears that changes of enzymatic activity can be correlated with various stages of root primordial development. This must indicate regulation of enzymes involved in the developmental process, and perhaps their regulation, in turn, of further steps in differentiation of root primordia.

It was suggested in the previous report (12) that both DNA and some protein synthesis must occur before cell division of the root initials can take place. Activities of particular fractions of this early synthesized protein may account for the developing pattern of enzyme activities.

Some of these proteins may exercise specific roles in the activation of root primordia. For example, peroxidase may be responsible for the destruction of some of the inhibitors responsible for blocking metabolic processes in the hydrangea stem leading to adventitious root formation.

Increase in activity of other enzymes may reflect only the resumption of rapid general cellular activity. Succinic dehydrogenase and cytochrome oxidase are integrated into the processes of cellular respiration, and the amylase activity is releasing sugars as general substrates for the continuing synthetic processes.

ACKNOWLEDGEMENTS

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LITERATURE CITED

1. Avers, C.J. 1958. Histochemical localization of enzyme activity in the root epidermis of *Phleum pratense*. Amer. J. Bot., 45: 609-612.

2. Bonner, J. and Varner, J.E. 1965. Plant Biochemistry. Academic Press, Inc., New York.

3. Briggs, D.E. 1962. Gel-diffusion method for the assay of alpha-amylase. J. Inst. Brew., 68: 27-32.

4. DeJong, D.W. 1967. An investigation of the role of plant peroxidase in cell wall development by the histochemical method. J. Histochem. Cytochem., 15: 335-346.

5. DeJong, D.W., Jansen, E.F. and Olson, A.C. 1967. Oxido-reductive and hydrolytic enzyme patterns in plant suspension culture cells. Exp. Cell Res., 47: 139-156.

6. Ekes, M. 1970. Electron-microscopic-histochemical demonstration of succinic dehydrogenase activity in root cells of Yellow Lupine. Planta (Berl.) 94: 37-46.

7. Galston, A.W. and Davies, P.J. 1969. Hormonal regulation in higher plants. Science, 163: 1288-1297.

8. Gates, J.W. and Simpson, G.M. 1968. The presence of starch and alpha-amylase in the leaves of plants. Can. J. Bot., 46: 1459-1462.

9. Jensen, W.A. 1962. Botanical Histochemistry. W.H. Freeman and Company, San Francisco.

10. Jensen, W.A. 1955. The histochemical localization of peroxidase in roots and its induction by indoleacetic acid. Plant Phys., 30: 426-432.

11. Mia, A.J. and Pathak, S.M. 1968. A histochemical study of the shoot apical meristem of *Rauwolfia* with reference to differentiation of sclereids. Can. J. Bot., 46: 115-120.

12. Molnar, J.M. and LaCroix, L.J. 1971. Studies of the rooting of cuttings of *Hydrangea macrophylla*: DNA and protein changes. Can. J. Bot., (submitted for publication).

13. Naylor, J.M. 1966. Dormancy studies in seed of *Avena fatua*. 5. On the response of aleurone cells to gibberellic acid. Can. J. Bot., 44: 19-32.

14. Simpson, G.M. and Naylor, J.M. 1962. Dormancy studies in seed of *Avena fatua*. 3. A relationship between maltase, amylases and gibberellin. Can. J. Bot., 40: 1659-1674.

15. Tremblay, G. 1962. The localization of amylase activity in tissue sections by a starch film method. J. Histochem. Cytochem., 10: 202-206.

16. Tremblay, G. and Charest, J. 1967. Modified starch film method for the histochemical localization of amylase activity. J. Histochem. Cytochem., 15: 147-148.

17. Van Fleet, D.S. 1959. Analysis of the histochemical localization of peroxidase related to the differentiation of plant tissues. Can. J. Bot., 37: 449-458.

18. Van Fleet, D.S. 1951. A comparison of histochemical and anatomical characteristics of the hypodermis with the endodermis in vascular plants. Amer. J. Bot., 37: 721-725.

