THESIS

PEROXIDASES AND DIFFERENTIATION IN CUCURBITS

Submitted by

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In partial fulfillment of the requirements

for the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

June, 1967

COLORADO STATE UNIVERSITY

June 1967

IT IS RECOMMENDED THAT THE DISSERTATION PREPARED BY

JAMES BRENT LOY

ENTITLED PEROXIDASES AND DIFFERENTIATION IN CUCURBITS

BE ACCEPTED AS FULFILLING THIS PART OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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Abstract of Dissertation

PEROXIDASES AND DIFFERENTIATION IN CUCURBITS

An investigation of the isoperoxidases in the family Cucurbitaceae and their relationship to genetic dwarfing was conducted. Histochemical, quantitative, and electrophoretic techniques were integrated in the study.

An examination of stem sections of squash and muskmelon revealed that peroxidase was strongly localized in the epidermis, the sclerenchymatous ring, and the xylem. No qualitative peroxidase differences were observed between the stem sections of bush and vine lines of squash and muskmelon. A gross analysis of the amount and distribution of lignin in the stem sections also revealed no apparent differences between the lignified areas and the regions of high peroxidase activity in the stem sections. The exception was the high peroxidase activity found in the non-lignified epidermal tissue. The results do not support peroxidase involvement in dwarfing mechanisms in the plants studied.

There were varietal differences in the levels of total peroxidase activity among the ten squash lines studied. However, no inverse relationship between peroxidase activity and the degree of stem elongation was found. There were no significant differences in peroxidase activity between the isogenic bush and vine lines of squash.

The anodic isoperoxidase patterns of the squash lines were found to vary considerably, while the cathodic isoperoxidases among the squash line were fairly uniform and much easier to discern than

the anodic isoperoxidases. There were no apparent consistent differences in the isoperoxidase patterns between the bush and vine lines. The isoperoxidase patterns of the isogenic bush and vine lines of squash were nearly identical. Thus, no relationships between the isoperoxidase patterns and the bush habit of growth were evident.

Both the anodic and the cathodic isoperoxidase patterns were quite uniform among the bush and vine watermelon lines. Although the growth responses of the GA-treated bush watermelon lines were different, their peroxidase patterns were identical. Hence, the watermelon isoperoxidases do not appear to be associated with dwarfing mechanisms.

The vine muskmelon lines exhibited much darker anodic bands than did the bush lines, and two of the cathodic isoperoxidases present in the vine muskmelon lines were absent from the bush lines. Gibberellin treatment stimulated growth in both the bush and vine muskmelon lines, and alleviated some of the isoperoxidase differences between them.

Comparisons of isoperoxidase patterns among the Cucurbita species and among Cucurbitaceae genera indicated a potential for such comparisons in assessing phylogenetic relationships among the species and particularly among the genera of Cucurbitaceae.

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June 1967

ACKNOWLEDGMENTS

The author would like to express his sincere gratitude to Dr. Donald W. Denna, Associate Professor of Horticulture, for his advice and guidance throughout the course of this study.

The author is also grateful to Dr. Cleon Ross, Associate Professor of Botany, and to Gestur Johnson, Professor of Chemistry, for their helpful suggestions and review of this dissertation.

Thanks are also due to Dr. R. L. Foskett, Chairman of the Horticulture Department, and Dr. Donald Wood, Professor of Agronomy, for their review of this dissertation.

Appreciation is extended to the Boettcher Foundation for the fellowship awarded to me during the terminal year of my doctoral work.

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INTRODUCTION

Peroxidases are a group of enzymes widely distributed in the plant kingdom. They are able to catalyze *in vitro* a large number of biologically important reactions (Burris, 1960). Differences in peroxidase activity have frequently been observed between normal and genetically dwarfed varieties of plants (Van Overbeek, 1935; Kamerbeek, 1956; McCune and Galston, 1959), and as such, peroxidase has been implicated in dwarfing mechanisms. Two hypotheses of peroxidase action are: (1) peroxidase could inactivate indoleacetic acid, resulting in dwarfing (Van Overbeek, 1935), and (2) peroxidase could bring about precocious lignification which by forming a rigid matrix in cell walls, could prevent complete cell elongation (McCune, 1961).

Two possible explanations for higher peroxidase levels in dwarfs as compared to non-dwarfs are: (1) gibberellin could act as a repressor of peroxidase synthesis so that high levels of peroxidase would be expected to occur in dwarf plants which appear from their response to exogenous gibberellin to be low in endogenous gibberellin, and (2) a mutation of a hypothetical regulator gene controlling peroxidase synthesis could occur, and this could result in constitutive peroxidase synthesis and consequent dwarfing.

There have been no reports in the literature concerning peroxidase relationships in dwarf and normal plants in Cucurbitaceae. The lack of research on peroxidases in cucurbits, and the availability of bush and vine varieties in this family, suggested that an investigation of the peroxidases in Cucurbitaceae would be a worthwhile project.

Peroxidases are known to exist in multimolecular forms in plants (Saunders, Holmes-Siedle, and Stark, 1965). It has also been found that peroxidase activity is not uniformly distributed in plant tissue (Van Fleet, 1952). It was therefore felt that an integration of histochemical, quantitative, and electrophoretic techniques for examining cucurbit peroxidases would be necessary to properly evaluate their physiological significance.

With the above information in mind, an investigation of the peroxidases in the Cucurbitaceae family was initiated with the following objectives:

1. To conduct a histochemical comparison of peroxidase as well as lignin distribution in stem tissue of bush and vine cucurbits.

2. To conduct a quantitative study of peroxidase activity in bush and vine lines of squash, muskmelon, and watermelon, using the following treatments:
   a. non-treated controls
   b. gibberellin-treated plants
   c. plants treated with growth retardants

3. To conduct an electrophoretic study of the peroxidase isozymes in cucurbits, using the same treatments as given above.

A considerable number of preliminary investigations were necessary to refine the methods used in the colorimetric and electrophoretic studies of peroxidase. A discussion of this research and of the methods adopted will be included in the methods and materials section.

The term isoperoxidase, as coined by Siegel and Galston (1967), will be employed in the results and discussion section in place of the terms peroxidase isoenzyme or peroxidase isozyme.

Abbreviations that will be adopted throughout the text include:

GA = GA_3 or gibberellic acid, and IAA = indoleacetic acid.

LITERATURE REVIEW

Peroxidases in Plants

General Action

Peroxidases (donor:H_2O_2 oxidoreductases) are a group of enzymes which are capable of catalyzing the oxidation by hydrogen peroxide of a number of classes or organic compounds (Saunders, Holmes-Siedle, and Stark, 1964). Among these compounds are phenols, aromatic amines, leuco dyes, and certain heterocyclic compounds (e.g. ascorbic acid and indole). The range of compounds capable of undergoing oxidation in the presence of a peroxidase system becomes even larger when coupled oxidations are considered (Burris, 1960; Saunders et al., 1964). These reactions and the direct oxidations by peroxidase, give it a degree of versatility unsurpassed by any other enzyme (Saunders et al., 1964).

Peroxidases have molecular weights of about 40,000, protohemin IX as their prosthetic group, and a carbohydrate moeity (Paul, 1963). Because of their high molecular activity and the fact that many of their hydrogen donors are oxidized to highly colored products (Saunders et al., 1964), peroxidases are easy to detect histochemically and electrophoretically.

A distinction should be made at this point between peroxidase and catalase. Catalase typically catalyzes the following reaction: $2H_2O_2 \longrightarrow 2H_2O + O_2$; whereas, peroxidase requires a hydrogen donor which is oxidized by hydrogen peroxide as follows: $AH_2 + H_2O_2 \longrightarrow A + 2H_2O$, where AH_2 is the hydrogen donor (Davies, Giovanelli, and

Ap Rees, 1964). This, of course, is a simplified scheme and doesn't include the intermediate compounds such as the peroxidase-H_2O_2 and peroxidase-substrate complexes (Saunders et al., 1964). Catalases have molecular weights of at least 220,000 and contain one heme group per molecule (Paul, 1963). It should be pointed out that under certain conditions catalase can function as a peroxidase, and peroxidase can function as a catalase (Saunders et al., 1964; Mahler and Cordes, 1966). The term "hydroperoxidases" is now being used by some authors to include both catalase and peroxidase (Mahler and Cordes, 1966).

Occurrence

Onslow, as early as 1921, made an extensive survey of peroxidase distribution in the plant kingdom. In her examination of 320 species representing 180 orders and 309 genera, she found that plants in all but 13 orders, 16 genera, and 16 species contained peroxidase. According to Burris (1960), Duquenois and his co-workers in 1953 listed over 100 plant species representing forty-three families which contained peroxidase. Chatelier and Duquenois (1954) later found in a study of 138 species representing 45 families, that all contained peroxidase.

Saunders et al. (1964) have divided plants into two main classes: (1) those that contain a powerful peroxidase, and (2) those that contain a strong polyphenoloxidase, but exhibit weaker peroxidase activity. Some of the plants noted by Onslow which did not give a peroxidase reaction might represent species which fall into the

latter group. At any rate, it appears that most, if not all, plants contain peroxidase.

Isolation and Purification

Theorell (1942) obtained the first crystalline peroxidase from horseradish. Since that time, several methods have been developed for purifying and isolating peroxidases, and crystalline peroxidases have now been obtained from a number of plant sources (Hosoya, 1960; Kavashima and Uritani, 1965; Beaudreau and Yasanobu, 1966).

When Theorell (1942) first purified peroxidase he obtained two fractions, only one of which he thought was a true peroxidase. Later, however, Jermyn and Thomas (1954) demonstrated that horseradish peroxidase contained two anodic and three cathodic components. Shannon, Kay, and Lew (1965) have subsequently separated and purified seven distinct peroxidases, three anodic and four cathodic, from horseradish. The seven isozymes could be divided into two general groups on the basis of their chromatographic behavior, electrophoretic migration, spectrophotometric properties, and amino acid and carbohydrate composition. It was further shown that all seven enzymes contained protohemin IX as the prosthetic group.

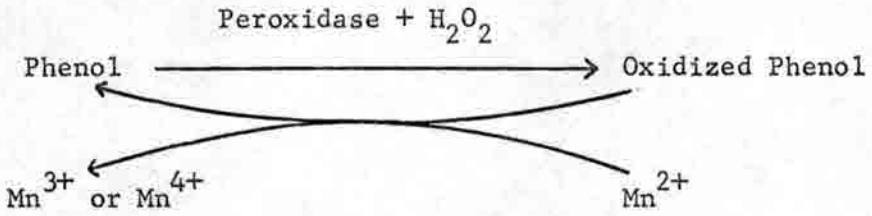
The multimolecular nature of peroxidase has now been described for a number of plant species, including corn (McCune, 1961), pea (Siegel and Galston, 1967), pineapple (Beaudreau and Yasunabu, 1966), rye (Siegel and Galston, 1966), sweet potato (Kavashima and Uritani, 1965), and tomato (Evans and Alldridge, 1965). The amino acid compositions of only a few peroxidases have now been determined (Shannon

et al., 1965; Kavashima and Uritani, 1965), but this list will probably be extended considerably in the next few years because of the development of more sophisticated equipment and better techniques for enzyme purification.

Functions of Peroxidase in Plants

Respiration. Although the potential activity of peroxidase in respiratory pathways in plants is very high, there is very little evidence to substantiate their role in respiration (Burris, 1960). The cytochrome system is usually assigned the chief role of terminal oxidation in plant respiration (Davies, et al., 1964).

Kenten and Mann (1950) demonstrated that peroxidase-hydrogen peroxide systems can, in the presence of certain phenols such as p-cresol, oxidize manganous ions to higher valency states. It was suggested that the reaction procedes in the following cyclic manner:



In turn, systems of peroxidase, H_2O_2, monophenol, and manganous ion have been found to mediate coupled oxidations of a number of organic acids and other compounds occurring in respiratory pathways.

Chance (1952) discovered that horseradish peroxidase could cause the peroxidatic oxidation of dihydroxyfumaric acid in the presence of Mn^{2+} ions. The following year, Kenton and Mann (1953) used a system involving peroxidase, hydrogen peroxide, p-cresol, and manganous ions

to oxidize oxalate, oxaloacetate, ketamalonate, and dehydroxytartrate. Shannon and Lew (1963) also demonstrated the oxidation of oxaloacetate by a similar system, and identified the reaction product as malonic acid. Shannon, Vellis, and Lew (1963) later partially purified an enzyme from bush bean roots capable of catalyzing the oxidative decarboxylation of oxaloacetate. The properties of the purified enzyme were nearly identical to those of other peroxidase catalyzed reactions and the oxidation of oxaloacetate by horseradish peroxidase.

The ability of HRP and co-factors (H_2O_2, Mn^{2+}, and monophenol) to oxidize diphosphopyridine and triphosphopyridine has been shown by Akazawa and Conn (1958).