19. Van Fleet, D.S. 1947. The distribution of peroxidase in differentiating tissues of vascular plants. Biodyn., 3: 125-140.

20. Van Fleet, D.S. 1952. Histochemical localization of enzyme in vascular plants. Botan. Rev., 18: 354-398.

21. Webster, P.L. and Van't Hof, J. 1970. DNA synthesis and mitosis in meristems: Requirements for RNA and protein synthesis. Amer. J. Bot., 57: 130-139.

22. Woodbury, W. 1971. Personal communication. Department of Plant Science, University of Manitoba.

GENERAL DISCUSSION

Prior to observation of the first anticlinal division of preformed root initials, the first anatomical sign of adventitious root development of hydrangea cuttings, a time sequence of biochemical events occurred. This included increased enzyme activity of peroxidase, cytochrome oxidase, succinic dehydrogenase and alpha-amylase, together with a rise in protein and DNA synthesis. Adventitious root formation is a complex process in which many factors are involved. These include the action of auxin, the utilization of carbohydrates, and special co-factors manufactured in the bud (97).

It was reported by Hess (42) that a phenolic compound, catechol, reacts synergistically with indoleacetic acid in root production in mung bean bioassay. Since, as he points out, catechol is readily oxidized to a quinone, and the mung bean itself is a good source of phenolase; it is possible that oxidation of an orthodihydroxyphenol is one of the first steps leading to root formation. Hartmann suggests that a phenolic cofactor from buds, combines with auxin (endogenous or applied) and forms an auxin-phenol complex. This may react at the base of the cutting with a specific enzyme, initiating cell division which leads to adventitious root formation (41).

It is not possible to draw a definite conclusion from the results presented regarding the first metabolic step which leads to DNA synthesis and cell division. However, the results indicate that the enzymes investigated play an important role in adventitious root development.

As was reported, two days after the cuttings were made there was a major increase in the quantities of cellular protein, in the root initial area. This was accompanied by changes in enzyme activity. These changes indicate regulatory controls of enzyme involvement in the developmental process, arising either from the pattern and extent of enzyme synthesis, and/or the contribution of the structural protein component.

The first changes found were in peroxidase, cytochrome oxidase and alpha-amylase activities, within the phloem ray cells, well before any obvious change in anatomy was noticeable. From the two respiratory enzymes investigated, increased cytochrome oxidase activity could be demonstrated one day earlier than that of succinic dehydrogenase. As previously mentioned, succinic dehydrogenase activity could have been masked in low concentrations by some inhibitors. This, unfortunately, is one of the disadvantages of microscopic histochemistry. Most of the reagents used to indicate enzyme activity are not completely specific and it is possible that some other

compounds may give similar reaction products. Lignified tissues usually give a very strong cytochrome oxidase reaction. However, a portion of this may be contributed by peroxidase and polyphenol oxidase. The tetrazolium staining reaction for succinic dehydrogenase may be inhibited by peroxidase and possibly catalase. Oxaloacetate is known also to inhibit succinic dehydrogenase. The problems in methodology, however, should not be of major concern in establishing the overall pattern of enzyme changes during the rooting process.

Alpha-amylase activity increased within two days in the periphery of the vascular bundles, especially in the phloem and xylem ray cells. This area was very high in starch content at the time the cuttings were taken. It is reasonable to assume that this hydrolyzed starch provides the energy and substrate required for the execution of the metabolic processes of root initiation. This supposition appears to be correct since there was a positive correlation between the number of roots initiated and the starch content of the cuttings.

The high amylase activity internal to the new root initials was reflected in the disappearance of starch from ray cells behind developing root initials in comparable areas of iodine stained sections.

Thus starch disappears from the endodermis, phloem and xylem rays and pith in close proximity to the developing root primordia. This suggests that starch plays an important role in adventitious root development. This observation is supported by that of Webster and Van't Hof (96) who indicated that the provision of sucrose increased rates of RNA and protein synthesis which they concluded were prerequisites for DNA synthesis and cell division.