In order for peroxidase to function in plants, there must be a system or systems for generating hydrogen peroxide. Flavoprotein systems such as glycolic acid oxidase in plants are a source of hydrogen peroxide (Davies et al., 1964). It is also possible that H_2O_2 may be furnished as a by-product of photosynthesis (Burris, 1960). The photolytic splitting of water can be pictured as yielding (H^+) and (OH^-) with two (OH^-) forming H_2O_2. Rabinowitch (1945) has questioned the peroxide hypothesis because of the substantial sacrifice of energy in the reaction sequence and the lack of evidence for it.

The observations that peroxidase systems can oxidize important respiratory compounds <u>in vitro</u> does not establish that they function as such in plants. Because peroxidase, catalase, and cytochrome oxidase all contain iron, it is difficult to find specific inhibitors for studying their functions separately <u>in vivo</u> (Burris, 1960).

Disease and wounding. High peroxidase activity has been frequently associated with disease infection and mechanical wounding of tissue. Brieger (1924) found strong reactions for peroxidase in wound zones and stated that peroxidase was a characteristic reaction associated with wounding. Van Fleet (1947) observed peroxidase in the cells underlying fresh wounds in the roots and stems of Sanaeveria zeylanica, and also found localized zones of peroxidase in natural wounds and lesions of Phaseolus and Smilax. High peroxidase activity appears to be associated with various plant disease infections, and is sometimes implicated in mechanisms of disease resistance (Uritani, 1961; Clare, Weber, and Stahman, 1966). At present no conclusions can be drawn on the physiological role of peroxidases in plant disease and wounding.

Indoleacetic acid oxidation. Probably the most widely investigated mechanism of peroxidase action is its ability to oxidize indoleacetic acid. This function of peroxidase was first suggested by Van Overbeek in 1935, but was not demonstrated conclusively in vitro until 1953 by Galston, Bonner and Baker, using horseradish peroxidase. The enzymatic oxidation of IAA has now been demonstrated with peroxidases from a number of plant sources (McCune, 1961; Morita, Kameda, and Mezuno, 1961; Shin and Kakamura, 1962; Beaudreau and Yasunobu, 1966). Although there are kinetic differences in the IAA-oxidase ability of different peroxidases (McCune, 1961; Morita et al., 1962; Shin and Kakamura, 1962), all of the peroxidases so far examined seem capable of oxidizing IAA to some extent.

Although several different mechanisms have been proposed to explain the enzymatic oxidation of IAA by peroxidase system, the process is usually interpreted as being cyclical and involving several reactions (Ray, 1961). The disappearance of each mole of IAA is accompanied by the consumption of one mole of oxygen and the production of one mole of carbon dioxide (Goldacre, 1961). Although added hydrogen peroxide is not a necessity for the IAA-oxidase system in vitro, its requirement is implied since catalase inhibits (Goldacre, 1951; Galston et al., 1953), and added hydrogen peroxide enhances the reaction (Ray, 1961). The addition of Mn^{2+} and a monophenol such as 2,4-dichlorophenol (DCP) to the reaction medium also greatly accelerates IAA oxidation (Hillman and Galston, 1956).

No specific peroxidase has been shown to function as an IAA-oxidase in situ. However, in at least two known cases where IAA-oxidase has been isolated and partially purified, it has also shown peroxidase activity (Stutz, 1957; Ray, 1960). Thus, as in the case of respiration, peroxidase has the potential to oxidize IAA, but its physiological significance has not been resolved.

Lignification. One of the more documented functions of peroxidase in plant tissue is its participation in the terminal enzymatically catalyzed step in lignin biosynthesis. Using coniferol alcohol and a semi-purified enzyme preparation, Freudenberg (1952) synthesized in vitro a polymer with lignin-like properties. Peroxidase was one of the enzymes present in the enzyme preparation, and the yield of polymer could be increased several fold with the addition of H_2O_2. Using thymol and eugenol as substrates, Siegel (1953)

synthesized lignin-like substances in the embryonic axis of Red Kidney bean seeds. A thermolabile, cyanide-sensitive, H_2O_2-requiring agency in the tissue was associated with oxidation of the phenols and lignin production, thus implicating peroxidase in the process. Moreover, the distribution of peroxidase, principally in the vascular tissues, corresponded to the distribution of lignin-like substances. Jensen (1955) noted that root cells of Vicia Faba containing high peroxidase activity were also capable of producing a compound from eugenol and H_2O_2, which appears to be an intermediate in lignin biosynthesis. In 1955, Siegel obtained a synthetic lignin using washed cell wall fragments from pea roots in the presence of eugenol and H_2O_2. The eugenol-lignin was shown to be chemically similar to spruce lignin, as well as giving characteristic lignin color reactions with the phloroglucinol-HCl and the chlorine-sulfite tests. In addition, the lignified pea root tissue exhibited subjective characteristics of heavily lignified tissues such as brittleness, ease of cutting, decreased shrinkage, and decreased wettability. Bland (1961) has since confirmed the fact that eugenol lignin is a lignin-like substance, but mentions that it differs from natural lignin in certain important respects.

The role of peroxidase in lignification was not substantiated in early work because in vitro model systems utilizing eugenol-H_2O_2-peroxidase in solution failed to form lignin-like products (Siegel, 1956b). The observation that washed cell-wall material (peroxidase still present, tightly bound) could effect polymerization, prompted Siegel to introduce cellulose matrices into his model system. Consequently, Siegel (1956a, 1957) was able to obtain large yields of

eugenol-lignin *in vitro* by using macromolecular matrices such as methyl cellulose, starch, and cellulose (cotton and filter paper). Wardrop and Davis, using the artificial lignification of oat coleoptiles, *Elodea* internodes, and other tissues in the same medium as used by Siegel in his experiments, studied lignification from a morphological point of view (Bland, 1961). They concluded from electron micrographs and staining reactions of the lignifying tissue that the artificial lignin was deposited between the cellulose microfibrils in the wall in the same way as natural lignin. Jansen, Jang, and Bonner (1960) observed that peroxidase was firmly bound to coleoptile cell walls, and their results suggested that peroxidase may be bound to the free carboxyl groups of the pectic substances of the cell walls.

Although eugenol is often used in model systems of lignification, it is not a precursor of natural lignin (Bland, 1961). The exact composition and structure of natural lignin is not known, and the composition varies among groups of plants such as the softwoods, hardwoods, and the monocotyledons (Davies, 1964). In a survey of 21 phenols for lignin forming properties, Siegel (1956) established that only hydroxyphenylpropanes such as eugenol, ferulic acid, and coniferyl alcohol can serve as lignin precursors. Freudenberg (1965) has recently reported that p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol seem to be the immediate precursors of lignin. According to Freudenberg (1965), a synthetic lignin resembling conifer lignin, can be prepared from a mixture of p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol (14:80:6 moles) in the presence of laccase or peroxidase. The enzymatic dehydrogenation of the

alcohols gives rise to free radicals which polymerize and undergo other changes resulting in lignin.

Although either laccase or peroxidase can catalyze the final enzymatic step in lignin biosynthesis, several lines of evidence as outlined by Davies (1964), support the role of peroxidase as at least an alternate to the laccase system: (1) hydrogen peroxide greatly increases the deposition of lignin in sections and in tissue cultures; (2) there is a correlation between the sites of lignin deposition and the sites of greatest peroxidase activity; and (3) peroxidase inhibitors inhibit the formation of lignin.

Localization of Plant Peroxidases

Tissues. According to Van Fleet (1952), Raciborski in 1898 was apparently the first one to examine peroxidase distribution in plant tissue. He reported between 1898 and 1903 that peroxidase (which he termed leptomin) activity occurred in the phloem, lenticels, and guard cells, gap cells of the endodermis, and the "absorption zone" of roots (Van Fleet, 1952).

In 1912 Keeble noted peroxidase activity in the epidermis and in the layers of cells neighboring the woody tissues of the vascular bundles of Primula sinesis. A few years later Wodzicyko (Van Fleet, 1947) reported a strong reaction of endodermal walls when benzidine and H_2O_2 were used to detect peroxidase.

Samisch (1935) studied localization of peroxidase in the apricot and observed high activity in the epidermis and the bundle sheath of the bundle tissue. Particularly high peroxidase activity was found in the stomatal guard cells. Hussein and Creuss (1940), and later

Rosoff and Creuss (1949), found high peroxidase activity in the epidermal and vascular tissues of grape and cauliflower respectively.

Van Fleet (1947) made an extensive study of the distribution of peroxidase in the differentiating tissues of a number of plants. Peroxidase was localized in the epidermis, endodermis, bundle sheath, and phloem, including the sieve tubes, companion cells, and the surrounding parenchyma. More recently, Van Fleet (1959) analyzed the peroxidase in the tissues of embryo sacs of Zea mays; the wound meristems of Coleus, Sanseviera, Cucurbita maxima, and Smilax hispida; root tips of Allium sativum; roots from many varieties of bulbs; and the primary roots of several kinds of seeds. Peroxidase was detectable either in advance of cell division or accompanying cell division in the proepidermis, proendodermis, protophloem, the primordial centers for origins of the buds and roots, wound meristems, the developing nucellus, and the embryo sac.

Siegel (1953), as previously mentioned, found peroxidase activity to be greater in the fibrovascular tissue of Red Kidney beans, and also associated its localization with the principal regions of lignification. Strong peroxidase activity in the vascular tissue was also noted by Jensen (1955) in Vicia faba roots.

Little mention is made of peroxidase activity in cortical parenchyma and pith tissue, but peroxidase is known to exist in these tissues in low amounts. Rosoff and Cruess (1949) noted peroxidase activity in the parenchyma cells of cauliflower in low but uniform amounts. Siegel (1953) observed low peroxidase activity in the cortex of Red Kidney bean embryonic axes, but found the pith to be almost devoid of peroxidase.

Intracellular localization. In 1928 Mangenot reported that peroxidase was localized in vacuoles and chondriosomes (mitochondria). Peroxidase activity in the vacuolar sap was also observed by Samisch (1935) in cells of apricot fruit. Tandlar (1952) reported that peroxidase activity in cells of Allium cepa was associated with several organelles including the mitochondria, proplastids, nucleus, and a new organelle which he suggested at the time as being equivalent to the golgi body. Although Yonetani and Ohnishi (1966) found cytochrome-C peroxidase in yeast mitochondria, they did not detect it in mung bean or potato mitochondria. Plesnicar, Bonner, and Storey (1967) at first thought they had detected peroxidase in mitochondria from etiolated mung bean hypocotyles and skunk cabbage spadices. However, the results were shown to be due to contamination of the isolated mitochondria by a denser particle fraction with high peroxidase activity.

It is well substantiated that peroxidase exists in the cell walls of some, if not all tissues. Siegel (1955) used cell wall macerates from pea roots as a source of peroxidase for synthesizing eugenol-lignin. Lipetz (1965) found peroxidase to be localized on the walls of lignifying tissues of Vinca minor and Kalenchoe daigremantiana. Peroxidase was observed by Jansen et al. (1960) to be firmly bound to the cell walls of oat coleoptiles.

The available information on the intracellular localization of peroxidase in plant tissues is rather incomplete. The localization of peroxidase in the cell in regions other than the cell wall remains to be conclusively demonstrated.

Developmental Changes in Peroxidases

Shcherbakov (1945) was apparently the first person to report on quantitative differences in peroxidase between and within organs. He found less peroxidase activity in the shoots than in the roots of pea seedlings. The highest activity in the root was found in the tissue of the young tip, whereas in the stem the greatest activity was in the older tissue. Jensen (1955) noted a general rise in peroxidase activity from 55 to 2,500 microns in the root tip of Vicia faba. As mentioned previously, Van Fleet (1959) detected high peroxidase activity in advance or accompanying cell division in roots from several plants, and noted a decline in activity at the end of the cell division phase. Nakai and Inaba (1951) found that peroxidase activity increased with growth of tobacco stems, and that this activity was also higher in leaves from the lower part of the stalk.

McCune (1961) separated and quantitatively analyzed six peroxidase isozymes (4 cathodic and 2 anodic) from expanding first leaf-sheaths of corn. Isozymes A_1, A_2, C_1, and C_2 increased during elongation of the sheath; C_3 was highest in the unexpanded leaf sheath; and C_4 increased slightly during elongation, but increased sharply as elongation ceased.

More recently, Evans and Alldridge (1965) studied the distribution of peroxidase in different organs and tissue of an extreme dwarf (d^x) and a normal tomato line. Using pyrogallol as the hydrogen donor, the activity of the normal pith, cortex, and root were essentially the same, but the dwarf root had about one-half the peroxidase activity of the dwarf pith and cortex. With guaiacol as

the hydrogen donor, the normal root activity was twice as great as the pith and cortex while peroxidase activities in the dwarf pith, cortex, and root were equal. The leaves had the lowest peroxidase activity of any of the organs. Using starch gel electrophoresis, qualitative differences were also observed in the different organs. The number of electrophoretic peroxidase bands in the different organs were: cortex-4, root-5, leaves-3, pith-2, fruit-1, and flower-4.

The isoperoxidases of Pisum sativum have been investigated by Siegel and Galston (1967). Using starch gel electrophoresis, they found three cathodic and eight anodic bands in roots, six cathodic and four anodic bands in shoots, and three cathodic and three anodic peroxidases in cotyledons. No relationships were necessarily drawn between the peroxidases from different organs since, for example, the cathodic peroxidases from cotyledons might not resemble the cathodic peroxidases from the shoots or roots. A limited survey of the pea isoperoxidases from three nodes of different ages revealed that some peroxidases increase while others decrease as a function of distance from the apical meristem. The shoots were treated as a single entity by Siegel and Galston since the isoperoxidases varied little among the organs composing the shoot.

The recent studies of peroxidase isozymes suggest that peroxidases might serve as a useful model for enzyme differentiation during plant growth and development. Qualitative and quantitative electrophoretic studies coupled with histochemical studies, should eventually

help to reveal the physiological functions of peroxidase, and the significance of the multimolecular forms of this enzyme.

Peroxidase and gene regulation. Since the distribution of peroxidase isozymes is influenced by plant growth and development (Evans and Alldridge, 1965; Siegel and Galston, 1967), it seems appropriate to briefly discuss some of the models for gene regulation in organisms, and how they might apply to the regulation of peroxidase. The classical system of gene regulation is that elucidated by Jacob and Monod (1961) to explain repression and induction of enzyme synthesis in bacteria. This system features genetic control mediated through two types of genes, regulator genes (R) and structural genes (Z), and an operator locus. The structural genes are mainly encoded for enzymes and other polypeptides, while the operator locus lies adjacent to a structural gene or a group of structural genes, and is the initiating point for their transcription. The operator region together with its adjacent structural gene or genes is called the operon. The regulator gene is located at a site different from that of the operon, and is responsible for making a protein repressor substance (Gilbert and Muller-Hill, 1966). This substance can associate with the operator locus to block RNA transcription and thus indirectly, protein synthesis. An effector compound (some metabolite) can attach to a specific site on the repressor protein, and can either prevent its association with the operator region (inducer), or cause it to associate with the operator locus (co-repressor). Inducers are usually substrates while repressors are generally the products of metabolic pathways (Jacob and Monod, 1961).

The above system provides explanations for certain observed phenomena of protein synthesis in bacteria, and may well apply in some cases to regulation of the synthesis of some proteins in higher plants and animals. Mutations can occur which result in non-functional repressors, and as such, protein synthesis can continue in a cell regardless of need. An operator mutation can occur which prevents the association of the repressor molecule with the operator locus; this also results in continuous RNA transcription for the affected operon. Mutations of this type are termed constitutive mutations.

On the other hand, mutations of the regulator or operator loci can occur which prevent protein synthesis. These mutations can be distinguished from those of structural genes because they can affect the production of more than one protein, and those of the regulator genes are dominant. Such a mutation of a regulator gene results in a repressor molecule with an inactive site for the attachment of the effector molecule.

Although enzyme induction and repression have been demonstrated in higher organisms (Jacob and Monod, 1961), there has been no genetic evidence for the existence of the above system in higher plants and animals. The investigation of such systems of induction and repression with respect to peroxidases is hindered because of the sparse knowledge of <u>in vivo</u> metabolic pathways in which peroxidase is involved.

As emphasized by Bonner (1965), a different kind of regulation than the preceding operates in higher plants and animals to determine the course of development. Hormones, possibly acting as effectors,

appear to regulate protein synthesis. Varner and Chandra (1964) have established that gibberellin triggers de novo RNA dependent synthesis of alpha-amylase in barley seeds. The dormancy of potato buds can be broken by gibberellic acid which apparently acts as an effector of DNA-dependent RNA synthesis (Bonner, 1965). Recent work by Nooden and Thimmann (1965, 1966) indicates that auxin stimulates growth by inducing formation of new enzymes and proteins. Although the action of ethylene is less clear, Abeles (1966) has reported that ethylene is capable of stimulating RNA and protein synthesis in bean abscission-zone explants prior to abscission.

Hormonal regulation of peroxidases. Since peroxidases seem closely tied to plant differentiation (Evans and Alldridge, 1965; Siegel and Galston, 1967), it seems likely that at least some of them are also regulated by the hormonal-controlled genetic system. Jensen (1955) reported that IAA induced the formation of peroxidase in the vascular tissue of Vicia faba root tips. In 1966 Ockerse, Siegel, and Galston reported an opposite effect of IAA. Young stem sections of dwarf peas (Progress No. 9) grown in light, were found to contain at least seven peroxidase isozymes separable by starch gel electrophoresis. As the tissue elongated and matured, an eighth isozyme appeared. The appearance of this isozyme in excised sections could be repressed by applications of IAA.

In 1956 Hayashi, Murakami, and Matsunaki observed an increase in peroxidase activity in GA-induced elongation of rice leaf-sheaths. McCune (1961) obtained six peroxidase fractions from corn leaf-sheath

preparations. The activity of these fractions in four different corn lines differed among the dwarf and normal segregates. "Gibberellic acid promoted growth in dwarfs d_1, d_3, and d_5, and reversed the effect of dwarfism on the peroxidase pattern" (McCune, 1961). In a more recent study, Raspevin (1964) found that peroxidase activity increased during increased growth of plants caused by the action of gibberellin.

Clare, Weber, and Stahman (1966) observed high peroxidase activity in extracts from healthy sweet potato tissue incubated with tissue infected with black rot (Ceratocystis fimbriata), and less activity in extracts from healthy tissue incubated with healthy tissue. The results indicated that a volatile material was responsible for inducing the greater peroxidase activity in the uninfected tissue. Subsequently, Stahman, Clare, and Woodbury (1966) reported that ethylene could induce an increase in peroxidase in sweet potato tissue. Infected sweet potato tissue was found to liberate more ethylene than healthy tissue, and thus, infected tissue could induce the greater peroxidase activity found in healthy tissue.

Stem Elongation, Growth Regulators, and Peroxidase

Stem Elongation

Stem growth in plants is a function of the number and length of individual internodes. In this review only the process of internode elongation will be discussed to any extent. Internode elongation is determined by both cell division and cell elongation, and the relative contribution of both of these factors has received

considerable attention (Sachs, 1965). Grisebach (1943) observed over a century ago that the first stage of internode elongation of plants is fairly uniform over its entire length, and is a period of rapid increase in cell number. Although the apical meristem proper is the site of the initial cells of the stem, according to Sachs (1965), Harting as well as Grisebach firmly stated that the subapical region is the site of formation of most of the cells that are to constitute the mature stem. This view has since been confirmed by a number of workers (Priestly, 1928; Bindloss, 1942; Sachs, 1959; Sachs et al., 1960; Sachs and Lang, 1961).

Genetic dwarfs. The genetically determined short internode or dwarf phenotype is a special case of retardation of stem elongation, and has provided important insight into both anatomical and physiological aspects of stem elongation. Examples of genetic dwarfism have been recorded for a number of higher plants, including rice (Kadam, 1937; Joden and Beachell, 1943), peas (von Rosen, 1958; Gorter, 1961), corn (Emersen, Beadle, and Frazer, 1935), tomato (MacArther, 1921; Clayberg et al., 1960), cotton (McMichael), squash (Whitaker and Davis, 1962; Denna and Munger, 1963), and muskmelon (Denna, 1962). The majority of genetic dwarfs are monorecessive (Kadam, 1937; Joden and Beachell, 1943; McMichael, 1942; Phinney, 1956; Anderson and Abbe, 1933; Denna, 1962; Denna and Munger, 1963; Shifriss, 1947). However, some cases of dominants (Kadam, 1937; Phinney, 1956a), double recessives (Kadam, 1937; Joden and Beachell, 1943), and even more complex types of inheritance (von Rosen, 1958) have been observed. Other abnormal morphological characters are

often associated with dwarfism such as stem brittleness in the case of dwarf Aquilegia and bush muskmelon (Anderson and Abbe, 1933; Denna, 1962); thick rugose, dark green leaves on tomato dwarfs (MacArther, 1921); twisted stem growth in bush muskmelon (Denna, 1962); and red vegetation in a dwarf cotton mutant (McMichael, 1942).

The anatomical basis for dwarfism has been studied by a number of workers. Some researchers have stressed cell length (Abbe, 1936; Van Overbeek, 1936; von Maltzahn, 1957), while others have stressed cell number (E. C. Abbe, 1942; Houghtaling, 1940; Bindloss, 1942; Denna, 1962) as being largely responsible for differences in internode length between dwarf and normal plants. Sinnott (1960) has reviewed the subject and states that "most genetically large plants are so because of more rather than larger cells." It should be emphasized, however, that a certain amount of decrease in cell size almost always accompanies the decrease in cell number in genetic dwarfs.

Auxin and Dwarfism

Auxinology had its beginning in 1928 when Went found a growth substance in diffusates from coleoptile tips, and was able to explain both the correlative nature of the tropistic response and the endogenous control of growth rates on the basis of this substance (Leopold, 1965).

In 1935 Van Overbeek found that normal corn mesocotyls contained more auxin than their dwarf sibs, and hypothesized that the lower auxin levels were responsible for the dwarf phenotype. A few years later (1938) Van Overbeek reported that auxin levels were lower for several corn dwarfs (d_1, d_2, d_3, d_7, and pigmy dwarf) as compared to their tall segregates. Harris and

Phinney (1953) supported Van Overbeek's work, showing that the d_1 dwarf exhibited both lower auxin and reduced auxin production than the normals. However, the dwarf plants failed to respond to exogenous applications of IAA, and in addition, normal coleoptile tips placed on decapitated dwarf and normal coleoptiles, elicited measurable elongation only in the normal coleoptiles.

According to Van Overbeek (1938), Hinderer demonstrated in 1936 that auxin production in Epilobium hybrids is proportional to size of the hybrid. In support of Hinderer's work, Ross (1939) found that abnormalities such as shortened internodes and leaf roll occurring in certain hybrid strains of Epilobium, were corrected by daily sprays with IAA solutions.

Von Abrahms (1953) obtained a 30 percent height increase in etiolated dwarfs treated with IAA as compared to controls, while growth of a tall variety was significantly repressed. Since no differences in diffusable auxin, free auxin, and the converstion of tryptophan to auxin were observed between tall and dwarf pea tissue, von Abrahms suggested that the partial response of dwarfs to exogenous IAA may not itself indicate the fundamental cause of dwarfing in the variety studies. Using non-etiolated dwarf and normal pea seedlings, Brian and Hemming (1955) noted a barely significant response of dwarf seedlings to applications of IAA.

Plummer and Tomes (1957) found that IAA applied to the young foliage of four dwarf (d_1) and four normal tomato lines gave no significant growth stimulation of either type.

In the case of cucurbits, Denna (1962) reported that the growth of a dwarf squash variety of Cucurbita pepo was slightly enhanced

by IAA applications, while a dwarf muskmelon gave no detectable response.

Gibberellins and Dwarfism

The most typical and striking response of plants to treatment with gibberellin is stem elongation (Stowe and Yamaki, 1957). The substances first shown to be produced by the rice disease fungus, Gibberella fujikuroi, by Kurosawa in 1926, were characterized by their ability to elicit marked stem elongation in rice plants (Stowe et al., 1961).

The anatomical basis for increased stem elongation resulting from gibberellin application appears to involve both cell elongation and cell division. In 1939 Yabuta and Hayashi reported that the stimulatory effects of GA on stem elongation in several species of plants were so pronounced, that they could not be explained by the cell-elongation theory alone. Other workers later reported that gibberellin exerted its effect through increased cell elongation (Hayashi, et al., 1953; Brian et al., 1953; Feucht and Watson, 1958). More recent evidence supports the supposition that gibberellin effects are mediated through increased cell division (Arney and Mancinelli, 1966; Greubach and Haesloop, 1958; Sachs, 1959; Sachs et al., 1960; Sachs and Lang, 1961). Sachs and Lang (1961) demonstrated in several caulescent plants that gibberellin increases cell division by increasing the region of subapical mitotic activity. In a study of the effect of GA on elongation of Meteor pea stems, Arney and Mancinelli (1966) found that the cell elongation caused by GA only occurred during the normal vacuolar phase of growth, and

there was no evidence of premature cell enlargement caused by GA. They suggested that the effects of GA on cell elongation are mediated through increased auxin levels resulting from the GA-enhanced cell division.