The first division of the root initials in the cuttings could be observed microscopically three to four days after the cuttings were made. While the DNA content of the cells does show some increase by the third day, it does not begin to increase rapidly until the sixth day. By comparing the estimated mean total protein content per cell with the DNA content per cell, it is clear that much protein synthesis precedes the large scale replication of nuclear DNA. The total protein per cell stabilized on or before the sixth day from when the cuttings were made. This indicated that the rate of protein synthesis relative to cell division was such that per cell protein remained relatively constant. While increased protein synthesis and doubling of DNA do not necessarily lead to cell division; in the tissue studied, it appeared that this was the sequence of events.

It was found that in the latent root initials of

hydrangea, the DNA content remained constant, since most cells were observed at the 2C level when the cuttings were made. This indicated that the process leading to DNA replication was blocked. This block was removed when the cuttings were made. The roots originated from a specialized group of cells within the phloem ray. Under normal growing conditions they do not divide and it is suggested that one or more conditions of their normal cellular environment provides such a block. If the stem is cut off and put into a rooting medium, these cells resume nuclear metabolism and cell division.

It is apparent from these studies that both DNA and some protein synthesis must occur before cell division of the root initials can take place. Activities of particular fractions of this early synthesized protein may account for the developing pattern of enzyme activities.

Some of the these proteins may exercise specific roles in the activation of root primordia. For example, peroxidase may be involved in the destruction of some of the inhibitors responsible for blocking metabolic processes in the hydrangea stem leading to adventitious root formation.

Increase in activity of other enzymes may reflect the resumption of rapid general cellular activity. Succinic dehydrogenase and cytochrome oxidase are integral

components of the processes of cellular respiration and the amylase activity is releasing sugars as substrates for the continuing synthetic processes. The importance of correlation of enzymatic activity with development becomes apparent. Activation and new synthesis of various enzymes and changes in metabolism, seem to be part of the regulatory mechanism of adventitious root development.

BIBLIOGRAPHY FOR GENERAL LITERATURE REVIEW

1. Alexander, A.G. 1965. Physiological study of enzymes catalyzing the synthesis and hydrolysis of sucrose, starch and phosphorylated hexose in sugar cane. J. Agr. Univ. Peurtro Rico, 49: 60-65.

2. Alvarez, M.R. 1968. Quantitative changes in nuclear DNA accompanying postgermination embryonic development in *Vanda (Orchidaceae)*. Amer. J. Bot., 55: 1036-1041.

3. Alvarez, M.R. and Sagawa, Y. 1965. A histochemical study of embryo development in *Vanda (Orchidaceae)*. Caryologia, 18: 251-261.

4. Argles, G.K. 1959. Root formation and root development in stem cuttings: A re-examination of certain fundamental aspects. Ann. Appl. Biol., 47: 626-627.

5. Audus, L.J. 1959. Plant growth substances. Leonard Hill Limited, London; Interscience Publishers, Inc., New York.

6. Avers, C.J. 1958. Histochemical localization of enzyme activity in the root epidermis of *Phleum pratense*. Amer. J. Bot., 45: 609-612.

7. Bagchi, M., Rothstein, H. and Stein, G.S. 1968. Synthesis of macromolecules in epithelial cells of the cultured amphybian lens. II. Effects of puromycin and actinomycin D on protein synthesis. Exp. Cell. Res., 50: 554-559.

8. Baker, J.R. 1943. The discovery of the uses of colouring agents in biological micro-technique. Jour. Quekett Miscroscop. Club. Ser. 4, 1: 256-275.

9. Barka, T. and Anderson, P.J. 1965. Histochemistry. Theory, practice and bibliography. Hoeber Medical Division. Harper and Row, Publishers, Inc. New York.

10. Beakbane, A.B. 1961. Structure of the plant in relation to adventitious rooting. Nature, 192: 954-955.

11. Bonner, J. 1959. Protein synthesis and the control of plant processes. Amer. J. Bot., 46: 58-62.

12. Bonner, J. and Varner, J.E. 1965. Plant Biochemistry. Academic Press, Inc., New York. p. 273.

13. Brandon, R. 1939. Seasonal variations in the starch content of the genus *Rosa*, and their relation to propagation by stem cuttings. J. Pom. Hort. Sci., 17: 233-253.

14. Briggs, D.E. 1962. Gel-diffusion method for the assay of alpha-amylase. J. Inst. Brew., 68: 27-32.

15. Brumfield, R.T. 1942. Cell growth and division in living root meristems. Amer. J. Bot., 29: 533-543.

16. Bucknall, R.A. and Sutcliffe, J.F. 1965. The nucleic acids and cellular differentiation in the root apex of *Pisum sativum*. II. The nucleic acids and cellular development patterns. J. Exp. Bot., 16: 587-603.

17. Burg, S.P. and Burg, E.A. 1967. Lateral transport in stems and roots. Plant Phys., 42: 891-893.

18. Carlson, M.C. 1933. Comparative anatomical studies of Dorothy Perkins and American Pillar Roses. I. Anatomy of canes. II. Origin and development of adventitious roots in cuttings. Contribution from Boyce Thompson Institute for Plant Research, Inc., 5: 313-329.

19. Carlson, M.C. 1938. The formation of nodal adventitious roots in *Salix cordata*. Amer. J. Bot., 25: 721-725.

20. Carpenter, J.B. 1961. Occurrence and inheritance of preformed root primordia in stems of citron *(Citrus medica* L.). Proc. Amer. Soc. Hort. Sci., 77:211-218.

21. Chrispeels, M.J. and Varner, J.E. 1967. Hormonal control of enzyme synthesis: On the mode of action of gibberellic acid and abscisin in aleurone layers of barley. Plant Phys., 42: 1008-1016.

22. Deeley, E.M. 1954. An integrating microdensitometer for biological cells. J. Sci. Inst., 32: 263-267.

23. Deeley, E.M., Davies, H.G. and Chayen, J. 1957. The DNA content of cells in the root of *Vicia faba*. Exp. Cell Res., 12: 582-591.

24. DeJong, D.W. 1965. Histochemical demonstration of extracellular distribution of acid phosphatase in onion roots. Oyton. 22: 141-146.

25. DeJong, D.W. 1967. An investigation of the role of plant peroxidase in cell wall development by the histochemical method. J. Histochem. Cytochem., 15: 335-346.

26. DeJong, D.W., Jansen, E.F. and Olson, A.C. 1967. Oxido-reductive and hydrolytic enzyme patterns in plant suspension culture cells. Exp. Cell Res., 47: 139-156.

27. Delisle, A.L. 1942. Histological and anatomical changes induced by indoleacetic acid in rooting cuttings of *Pinus strobus*. Va. J. Sci., 3: 118-124.

28. Dwivedi, R.S. and Naylor, J.M. 1968. Influence of apical dominance on the nuclear proteins in cells of the lateral bud meristem in *Tradescantia paludosa*. Can. J. Bot., 46: 289-298.

29. Ekes, M. 1970. Electron-microscopic-histochemical demonstration of succinic dehydrogenase activity in root cells of Yellow Lupine. Planta (Berl.) 94: 37-46.

30. Esau, K. 1960. Anatomy of seed plants. John Wiley and Sons Inc., New York.

31. Feder, N. and O'Brien, R.P. 1968. Plant microtechnique: Some principles and new methods. Amer. J. Bot., 55: 123-142.

32. Gagnon, C. 1968. Peroxidase in healthy and diseased elm trees investigated by the benzidine histochemical technique. Can. J. Bot., 46: 1491-1494.