The first observation of gibberellin producing dramatic growth effects on dwarf plants was reported by Brian and Hemming (1955). In their experiments dwarf varieties of peas responded much more to applications of gibberellic acid than did tall varieties. It was later demonstrated (Gorter, 1961) with three different dwarf varieties of pea that exogenously applied gibberellin increased their growth rate to that of GA-treated normal varieties.

Phinney (1956b) reported that five out of seven dwarf corn mutants responded to gibberellic acid treatment by assuming normal growth. He emphasized that "the lack of response of two of the mutants to GA indicates that the mechanism of dwarfing can vary, depending on the gene controlling the expression of the dwarf character."

Bukovac and Wittwer (1956) found that bush beans responded dramatically to gibberellin. They further observed that foliar application of gibberellin under field conditions resulted in longer internodes in bush summer squash and cucumber. Lockhart (1958) also reported that gibberellin induced elongation in cucumber and a bush squash (Cucurbita pepo). Denna (1963) found that isogenic bush and vine lines of Cucurbita pepo exhibited nearly identical growth curves when they both were treated with gibberellin. The bush form of C. maxima also responded to GA treatments, but more sluggishly.

Significant responses of dwarf phenotypes to gibberellin have been reported for other species including tomato (Plummer and Tomes, 1957), cotton (Ergle, 1957), and Lathyrus odoratus (Brian et al., 1959). It should be emphasized that not all dwarf types of plants respond dramatically to gibberellin (Phinney, 1956b), and that in some cases the non-dwarf responds as much (tomato - Plummer and Tomes, 1957) or more (muskmelon - Denna, 1962) than its dwarf counterpart.

In all of the cases reported above, GA_3 (gibberellic acid) was used for demonstrating gibberellin effects on genetic dwarfs. There is now unequivocal evidence for the existence of gibberellins A_1, A_3, A_4, A_5, A_6, A_7, and A_8 in higher plants, and a reasonable certainty that yet other gibberellins remain to be characterized (Brian, 1966). It has been observed that the different gibberellins produce differential growth responses (Thimann, 1963). The possibility exists that some dwarf plants not responding to GA_3 might show significant growth responses to other gibberellins.

There is presently little evidence to show that endogenous levels of gibberellin are lower in dwarf than in normal plants. Gibberellin-like substances were found in extracts from dwarf and tall pea seedlings by Radley (1958), but the two varieties differed little in content of the GA-like substances. According to Brian (1966), Radley found that if the gibberellin contents of the apical buds were compared, more marked and more consistent differences were found. In extracts from dwarf and tall pea varieties, Kende and Lange (1964) found gibberellin-like activity in two fractions corresponding closely to GA_1 and GA_5. No quantitative differences were observed between the tall and dwarf varieties.

According to Brian (1966), Ogawa (1962) found two endogenous gibberellin-like compounds in hypocotyls of dwarf and normal varieties of Pharbitis nil. The compounds reached maximum levels of 0.09 and 0.05 ug GA_3 equivalents in the normal variety, while the same two gibberellins in a dwarf variety failed to exceed 0.02 ug GA_3 equivalents.

Phinney (1961) detected two gibberellin-like substances in extracts from normal maize, and found these substances to be lacking in mutant dwarfs d_3, d_5, and an_1. Although the same two gibberellin-like substances were present in mutants d_1 and d_2, they were present in less than half the concentration found in the normal seedlings.

Growth Retardants

The terms "growth retardant" will be applied here according to Cathey's definition (1964), "for those chemicals that slow cell division and cell elongation in shoot tissues and regulate plant height physiologically without formative effects." Of course, it is realized that high concentrations of growth retardants can result in side effects (Cathey, 1964). Four major growth retardants which fall under Cathey's definition and have been used extensively in research are: 2'-isopropyl-4'-(trimethylammonium chloride)-5'methylphenyl 1-piperidine carboxylate (Amo-1618); tributyl-2,4-dichlorobenzyl-phosphonium chloride (Phosphon-D); (2-chloroethyl)trimethylammonium chloride (CCC); and N-dimethylamino succinamic acid (B-995).

Plant inhibition. In 1949 Wirwille and Mitchell reported that a series of ammonium carbamates caused a marked reduction in growth of snap beans without development of organ malformations. The most active of the compounds was Amo-1618. Other growth retardants have

since been discovered such as Phosphon-D, CCC, and B-995 which retard growth in a similar manner to Amo-1618 (Cathey, 1964). However, the growth retardants vary markedly in the range of plants responsive to them. Cathey and Stuart (1961) observed that only 6 out of 54 species of plants tested were retarded by Amo-1618, while 19 out of 54 species were retarded by Phosphon-D. The growth retardant CCC was effective on even a wider range of plants than Phosphon-D. The newest growth retardant, B-995, also appears to be effective on a wide range of plants (Cathey, 1964).

Within the cucurbit family, Denna (1962) reported that Amo-1618 and CCC retarded the growth of squash and cucumber, but had little effect on muskmelon. Preston and Link (1958) found that cucumber was retarded by Phosphon-D.

Anatomical effects on stems. Sachs and co-workers (1959, 1960, 1961) demonstrated with a number of caulescent and rosette type plants that Amo-1618 exerts its effect mainly by reducing the number of cell divisions in the subapical meristem.

According to Cathey (1964), Scheff observed that parenchymatous cortical cells in the first internode of Amo-1618 treated bean plants were 69 percent shorter than similar cells in untreated plants. In the case of 14 day old pea seedlings, Wheaton (Cathey, 1964) found that pith cells in the mature third internode were significantly shorter than those in normal plants. Zeevaart (1964) noted that the mature stem of Pharbitis, treated in the seedling stage with CCC, contained one third as many cells as the untreated stems.

Thus, from the limited data, it appears that both cell elongation and cell division are involved in the dwarfing effect of growth retardants.

Physiological mode of action. It has been observed in a number of instances that gibberellin and growth retardants, particularly Amo-1618, are mutually antagonistic (Sachs, 1956; Halevy, 1962; Lockhart, 1962; Tolbert, 1961). Lockhart (1962) studied the kinetics of certain "antigibberellins," and concluded that CCC and Phosphon act as competitive inhibitors of GA-induced growth.

In 1963 Kende, Ninneman, and Lang demonstrated that Amo-1618 and CCC inhibited the biosynthesis of gibberellin in the fungus Fusarium moniliforme. Although Phosphon-D did not inhibit GA synthesis by the fungus, it was shown that it was rapidly degraded by the organism; this possibly accounted for its ineffectiveness (Harada and Lang, 1965). The results of Dennis, Upper, and West (1965) indicate that Amo-1618 and Phosphon, but not CCC and B-995, block the synthesis of certain kaurene intermediates in gibberellin biosynthesis. Paleg and co-workers (1965) found that Amo-1618, CCC, Phosphon-D, and B-995 failed to prevent GA-induced reducing sugar release from barley endosperm. This ruled out three other possible mechanisms of growth retardant action: (1) a possible destruction of gibberellin, (2) an action which prevents gibberellin from fulfilling its primary role, or (3) a prevention of one of the GA-induced reactions from taking place.

Kuraishi and Muir (1963) have presented less convincing evidence that the effect of CCC is mediated through a reduction of auxin

levels. Reed et al. (1965) have attributed the growth retarding action of B-995 to the formation of 1,1-dimethylhydrazine *in vivo*. This hydrazine strongly inhibited tryptamine oxidation in pea epicotyl homogenates, and thus, could result in lower auxin levels.

Although the mode of action of growth retardants remains an open question, the available evidence indicates that at least some of them probably inhibit gibberellin biosynthesis.

Peroxidase and Dwarfism

The association of dwarfism or stunted growth with higher peroxidase activity has often been reported in the literature. Van Overbeek (1935) was the first to report such a relationship. He observed that dwarf corn (nana) segregates had higher peroxidase activity per mesocotyl than the normal sibs. Ross (1941) found that peroxidase activity was directly related to the degree of growth inhibition in crosses of *Epilobium* species.

In 1956 Kamerbeek compared peroxidase activity on a fresh weight basis in dwarf and normal types of several species. A necrotic, severely stunted line of tobacco exhibited much higher activity than two taller lines. Mesocotyls of dwarf corn had higher peroxidase activity than normal segregates. In crosses between *Phaseolus vulgaris* and *P. multiflorus*, giant and dwarf plants were observed among the progeny. The giant plants had slightly less peroxidase activity than the mean of the two parents; whereas, the peroxidase activity of the dwarfs was greater than 25 fold that of the parents. Even more unusual, the F_2 dwarfs examined had only one-fourth the activity of the F_1 dwarfs.

Using pyrogallol as the peroxidase substrate, McCune and Galston (1959) reported that dwarf corn (d_1) and pea (Progress No. 9) had greater peroxidase activity per unit of protein than their respective normals. However, using guaiacol as the substrate, leaf-sheaths of dwarf corn had less peroxidase activity than normal corn (Galston and McCune, 1961). Electrophoretic separation and quantitative analysis of the peroxidase isozymes, revealed that the contrasting results with each substrate were due to differences in specificity among certain of the isoperoxidases.

In a later report (McCune, 1961), it was shown that the leaf-sheaths of corn contained six distinct, electrophoretically separable peroxidases. Using guaiacol as the hydrogen donor, peroxidase activity was determined for all six isozymes in four corn lines segregating for normal corn and the dwarf mutants d_1, d_3, d_5, and d_8. In most cases the isoperoxidase activities of the normal segregates were greater than those of the dwarf sibs.

Evans and Alldridge (1965) found that the extreme dwarf (d^x) tomato line contained three times as much peroxidase activity per unit of total plant protein as a normal tomato variety. Using starch gel electrophoresis, they found no qualitative differences between the dwarf and normal lines.

The often observed differences in peroxidase activity between dwarf and normal plants has led to three hypotheses as to how peroxidase might be involved in dwarfing mechanisms. These schemes are based on postulated <u>in vivo</u> or known <u>in vitro</u> reactions catalyzed by peroxidase, and are as follows: (1) peroxidase levels could affect growth as a result of peroxidase being involved in important

respiratory functions (McCune, 1961); (2) high peroxidase activity could limit growth through the increased oxidation of indoleacetic acid (Van Overbeek, 1935); or (3) peroxidase could be involved in precocious lignification, resulting in a rigid lignin matrix which could limit growth (McCune, 1961). The last two hypotheses will be discussed further in the sections to follow.

Relationships of Peroxidases and Growth Regulators to Stem Elongation

Thimann (1934) first reported evidence for an IAA destroying enzyme in plants. The following year Van Overbeek (1935) demonstrated that auxin was destroyed faster, and peroxidase levels were higher in dwarf as compared to normal corn. He postulated that peroxidase might be responsible for auxin degradation, and that the lowering of auxin levels by high peroxidase activity could result in dwarfism. Van Overbeek (1938), as mentioned previously, later reported that auxin levels of a series of corn dwarf mutants were lower than their normal sibs.

According to Van Overbeek (1938), Hinderer showed in 1936 that auxin production in Epilobium hybrids was directly proportional to plant size. Ross (1939) subsequently found that abnormalities such as short internodes and leaf roll occurring in Epilobium hybrids could be corrected by daily sprays with solutions of IAA. In support of Van Overbeek's hypothesis, Ross (1941) observed that peroxidase activity was directly correlated with the degree of growth retardation in Epilobium hybrids.

In 1947 Tang and Bonner found an indoleacetic acid oxidase in etiolated pea seedlings which appeared to be an iron containing

enzyme. A few years later Goldacre (1951) showed that catalase strongly inhibited IAA oxidation, guaiacol competed with IAA for IAA-oxidase, and low concentrations of hydrogen peroxide enhanced the enzymatic oxidation of IAA. Galston et al. (1953) confirmed Goldacre's work using the IAA-oxidase system of peas, and they also demonstrated that horseradish peroxidase can attack and destroy IAA in the presence of H_2O_2. Stutz (1957), using electrophoresis, separated an IAA-oxidase from Lupinus albus and found it to be indistinguishable from peroxidase. Ray (1960) later observed that the IAA oxidizing enzyme from the fungus, Omphalia flavida, exhibited peroxidase activity.

In 1955 (Brian and Hemming), and several times thereafter (Phinney, 1956ab; Bukovac and Wittwer, 1956; Brian et al., 1959; Denna, 1962), it was shown that gibberellin (GA_3) could reverse the growth of many genetic dwarfs to the normal phenotype. However, researchers were reluctant to throw out the hypothesis that auxin was somehow involved in the dwarfing mechanism. Brian and Hemming (1957, 1958) and later other workers (Kato, 1961; Galston and McCune, 1961) demonstrated that IAA is necessary for GA to elicit appreciable growth in green pea stem sections. In addition, anti-auxins such as 3-chlorophenoxyisobutyric acid suppress the growth responses of plant tissue to gibberellin (Kato, 1961; Kefford, 1962).