33. Galston, A.W. and Davies, P.J. 1969. Hormonal regulation in higher plants. Growth and development are regulated by interactions between promotive and inhibitory hormones. Science, 163: 1288-1297.

34. Gaspar, T. and Lacoppe, J. 1968. The effect of CCC and Amo-1618 on growth, catalase, peroxidase and indoleacetic acid oxidase activity of young barley seedlings. Physiol Planta., 21: 1104-1109.

35. Gates, J.W. and Simpson, G.M. 1968. The presence of starch and alpha-amylase in the leaves of plants. Can. J. Bot., 46: 1459-1462.

36. Gifford, E.M. and Tepper, H.B. 1962. Histochemical and autoradiographic studies of floral induction in *Chenopodium album*. Amer. J. Bot., 49: 706-714.

37. Girouard, R.M. 1967. Initiation and development of adventitious roots in stem cuttings of *Hedera helix*. Anatomical studies of the juvenile growth phase. Can. J. Bot., 45: 1877-1882.

38. Girouard, R.M. 1967. Initiation and development of adventitious roots in stem cuttings of *Hedera helix*. Anatomical studies of the mature growth phase. Can. J. Bot., 45: 1883-1886.

39. Girouard, R.M. 1967. Anatomy of adventitious root formation in stem cuttings. Proc. Int. Plant Prop. Soc. Ann. Meet. 17: 289-302.

40. Hare, R.C. 1964. Indoleacetic acid oxidase. Botan. Rev., 30: 129-165.

41. Hartmann, H.T. and Kester, D.E. 1968. Plant Propagation. Principles and Practices. Second Edition. Prentice-Hall, Inc., New Jersey.

42. Hess, C.E. 1967. Root initiation and juvenility - a possible implication of terpene compounds. Proc. XVII Int. Hort. Cong., 3: 443-451.

43. Hiatt, A.J. 1960. preparation and some properties of soluble succinic dehydrogenase from plants. Plant Physiol., 35: Supp. XI.

44. Jakob, K.M. and Bovey, F. 1960. Early nucleic acid and protein synthesis and mitoses in the primary root tips of germinating *Vicia faba*. Exp. Cell Res., 54: 118-126.

45. Jensen, W.A. 1962. Botanical Histochemistry. W.H. Freeman and Company, San Francisco.

46. Jensen, W.A. 1955. The histochemical localization of peroxidase in roots and its induction by indoleacetic acid. Plant Phys., 30: 426-432.

47. Kawase, M. 1967. Effect of centrifugation on rooting of softwood cuttings. Proc. XVII Int. Hort. Cong., 3: 453-467.

48. Key, J.L. and Shannon, J.C. 1964. Enhancement by auxin of ribonucleic acid synthesis in excised Soybean hypocotyl tissue. Plant Physiol., 39: 360-364.

49. Lanphear, F.O. and Meahl, R.P. 1966. Influence of the stock plant environment on the rooting of *Juniperus horizontalis 'Plumosa'*. Amer. Soc. Hort. Sci., 89: 666-671.

50. Laurie, A., Kiplinger, D.A. and Kennard, S.N. 1958. Commercial Flower Forcing. Sixth Edition. McGraw-Hill Book Company, New York.

51. Maksymowych, R. and Kettrick, M.A. 1970. DNA synthesis, cell division and cell differentiation during leaf development of *Xanthium pennsylvanicum*. Amer. J. Bot., 57: 844-849.

52. McCahon, W. 1964. Propagation of Hydrangeas. Proc. Int. Plant Prop. Soc., 14: 253-254.

53. McLeish, J. and Sunderland, N. 1961. Measurements of deoxyribosenucleic acid (DNA) in higher plants by Feulgen photometry and chemical methods. Exp. Cell Res., 24: 527-540.

54. Mendelsohn, M.L. and Richards, B.M. 1958. A comparison of scanning and two-wave length microspectrophotometry. J. Biophys. Biochem. Cytol., 4: 707-709.

55. Mia, A.J. and Pathak, S.M. 1968. A histochemical study of the shoot apical meristem of *Rauwolfia* with reference to differentiation of sclereids. Can. J. Bot., 46: 115-120.

56. Naylor, J.M. 1958. Control of nuclear processes by auxin in axillary buds of *Tradescantia paludosa*. Can. J. Bot., 36: 221-232.

57. Naylor, J.M. 1966. Dormancy studies in seed of *Avena fatua*. 5. On the response of aleurone cells to gibberellic acid. Can. J. Bot., 44: 19-32.

58. O'Brien, T.P. and McCully, M.E. 1969. Plant Structure and Development. MacMillan Company. Collier-MacMillan Canada, Ltd., Toronto, Ontario.

59. Overbeek, J. van. 1966. Plant hormones and regulators. Science, 152: 721-730.

60. Pawelek, D. 1968. Histochemical investigations of acid deoxyribonuclease activity as related to Pea root growth. Folia Biologica, 16: 391-400.

61. Priestley, J.H. and Swingle, C.F. 1929. Vegetative propagation from the standpoint of plant anatomy. USDA Tech. Bull., 151.

62. Purvis, M.J., Collier, D.C. and Walls, D. 1966. Laboratory Techniques in Botany. Second Edition. Butterworths, London.

63. Reed, H.S. 1942. A short history of plant sciences. Chronica Botanica Co., Waltham, Mass.

64. Rickson, F.R. 1968. Anatomy, histochemistry and fine structure of the germinating embryo of *Paulownia tomentosa*. Amer. J. Bot., 55: 280-290.

65. Ridge, I. and Osborne, D.J. 1971. Role of peroxidase when hydroxyproline-rich protein in plant cell walls is increased by ethylene. Nature, 229: 205-208.

66. Sadik, S. and Ozbun, J.L. 1967. Histochemical changes in the shoot tip of cauliflower during floral induction. Can. J. Bot., 45: 955-960.

67. Salisbury, F.B. and Ross, C. 1969. Plant Physiology. Wadsworth Publishing Company, Inc., California.

68. Sass, J.E. 1968. "Botanical Microtechnique". Third Edition. Iowa: The Iowa State University Press.

69. Saurer, W. 1969. Effect of fixation on histochemical determination of deoxyribonucleic acid in plant material. I. Feulgen spectrophotometric and fluorometric measurements. Mikroskopie, 24: 284-290.

70. Saurer, W. 1969. Effect of fixation on histochemical determination of deoxyribonucleic acid in plant material. II. Ultraviolet microspectrophotometry. Mikroskopie, 24: 291-295.

71. Schrader, F. and Leuchtenberger, C. 1950. A cytochemical analysis of the functional interrelations of various cell structures in *Arvelius albopunctatus* (De Geer). Exp. Cell Res., 1: 421-452.

72. Setterfield, G., Schreber, R. and Woodard, J. 1954. Mitotic frequency determinations and microphotometric Feulgen dye measurements in root tips. Stain Tech., 29: 113-120.

73. Setterfield, G. 1961. Structure and composition of plant-cell organelles in relation to growth and development. Can. J. Bot., 39: 469-489.

74. Siegel, S.M. and Galston, A.W. 1955. Peroxide genesis in plant tissues and its relation to indoleacetic acid destruction. Arch. Biochem. Biophys., 54: 102-112.

75. Simpson, G.M. and Naylor, J.M. 1962. Dormancy studies in seed of *Avena fatua*. 3. A relationship between maltase, amylases and gibberellin. Can. J. Bot., 40: 1659-1674.

76. Stangler, B.B. 1956. Origin and development of adventitious roots in stem cuttings of chrysanthemum, carnation and rose. Cornell Agr. Exp. Sta. Memoir 342.

77. Starring, C.C. 1923. Influence of the carbohydrate-nitrate conent of cuttings upon the production of roots. Proc. Amer. Soc. Hort. Sci., 20: 288-292.