Three theories of GA mode of action were presented by Brian and Hemming (1958): (1) gibberellin may combine with auxin or auxins in the plant to form a more active compound; (2) gibberellin may accelerate the effective level of auxin in plants by increasing the

amount formed or by retarding destruction; or (3) gibberellin may affect the same metabolic process which normally limits growth even though auxin is present in non-limiting amounts. There is no evidence for the first hypothesis, and it seems unlikely since the spectra of activity of auxins and gibberellins are not identical (Brian, 1966).

Much controversy has arisen with respect to the second theory. Some workers have demonstrated that gibberellin treatment of plants results in a decrease in IAA-oxidase activity (Housley and Deverall, 1961; Halevy, 1963), while others have found that gibberellin has little effect on IAA-oxidase activity (Brian and Hemming, 1958; Kefford, 1962; Kuraishi and Muir, 1962). This same discrepancy exists in the case of peroxidase levels in plants treated with gibberellin. In 1956 Hayashi, Murakami, and Matsunaka reported an increase in peroxidase activity in GA-induced elongation of rice leaf sheaths. Using pyrogallol as the peroxidase substrate, McCune and Galston (1959) observed that GA increased the growth rate and decreased peroxidase activity in dwarf varieties of corn and peas. It was later reported (Galston and McCune, 1961) that by using guaiacol as the enzyme substrate, peroxidase activity increased in dwarf corn plants treated with gibberellin. Halevy (1962, 1963) found that GA stimulated cucumber hypocotyl elongation while inhibiting peroxidase and IAA-oxidase activity. Amo-1618 had the exact opposite effect on both growth and enzyme activity.

Increased auxin production rather than decreased inactivation has also been postulated in schemes of gibberellin action on growth.

In a comparison of gibberellin-treated and non-treated sumac plants, Nitsch (1957) found higher levels of endogenous auxin as well as increased growth in treated plants. In dwarf pea Kuraishi and Muir (1964) reported that GA stimulated growth, but did not affect the activity of IAA-oxidase. On the other hand, they did note higher auxin levels in GA-treated plants, and ascribed the mode of action of gibberellin to its effect on auxin production. In more recent work on gibberellin-auxin interactions in pea stem elongation, Ockerse and Galston (1967) concluded that gibberellin-induced growth did not appear to be mediated through its effect on auxin synthesis.

Katsumi, Phinney, and Purves (1965) studied the effect of GA and IAA on cucumber-hypocotyl growth. They found that endogenous gibberellin was needed for the optimum IAA response as well as IAA needed for the GA growth response. Endogenous auxin is apparently not limiting in most genetic dwarfs since they fail to respond dramatically to exogenous applications of IAA. Although auxin levels may increase concomitantly with increased stem elongation in gibberellin-treated plants, this does not appear to be the principal mode of action of gibberellin. Considering the three theories of gibberellin mode of action presented by Brian and Hemming (1958), the third theory seems to be best represented. The theory stated that "gibberellin may affect the same metabolic process which normally limits growth even though auxin is present in non-limiting amounts." Because of the lack of knowledge concerning the metabolic processes regulated by plant hormones which limit growth, it appears that this hypothesis could be altered as follows: gibberellin may affect metabolic processes which normally limit growth even though auxin is present in

non-limiting amounts. In other words, GA could affect different growth processes than IAA, but both plant hormones would appear dependent on one another for normal growth since either one could be limiting.

Lignification and Dwarfism

Anderson and Abbe (1933) conducted a comparative anatomical study of a normal and a dwarf type of Aquilegia vulgaris. The "compacta mutant" was characterized by a bushier, more branched habit of growth and by brittle stems. It was observed in the stems that the secondary walls of the cortex schlerenchyma were much thicker in "compacta" than in normal plants, and the metaxylem elements also seemed somewhat thicker in the dwarf. The hypothesis was presented that precocious secondary thickening of cell walls in "compacta" prevented complete elongation, resulting in the bushy, compact habit of growth.

Anderson and Abbe did not discuss the compounds which might be involved in the precocious secondary thickening of "compacta" cell walls. Several substances are synthesized during cell wall formation including pectins, celluloses, hemicelluloses, and lignins. However, secondary cell walls are characterized by greatly increased amounts of cellulose and by considerable quantities of lignin (Albersheim, 1965). Brittleness of stems was an additional characteristic of the dwarf mutant studied by Anderson and Abbe. Although the brittle characteristic may not be associated with the dwarfing mechanism, it suggests lignin as a likely suspect in the precocious secondary thickening of "compacta" cell walls, because brittleness is one of the characteristics of heavily lignified tissues (Siegel, 1955).

Using eugenol as a lignin precursor, Siegel (1955) was able to synthesize a synthetic lignin in pea root tissue. Such tissue exhibited the brittle characteristic of heavy lignification. Solberg and Higinbothan (1957) found that application of eugenol to etiolated pea epicotyles resulted in thickened cortical walls, increased dry weight, and marked inhibition of cell elongation.

As mentioned previously, peroxidase has often been implicated in dwarfing mechanisms. McCune (1961) mentions the possibility that peroxidase could limit growth by involvment in the formation of a rigid lignin matrix. Theoretically, peroxidase could be a limiting factor in lignification since it is probably involved in the final enzymatic step in lignin biosynthesis (Siegel, 1956, 1957; Freudenberg, 1965). It could therefore be postulated that an early initiation of peroxidase synthesis in dwarf plants could result in a precocious lignification which could then limit cell elongation. No evidence for this type of a mechanism in dwarf plants has been reported, and in addition, the brittle characteristic in dwarfs has been reported in only a few cases (Anderson and Abbe, 1933; Denna, 1962).

MATERIALS AND METHODS

Plant Materials

When discussing the phenotypes of the cucurbits and other plant species in the sections to follow, the terms vine, non-dwarf, and normal will be used interchangeably, and the terms bush and dwarf will have the same meaning. The term "bush" is usually applied to short internode mutants of species which otherwise have the vine habit of growth.

Squash Lines

Squash lines provided most of the experimental material for the following reasons: (1) seeds of the four major Cucurbita species, including several varieties of each species, were readily available; (2) a number of bush lines, including isogenic bush (Cornell 60-4) and vine (Cornell 51-26-7), were on hand; (3) squash dwarfs were known to be responsive to gibberellin (Denna, 1963); and (4) preliminary investigations indicated that squash plants had high peroxidase activity and possessed multimolecular components of peroxidase.

Twenty four squash lines, including a representative range of the four major squash species, were analyzed for peroxidase activity in a preliminary experiment. Ten of these lines, representing bush and vine types of pepo and maxima, and vine types of moschata and mixta, were then selected for a more comprehensive study. A list of the squash lines is given in Table 1, along with their classification according to species, variety, growth habit, and numerical designation.

Table 1. Squash lines used in experiments, including phenotype and source

Line Number	Species	Variety or Line	Phenotype	Ten Selected Lines	Source
S-1	pepo	Cornell 51-26-7	Vine	X	H. M. Munger, Cornell Univ.
S-2	pepo	Cornell 60-4	Bush	X	H. M. Munger, Cornell Univ.
S-3	pepo	(S-1 x S-2)F_1	Intermediate		D. W. Denna, Colo. State U.
S-4	pepo	Seneca Prolific	Bush		Robson Seed Co.
S-5	maxima	Minnesota 503.58	Bush	X	Hutchins, Minnesota Univ.
S-6	pepo	Early Golden Summer Crookneck	Bush		Burpee Seed Co.
S-7	moschata	Butternut 77	Vine		Lawrence, Robinson & Sons
S-8	maxima	Bush Buttercup	Intermediate		Burgess Seed Co.
S-9	maxima	Blue Hubbard	Vine	X	Harris Seeds
S-10	pepo	Connecticut Field	Vine	X	Harris Seeds
S-11	Lagenaria*	Zucca de Pergola	Vine	X	Vaughan's Seeds
S-12	maxima	Cornell 59-166-9	Bush	X	H. M. Munger, Cornell Univ.
S-13	pepo	Zucchini	Bush	X	Asgrow Seeds
S-14	pepo	Early White Bush Scallop	Bush		Cornelli Seeds
S-15	maxima	Warren Turban	Vine		Lawrence, Robinson & Sons
S-16	pepo	Caserta	Bush		Gill Bros. Seed Co.
S-17	moschata	Dickinson	Vine		Lawrence, Robinson & Sons
S-18	mixta	Japanese Pie	Vine	X	Lawrence, Robinson & Sons
S-19	moschata	Kentucky Field	Vine	X	Lawrence, Robinson & Sons
S-20	mixta	Striped Cushaw	Vine		Lawrence, Robinson & Sons
S-21	maxima	Golden Nugget	Bush		Will's Bismarck Seed House
S-22	pepo	Mammoth Table Queen	Vine		Northrup, King & Co.
S-23	pepo	Burpee's Bush Table Queen	Bush		Burpee Seed Co.
S-24	moschata	Large Cheese	Vine		Lawrence, Robinson & Sons

*Lagenaria siceraria - White-flowered Gourd

Table 2. Muskmelon lines used in experiments, including phenotype and source

Line Number	Species	Variety or Line	Phenotype	Source
M-1	Cucumis melo	#10	Bush	M. Hardin, Geary, Oklahoma
M-3	Cucumis melo	#30	Bush	M. Hardin, Geary, Oklahoma
M-5	Cucumis melo	#50	Bush	D. W. Denna, Colorado State Univ.
M-6	C. melo	Rocky Ford	Vine	Cornelli Seed Company
M-7	C. melo	Texas No. 1	Vine	Willhite Melon Seed Farms

Table 3. Watermelon lines used in experiments, including phenotype and source

Line Number	Species and Genus	Variety or Line	Phenotype	Source
W-1	Citrullis vulgaris	Vine Desert King	Vine	Willhite Melon Seed Farms
W-2	C. vulgaris	Bush Desert King	Bush	Willhite Melon Seed Farms
W-3	C. vulgaris	Charleston Grey	Vine	Cornelli Seed Company
W-4	C. vulgaris	Purdue 61-5-651-4	Bush	C. Jones, Purdue Univ.

Muskmelon Lines

Three bush and two vine muskmelon lines or varieties were grown for experimental use (Table 2). The bush muskmelon lines in order of decreasing internode length were #50, #30, and #10. Rocky Ford and Texas No. 1 were the vine types grown. The bush muskmelon lines were of particular interest because of their brittle and twisted stems, suggestive of lignin involvement in the dwarfing mechanism.

Watermelon Lines

The vine lines, Vine Desert King and Charleston Grey, and the bush lines, Bush Desert King (probably isogenic to Vine Desert King) and Purdue (Dwarf) 61-5-651-4, were used in the watermelon experiments (Table 3). Earlier greenhouse observations of Bush Desert King confirmed that it possessed brittle and twisted stems as did the bush muskmelon lines, while Purdue Dwarf was normal in all respects other than the short internode habit of growth.

Growing Conditions

Plant material used for the quantitative and electrophoretic examination of peroxidase was grown in the greenhouse during the months of July, August, and September, 1967. Plant material for the preliminary experiments was obtained from greenhouse grown plants in the fall of 1965 and the spring and summer of 1966.

The seeds were sown in flats containing a soil mixture of approximately one-half soil, one-fourth sand, and one-fourth peat. Each flat contained 20 seedlings, 10 each of two varieties, or six each of four varieties, depending on the experiment.

The greenhouse temperatures were maintained at an approximate day range of 70 to 80° F, and a night range of 65 to 70° F.

Sampling, Harvesting, and Storage of Plant Material

Several factors had to be considered before arriving at a suitable procedure for obtaining plant samples for analysis. The most pertinent considerations were the most desirable age at which to analyze dwarf and non-dwarf; the organ or organs to be analyzed for peroxidase; and whether to compare peroxidase in plants of the same age or at the same morphological stage of growth.

Plants at the four or five internode stage of growth were found to be the most suitable for analysis. The dwarf plants could be readily distinguished from the non-dwarfs at this stage, only a few plants were necessary to provide large enough samples for efficient extraction, and the growing period for such plants was conveniently short.

Stem tissue was selected in preference to leaf tissue; the expanded leaf did not appear to be a likely site for the dwarfing mechanism since there were little size differences between leaves of bush and vine cucurbits. Moreover, it was found that the leaf extracts inhibited polymerization of the sample gels used in electrophoresis.

Analysis of the whole plants was considered undesirable for a number of reasons: the leaf material in the whole-plant extracts inhibited polymerization of the sample gels, the leaves and stems together resulted in large samples which were difficult to extract, and the leaf peroxidases might be expected to mask differences in the stem peroxidases.

Since the dwarfing effect of the bush genes is restricted to stem internodes in squash, muskmelon, and watermelon, the stem seemed to be the most suitable plant part to analyze. In addition, the stem extracts did not inhibit polymerization of the sample gels to any extent. Although the principle site of the dwarfing mechanism may exist in the apical region as opposed to the lower regions of the stem, these experiments were limited to examining the whole stem minus the apex. Considering the large number of plant species and varieties to be analyzed, it was not feasible to conduct a complete developmental analysis of peroxidase distribution in stems.

There was some question as whether to harvest plants at the same morphological stage of growth (i.e. those having the same number of internodes), or to harvest plants of the same age. In a preliminary experiment plants from 24 squash lines were harvested at the five internode stage of growth, and the stems were analyzed for peroxidase activity. Two samples of four plants each per variety were harvested for this experiment. Because of the differences in growth rates of plants within a variety, the two replications of each variety were harvested anywhere from zero to four days apart to obtain plants at the same stage of growth. The squash lines varied considerably in growth rate. Some lines required as little as 18 days to reach the five internode stage of growth, while others required up to 25 days.

Because of early difficulties in maintaining constant temperatures in the laboratory when measuring peroxidase activity, data from nine of the lines had to be discarded. The peroxidase activity

of the other 15 lines was measured at 20° C in a constant temperature room. In Figure 1 peroxidase activity is plotted against age of the squash plants when harvested. There is a general trend toward a rise in peroxidase activity with increased age of the squash plants. There was almost invariably greater peroxidase in the older replication of those harvested two or more days apart (Appendix A). Varietal differences are also reflected in the broad scattering of the points in Figure 1. In subsequent experiments the plants were harvested at the same age. It would have been desirable to grow enough plants so that they could have been harvested at both the same age and internode number. This was not feasible with the limited bench space and differences in growth rates among the lines.

The plants to be used for analysis were cut off at the cotyledon level and placed in polyethylene bags. This material was then transferred to the laboratory where the petioles, leaves, and the apices were removed, leaving only the stems. The stems were immediately measured lengthwise, weighed, wrapped in polyethylene bags, and stored at a minus 20° C until needed for laboratory analysis--a period of between two and one-half to four and one-half months. Peroxidase was found to be quite stable under these conditions.

Growth Regulators

Five different treatments were included under this section, and they provided most of the plant material for peroxidase analysis. Ten lines of squash, five lines of muskmelon, and four lines of watermelon were included in this group of experiments. The treatments were: (I) control, (II) gibberellin - GA_3, (III)

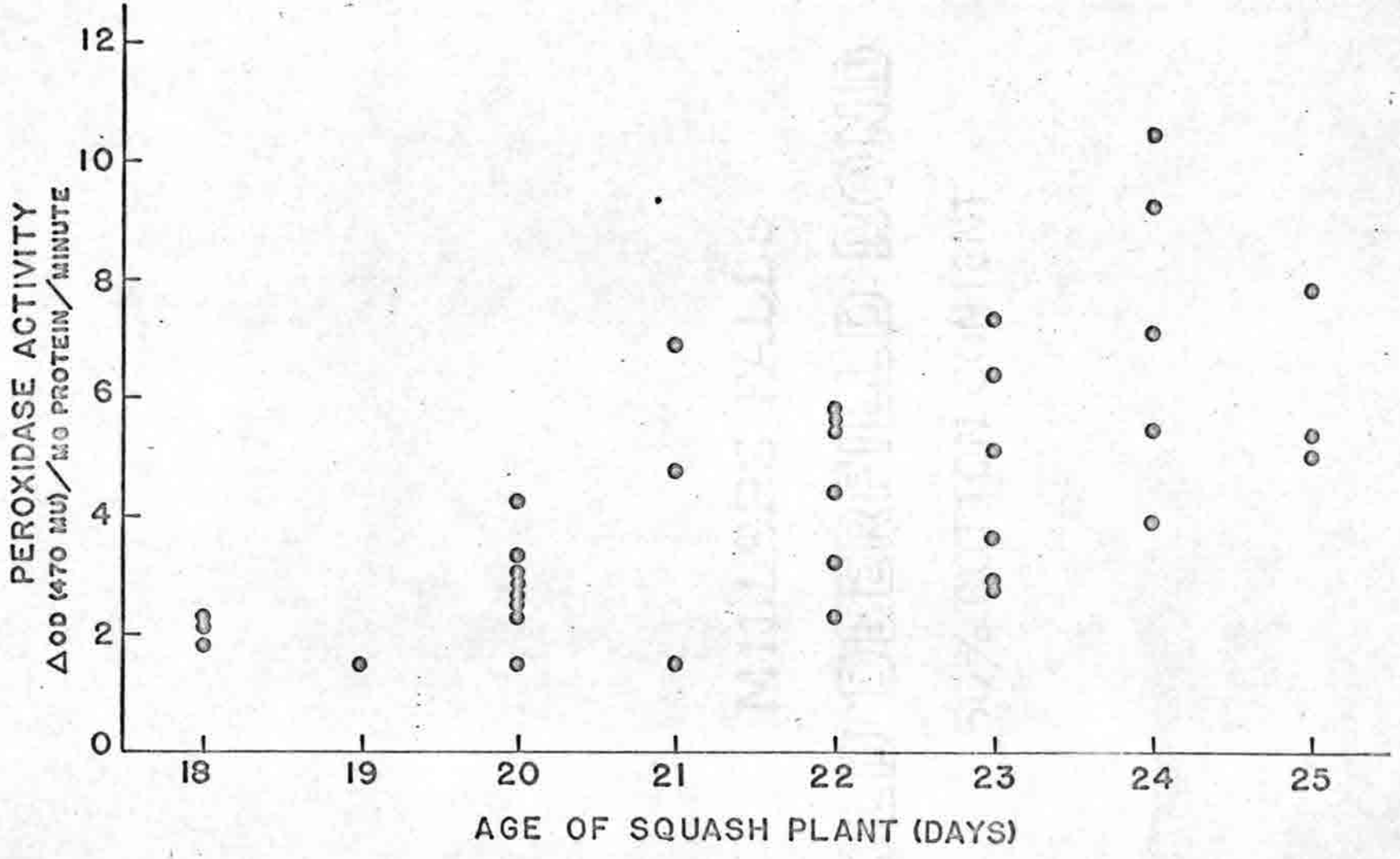


Figure 1. The effect of age on peroxidase activity in stems of 15 squash lines. The reaction medium consisted of 4.2×10^{-2} M guaiacol and 5×10^{-3} N hydrogen peroxide in a 0.1 M sodium acetate buffer, pH 5.0.

N-dimethylamino succinamic acid (B-995), (IV) tributyl-2,4-dichlorobenzylphosphonium chloride (Phosphon-D), and (2-chloroethyl)trimethylammonium chloride (CCC). The concentrations and methods of application of the growth regulators are given in Table 4.

Histochemical Techniques

Tissue sections from fresh stems at the four to six internode stage of growth were analyzed for peroxidase and lignin localization. The sections were taken from the middle portion of each internode. Sectioning was done with a freezing microtome (Model 880, American Optical Company). Powdered dry ice rather than liquid carbon dioxide was used for freezing the plant material. The sections to be examined were placed on glass slides, and then immersed with a few drops of the appropriate indicator solutions. For detecting peroxidase a solution containing 5×10^{-3} N hydrogen peroxide and either 0.5 percent o-anisdine (own preference) or 0.5 percent guaiacol (Jensen, 1962) was employed. A one percent solution of phloroglucinol in 20 percent HCl was employed for lignin staining (Jensen, 1962). Development of a bright red color indicated the presence of lignin.

Plant Extraction

Several methods of extraction of plant material were tried before arriving at a suitable method for this study. Extractions were first carried out by macerating tissue in a Waring Blendor, and then filtering out the rough debris using gauze faced filter disks (Johnson and Johnson) with a Buchler funnel under vacuum. This method had two disadvantages: (1) large amounts of plant material were required, and (2) the plant material had to be diluted with a

Table 4. Concentrations and methods of application of the growth regulators

Growth Regulators	Concentrations	Methods of Application	Source
Gibberellin - A_3 (K-salt)	1×10^{-3} M	Daily spray	Merck and Co., Inc.
N-dimethylamino succinamic acid (B-995)	5×10^{-3} M actual	One spray	United States Rubber Co.
tributyl-2,4-dichlorobenzyl-phosphonium chloride (Phos-phon-D)	1×10^{-4} M actual	Soil drench (5 liters/cu.ft.)	Virginia-Carolina Chemical Corp.
(2-chloroethyl)trimethyl-ammonium chloride (CCC)	5×10^{-4} M actual	Soil drench (5 liters/cu.ft.)	American Cyanamid Co.

Gibberellin and B-995 solutions contained 0.1 percent polyoxyethylene sorbitan monolaurate (Tween 20) as a wetting agent.

fairly large volume of water or buffer, roughly 5 to 10 times on a weight to volume basis, for the Waring Blendor to operate effectively. Dilution of the extracts was no problem for the gross quantitative peroxidase determinations; however, a maximum concentration of peroxidases was desirable for the electrophoretic studies. Plant samples of muskmelon and watermelon were often in the one to five gram range, making extraction in the Waring Blendor impractical. A Sorvall omni-mixer with a small chamber assembly was briefly tested, but it also required a relatively large volume of solvent for effective extraction.

For samples weighing one gram or less, a "vice-grip," modified so as to hold small amounts of plant material, was found useful. However, it was difficult to quantitate the results using this device, and it was a very time-consuming method for medium to large samples. In addition, it was felt that any tightly bound peroxidase would not be adequately extracted by this method.

The most suitable technique was grinding the tissue with a mortar and pestle. This technique was rapid, required only a small addition of water or buffer for extraction, and could be applied to samples as small as one gram. Since peroxidase is an extremely thermal-stable enzyme (Saunders et al., 1964), the extraction procedures could be carried out at room temperatures.

The steps followed in the complete extraction procedure were: (1) the plant material was extracted with water (1:1 w/v); (2) the macerate was then squeezed through a few layers of cheesecloth; (3) the resulting extract was centrifuged in a cold room (1^{o} C)

at 14,350 x g for 25 minutes to remove the larger particles. The resulting supernatant was used for the quantitative and electrophoretic analysis of peroxidase. Table 5 lists the various extraction techniques and their versatility.

Bound peroxidase. Siegel (1955) and later Jansen et al. (1960) reported that peroxidases were tightly bound to cell walls. Lipetz and Garro (1965) found that cell wall fractions could be exhaustively washed until the supernatants contained little or no peroxidase activity. If the cell wall fractions were then resuspended in a calcium salt solution, followed by centrifugation, the resulting supernatant exhibited peroxidase activity. Other divalent, alkali-metal salts could be used in place of calcium, but were less effective in releasing the bound peroxidases. A salt concentration of at least 2.5×10^{-2} M was needed for appreciable release of the bound peroxidases.

The results of Lipetz and Garro (1965) suggested that extraction with a calcium salt might be useful for increasing the peroxidase activity in extracts and for obtaining additional isoperoxidase. The extraction of squash stems with 0.1 M $CaCl_2$ did not result in an increase in peroxidase activity in the extracts. However, when the filtered debris were re-extracted, extraction with a 1 M $CaCl_2$ solution resulted in over a two-fold increase in peroxidase activity as compared to the water-extracted control (Table 6). Another extraction comparison using 2 M $CaCl_2$ gave similar results (Table 7).

A further investigation was conducted to determine the $CaCl_2$ concentration resulting in the greatest peroxidase activity in plant

Table 5. Versatility of various extraction techniques

Method of Extraction	Sample Size	Dilution Factor	Efficiency	Reproducibility
Waring Blendor	Large	5 to 10X (w/v)	Good	Good
Omni-mixer	Large to small	5 to 10X (w/v)	Good	Good
Modified Vice-grips	Very small	0	Fair	Poor
Mortar and Pestle	Large to small	1X (w/v)	Good	Good

Table 6. Comparison of water and 1 M calcium chloride extraction (1:1 w/v) of squash stems with a mortar and pestle. The reaction medium consisted of 4.2×10^{-2} M guaiacol and 5×10^{-3} N hydrogen peroxide in a 0.1 M sodium acetate buffer, pH 5.0.

	Peroxidase Activity Δ OD (470 mμ) per ml extract per minute	
	First Extraction	Second Extraction
Water	16.30	3.78
$CaCl_2$ (0.1 M)	16.44	--
$CaCl_2$ (1 M)	--	9.11

Table 7. Comparison of water and 2 M calcium chloride extraction (1:1 w/v) of squash stems with a mortar and pestle. The reaction medium consisted of 4.2×10^{-2} M guaiacol and 5×10^{-3} N hydrogen peroxide in a 0.1 M sodium acetate buffer, pH 5.0.

	Peroxidase Activity Δ OD (470 mμ) per ml extract per minute	
Replications	Water	2 M $CaCl_2$
1	10.63	17.54
2	12.46	20.81

extracts. As illustrated in Figure 2, one to two molar calcium chloride solutions seemed to be optimum. Although concentrations of five molar and above are inhibitory, peroxidase appears to be quite tolerant to high concentrations of calcium chloride.