78. Steel, R.G.D. and Torrie, J.H. 1960. Principles and Procedures of Statistics. McGraw-Hill Book Company, Inc., New York.

79. Steeves, R.A., Hicks, M.A. and Naylor, J.M. 1969. Analytical studies on the shoot apex of *Helianthus annuus*. Can. J. Bot., 47: 1367-1375.

80. Sunderland, N. and McLeish, J. 1961. Nucleic acid content and concentration in root cells of higher plants. Exp. Cell Res., 24: 541-554.

81. Symposia of the Society for Experimental Biology. Number XIII. Utilization of nitrogen and its compounds by plants. Cambridge: 1959. University Press, Cambridge, England.

82. Sykes, J.T. and Howard, B.H. 1967. The effect of temperature, light intensity and carbohydrate content on the rooting of cuttings under mist. Proc. XVII Int. Hort. Cong., 3: 433-441.

83. Tata, J.R. 1966. Progress. Nucleic acid research. Molec. Biol., 5: 191

84. Tremblay, G. 1962. The localization of amylase activity in tissue sections by a starch film method. J. Histochem. Cytochem., 10: 202-206.

85. Tremblay, G. and Charest, J. 1967. Modified starch film method for the histochemical localization of amylase activity. J. Histochem. Cytochem., 15: 147-148.

86. Vanden Born, W.H. 1963. Histochemical studies of enzyme distribution in the shoot tips of white spruce. Can. J. Bot., 41: 1509-1527.

87. Van Fleet, D.S. 1959. Analysis of the histochemical localization of peroxidase related to the differentiation of plant tissues. Can. J. Bot., 37: 449-458.

88. Van Fleet, D.S. 1951. A comparison of histochemical and anatomical characteristics of the hypodermis with the endodermis in vascular plants. Amer. J. Bot., 37: 721-275.

89. Van Fleet, D.S. 1947. The distribution of peroxidase in differentiating tissues of vascular plants. Biodyn., 3: 125-140.

90. Van Fleet, D.S. 1955. The significance of the histochemical localization of quinones in the differentiation of plant tissues. Phytomorph., 4: 301-310.

91. Van Fleet, D.S. 1952. Histochemical localization of enzyme in vascular plants. Botan. Rev., 18: 354-398.

92. Van't Hof, J. 1965. Relationship between mitotic cycle duration, S. period duration and the average rate of DNA synthesis in the root meristem cells of several plants. Exp. Cell Res., 39: 48-58.

93. Van't Hof, J. 1966. Experimental control of DNA synthesizing and dividing cells in excised root tips of *Pisum*. Amer. J. Bot., 53: 970-976.

94. Van't Hof, J. and Sparrow, A.H. 1963. A relationship between DNA content, nuclear volume and minimum cycle time. Botany. Proc. N.A.S. 49: 897-902.

95. Wardlaw, C.W. 1952. Morphogenesis in Plants. John Wiley and Sons, Inc., New York.

96. Webster, P.L. and Van't Hof, J. 1970. DNA synthesis and mitosis in meristems: Requirements for RNA and protein synthesis. Amer. J. Bot., 57: 130-139.

97. Went, F.W. 1934. A test method for rhyzocaline, the root forming substance. Proc. Kon. Ned. Akad. Wet., 37: 445-455.

98. Wied, G.L. 1966. Introduction to Quantitative Cytochemistry. Academic Press, New York.

99. Wied, G.L. and Barr, G.F. 1970. Introduction to Quantitative Cytochemistry. - II. Academic Press, New York.

100. Woodard, J., Rasch, E. and Swift, H. 1961. Nucleic acid and protein metabolism during the mitotic cycle in *Vicia faba*. J. Biophys. Biochem. Cytol., 9: 445-462.

101. Woodbury, W. 1971. Personal communication. Department of Plant Science, University of Manitoba.