According to Rosoff and Cruess (1949), Smirnov in 1925 reported an enhancement of peroxidase activity with alkaline chloride salts. Rosoff and Cruess (1949), themselves, found that calcium and magnesium salts inhibited peroxidase activity at 10^{-7} to 1 M concentrations. To test the possibility that $CaCl_2$ might be enhancing the peroxidative reactions rather than releasing bound peroxidases, the calcium chloride extracts used in the preceding experiment were dialyzed for 24 hours in distilled water. The curve of peroxidase activity for the dialyzed extracts was similar to that for the non-dialyzed extracts (Figure 2). The drop in activity of the dialyzed extracts as compared to the non-dialyzed extracts is difficult to explain. Some loss of activity could have occurred during the 48 hours between measurements of the dialyzed and non-dialyzed extracts, and it also appears that the high molar concentrations of $CaCl_2$ had a denaturing effect on peroxidase during this period. Other possibilities are that natural peroxidase cofactors might have been dialyzed out of the extracts, or some leakage of proteins during dialysis might have occurred through the tied ends of the dialyzing tubes.

Water and 1 M calcium chloride extracts of muskmelon stems were subjected to disc electrophoresis for the purpose of examining any qualitative peroxidase differences between the two methods of extraction. Although the $CaCl_2$ extracts exhibited twice as much

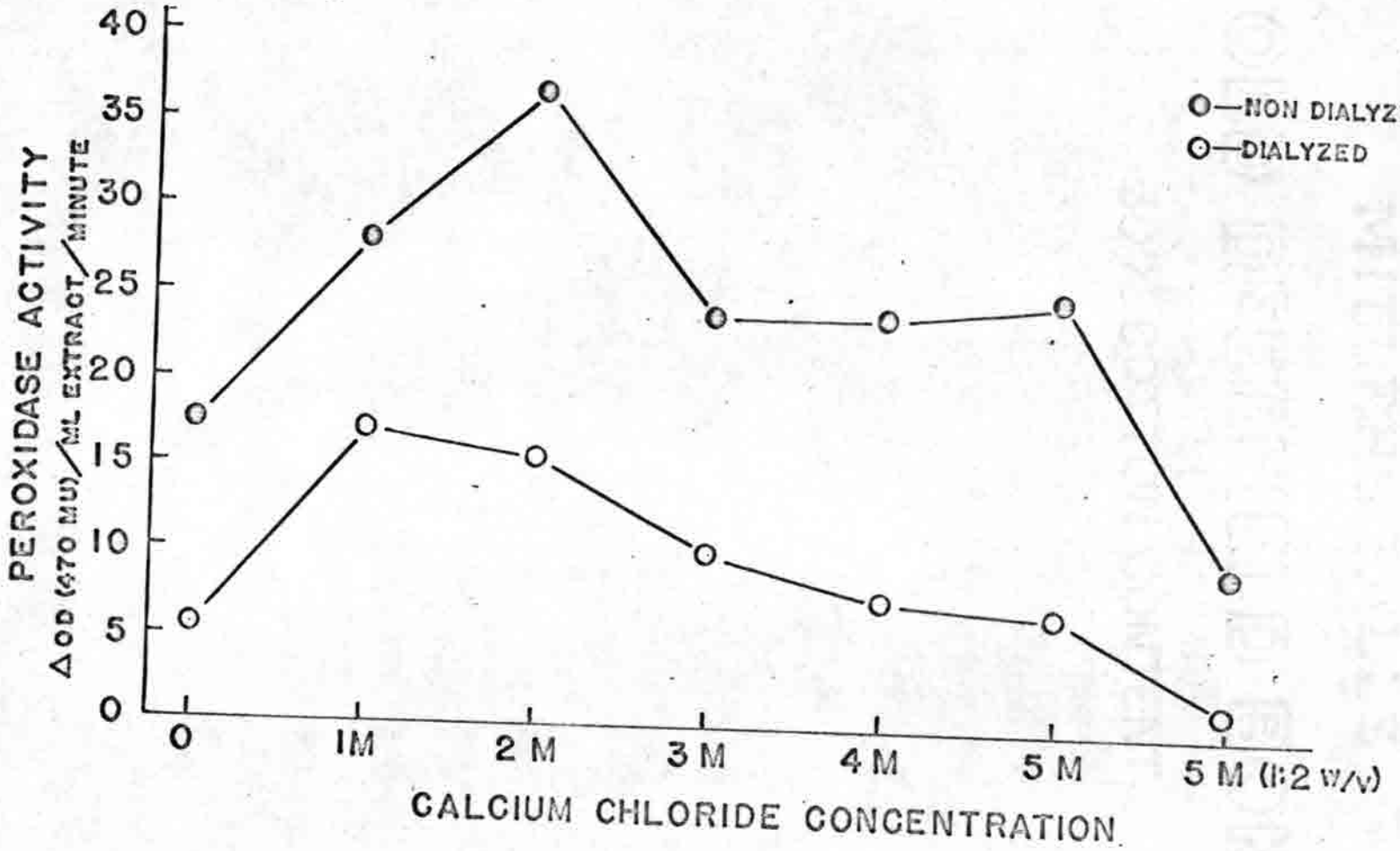


Figure 2. The peroxidase activity of muskmelon stems extracted with different molar concentrations of calcium chloride (1:1 w/v). The reaction medium consisted of 4.2×10^{-2} M guaiacol and 5×10^{-3} N hydrogen peroxide in a 0.1 M sodium acetate buffer, pH 5.0.

peroxidase activity as the water extracts when measured quantitatively, there were little differences in the electrophoretic patterns of the two extracts (Figure 3). The only appreciable difference in iso-peroxidase intensities was in the first cathodic band.

More experimentation is needed to determine the merit of using $CaCl_2$ in extraction procedures; however, this was not one of the objectives of this thesis research. Not enough replications of the above experiments were performed to confirm that $CaCl_2$ extraction is worthwhile. Since 1 M $CaCl_2$ solutions increased peroxidase activity in the preliminary investigations, such solutions were employed in subsequent extractions of plant material with the notion that bound peroxidases were being released.

Protein Determination

The early comparisons of peroxidase activity in dwarf and normal plants were based on fresh weight (Kamerbeek, 1956) or individual plant comparisons (Van Overbeek, 1935). Since size of plants can vary considerably, individual plant comparisons of enzyme activity are not always accurate. Fresh weights are largely a water component of plants, and the water content of plants can vary under a number of environmental conditions (Strafford, 1965). It is generally recommended that enzyme assays be based per unit of protein (Dixon and Webb, 1964).

The Folin-Ciocalteau method (Lowry et al., 1951) of protein determination was adopted here because of the reported simplicity, rapidity, and sensitivity of the technique. Because of the difficulty in extracting relatively purified protein samples from squash,

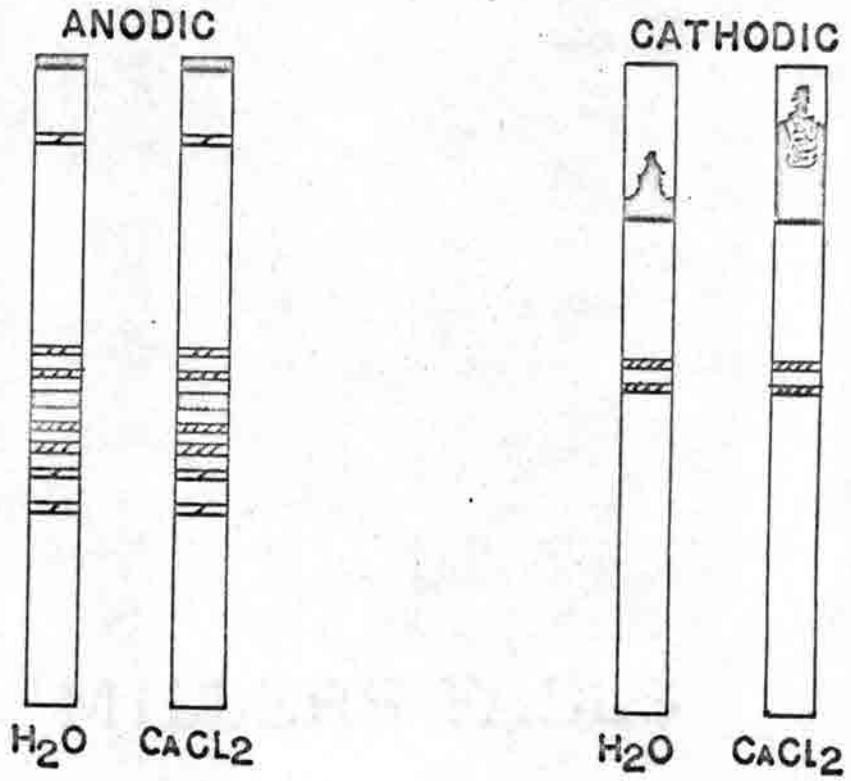


Figure 3. The isoperoxidase patterns of water and 1 M calcium chloride extracts of muskmelon stems as revealed by polyacrylamide-gel, disc electrophoresis. A medium of 4.2×10^{-2} M guaiacol and 5×10^{-3} N hydrogen peroxide in a 0.1 M sodium acetate buffer, pH 5.0, was used for peroxidase detection.

muskmelon, and watermelon plants, alpha-globulin protein, fraction IV (Pentex Incorporated) was used as a standard.

Lowry and co-workers (1951) mention that the Folin-Ciocalteau reagent is sensitive toward free aromatic amino acids and phenolic compounds. A preliminary experiment (Table 8) indicated that the Folin-Ciocalteau method was unreliable for estimating the protein content of the crude extracts employed for the peroxidase measurements since other compounds apparently interfered with the determinations.

Table 8. Comparison of protein determinations (Folin-Ciocalteau) on crude squash extracts, and on squash extracts in which the protein was precipitated with 80 per cent acetone and then taken up in 0.5 M NaOH

	µg protein per 0.1 ml of extract			
Squash Varieties	S-4	S-5	S-5 (1)	S-6
Crude Extracts	118	146	200	282
NaOH Extracts	56	79	130	159

In an attempt to alleviate the problem of other compounds interfering with the protein measurements, additional extraction procedures were conducted in a cold room (1° C) as follows: (1) 4 ml of acetone were added to 1 ml of centrifuged extract (crude extract used for peroxidase measurements) to precipitate the proteins; (2) after allowing the mixture to set for 15 minutes, the extract was centrifuged at 5,400 x g for 20 minutes to pellet the proteins; (3) the supernatant was poured off, and the proteins were taken up in 10 ml of 0.5 M NaOH with the aid of a Tri-R, Model S63 homogenizer;

(4) the NaOH-protein extract was allowed to set for at least one hour before making the protein test to assure complete solublization of the proteins.

Although the NaOH-protein extracts may still have contained some compounds interfering with the Folin-Ciocalteau test, this method seemed to provide reliable comparisons of the plant samples.

Electrophoresis Procedure

Polyacrylamide disc electrophoresis was used for separation of the peroxidases. Electrophoresis was performed using a Canalco Model-6 disc electrophoresis unit along with a Canalco Model 200 power supply. The basic features of disc electrophoresis are reviewed by Reisfield, Lewis, and Williams (1962): "Disc electrophoresis is carried out in columns of polyacrylamide gel consisting of three sections: (1) a large-pore anticonvection gel containing the protein sample; (2) a large-pore spacer gel in which electrophoretic concentration takes place; (3) a small-pore gel in which electrophoretic separation is accomplished."

The procedure for disc electrophoresis used here was basically the same as that outlined by Davis (1964) for human serum protein. However, certain modifications were needed for working with the more dilute plant proteins. The sample gels were prepared according to the procedures given for very dilute proteins in the Canalco (Canal Industrial Corporation) instructions in 1965 for the "Model 12 System." The procedure was modified slightly by using one part sample to five or more parts working solution instead of the recommended three parts to five parts proportion. In the squash

studies one part sample on a fresh weight basis was added to six parts working solution. For the muskmelon experiments the proportion was one part sample to seven parts working solution. In the case of watermelon, the proportions of sample to working solution were adjusted so as to give 52 µg of protein per sample gel for all of the samples.

Four inch, rather than the standard two and one-half inch, glass tubes were used for the electrophoretic runs. This was done because the colored reaction products indicative of the peroxidase isozymes diffused in the gels, resulting in much wider bands than those of the actual proteins. The longer electrophoretic runs further separated the proteins without appreciably affecting the widths of the protein bands. Five inch tubes also gave good separation, but the gels were harder to remove from them, and to prevent heat buildup within the columns they required a low current during runs, and hence a much longer development period.

All electrophoresis was carried out in a cold room (1^{o} C) as an extra precaution against heat buildup during the runs. Using four inch tubes, the runs were made at a current of one and one-half milliamps per tube. Six tubes could be run simultaneously, and the runs required from four to six hours.

For anodic runs a 7 percent acrylamide separating gel was used which ran at a pH of 9.5. For the cathodic runs a 7 percent acrylamide gel was used which ran at a pH of 4.3. The gel formulations employed were those suggested by Davis (1964) for the basic gels, and by Reisfield et al. (1962) for the acidic gels.

Following the completion of an electrophoretic run, the gels were gently removed from the glass columns under water using a dissecting needle. The gels were then placed in test tubes containing the appropriate solution for detecting the isoperoxidases.

Detection of Peroxidases

The substrates commonly used for quantitative measurements of peroxidase activity, such as guaiacol and pyrogallol, have previously been used in electrophoretic studies (Galston and McCune, 1961 McCune, 1961; Evans and Alldridge, 1965). Because of its wide use, guaiacol was also initially used here as the peroxidase hydrogen donor for both quantitative and qualitative purposes. However, the research of Saunders and co-workers (1965) suggested that there might be better substrates for detecting peroxidase. An experiment was set up to test various hydrogen donors for their ability to detect peroxidase isozymes separated by electrophoresis. Eight hydrogen donors chosen for this experiment were aminobenzene (aniline); 2-amino-1,3,5-trimethylbenzene (mesidine); 2-methoxyaniline (o-anisidine); 4,4'-diamino-3,3'-dimethoxybiphenyl (o-tolidine); 4-amino-3-methoxy diphenylamine (AMDPA); 1,2,3-trihydroxy benzene (pyrogallol); 4-methoxyphenol (guaiacol); 4-allylguaiacol (eugenol). Internodes from the base of 80 day-old muskmelon plants were used for the enzyme extract. Five inch tubes were used for the anodic run, and four inch tubes were used for the cathodic run. The reaction mediums used for peroxidase detection consisted of ten parts 0.1 M sodium acetate buffer (pH 5.0), one part 0.05 N H_2O_2, and one part 0.05 percent substrate.

The results of the hydrogen donor experiment are shown in Figures 4 and 5. It is evident that o-tolidine (7) and the diphenylamine (8) compound are superior to the other compounds for the detection of peroxidase under these conditions. Eugenol, guaiacol, and pyrogallol yielded similar results in the anodic run, while two additional bands were detected by eugenol and guaiacol as opposed to pyrogallol in the cathodic run. The aromatic amines, mesidine, and o-anisidine, were fair hydrogen donors while aniline was the poorest hydrogen donor for peroxidase detection.

Not a lot can be said with certainty about hydrogen donor specificities since the relative color intensities of the various products were not known. In other words, some of the lighter bands detected with tolidine might have been detected with this hydrogen donor simply because the color of the product was very intense, and as such, small quantities of the product could be detected. On the other hand, it appears that there is a definite hydrogen donor specificity in the case of the anodic peroxidases 7, 8, and 9, and the cathodic peroxidases 3, 6, and 7. These peroxidase bands were quite intense using tolidine and AMDPA, but were not detected or were barely detected by the majority of the hydrogen donors. Other cases of hydrogen donor specificity for peroxidases have been reported with respect to guaiacol and pyrogallol (Galston and McCune, 1961; Evans and Alldridge, 1965), and guaiacol and 2,6-dimethoxyphenol (Siegel and Galston, 1967).

Saunders et al. (1964) state that "in general the hydrogen donors (particularly mesidine) which are used also for the

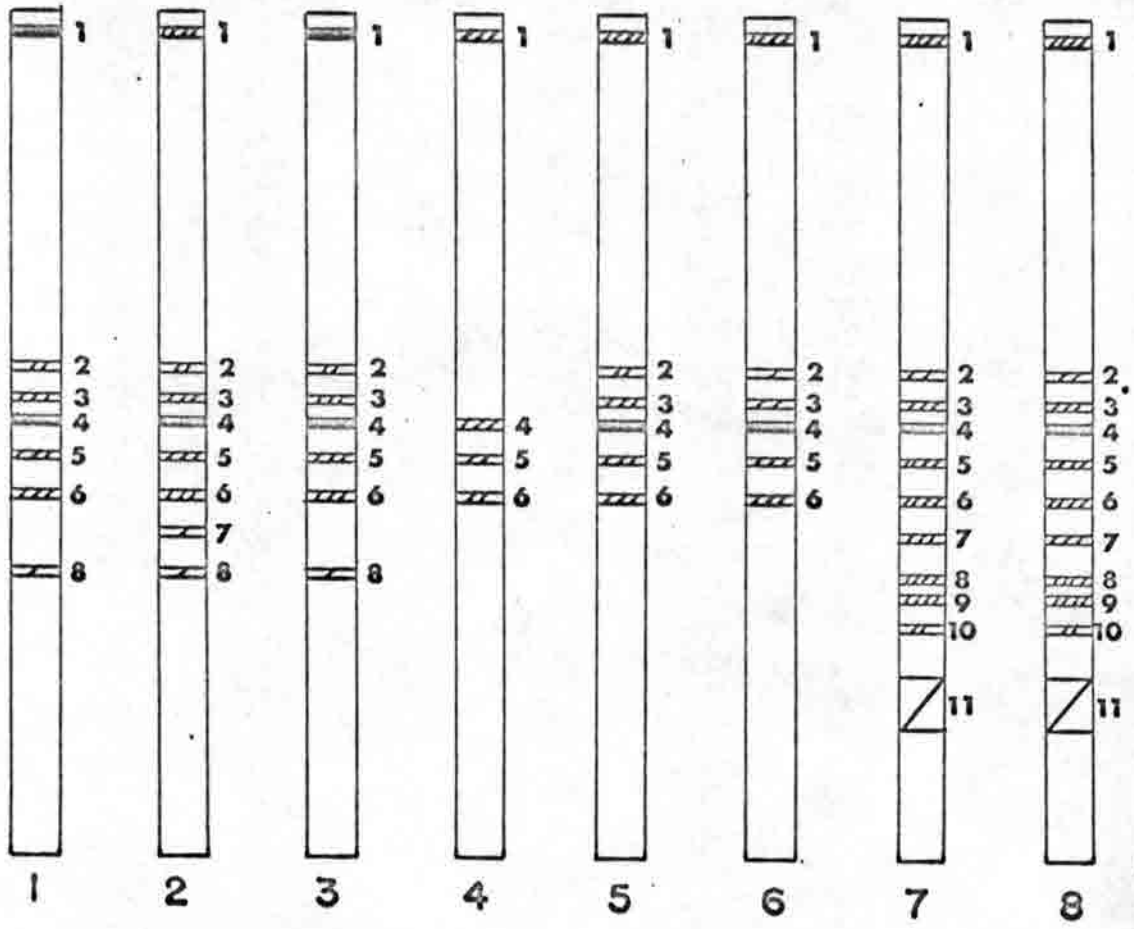


Figure 4. The ability of different compounds to act as hydrogen donors for the anodic isoperoxidases of muskmelon stems. Symbols: 1 = guaiacol, 2 = pyrogallol, 3 = eugenol, 4 = aniline, 5 = o-anisidine, 6 = mesidine, 7 = o-tolidine, and 8 = p-amino-3-methoxy diphenyl amine. The reaction mediums consisted of hydrogen donor concentrations of 0.05 per cent and 0.05 N hydrogen peroxide in 0.1 M sodium acetate buffers at pH 5.0.

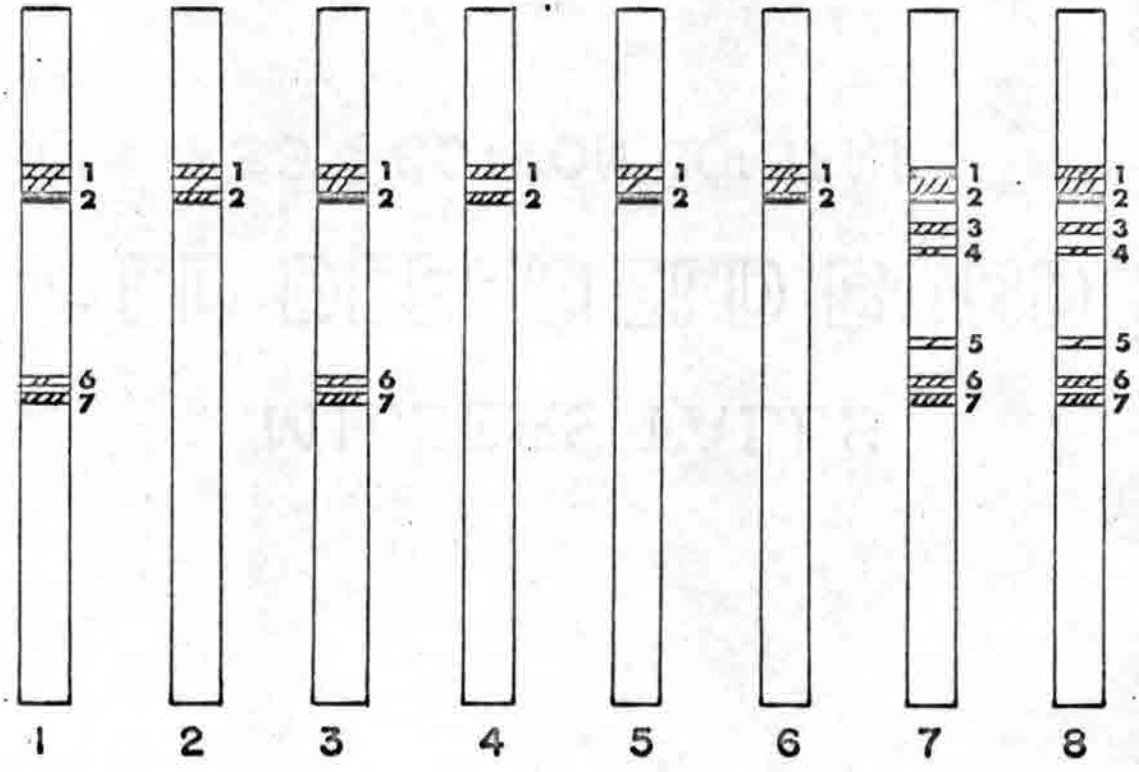


Figure 5. The ability of different compounds to act as hydrogen donors for the cathodic isoperoxidases of muskmelon stems. Symbols: 1 = guaiacol, 2 = pyrogallol, 3 = eugenol, 4 = aniline, 5 = o-anisidine, 6 = mesidine, 7 = o-tolidine, and 8 = p-amino-3-methoxy diphenyl amine. The reaction mediums consisted of hydrogen donor concentrations of 0.05 per cent and 0.05 N hydrogen peroxide in 0.1 M sodium acetate buffers at pH 5.0.

quantitative estimation of peroxidase happen to be the most suitable for actual detection of the enzyme since they are generally the most sensitive and the most specific for peroxidase." The results here do not agree with the above statement. Because of the multimolecular nature of peroxidase (Figures 4 and 5), it appears impossible to determine the most specific hydrogen donors on the basis of colorimetric measurements alone. Peroxidase substrates subject to narrow pH ranges are not suitable for detecting peroxidases separated by electrophoresis in gel systems which vary widely in pH. Siegel and Galston (1967) reported that mesidine was not satisfactory in their experiments because the oxidation products were extremely pH sensitive, and thus unstable. Mesidine was not a good substrate in these experiments -- probably for the same reason.

For colorimetric determinations of peroxidase, the hydrogen donor and oxidation product should be fairly soluble in the reaction medium (Saunders et al., 1964). The opposite characteristic is desirable for peroxidase detection on gels since a soluble product will diffuse rapidly, resulting in wide bands. Siegel and Galston (1967) listed pyrogallol as being unsuitable for this reason. There appeared to be little differences in widths of the anodic bands with respect to the hydrogen donors used in this experiment. In the cathodic run the products of tolidine and AMDPA were produced in such a large yield that diffusion resulted in an extremely broad band (numbers 1 and 2). With the other hydrogen donors, this band appeared to be composed of two isoperoxidases. The increased

sensitivity of these two hydrogen donors for peroxidases more than compensates for their disadvantage--the solubility of their products.

Tolidine was used as the hydrogen donor for peroxidase detection in subsequent experiments. It was favored over 4-amino-3-methoxy diphenylamine (AMDPA) because its absorption spectrum was known (Salomon and Johnson, 1959) so that it could be used for quantitative measurements, and because it was readily available at a low cost. This hydrogen donor was not ideal for quantitative determinations, but it was adequate. It was not oxidized by any substances in the plant extracts without the addition of hydrogen peroxide. Although it was oxidized by hydrogen peroxide alone, the rate of the reaction was so slow that it did not interfere with the peroxidase activity measurements. Secondary reaction products were formed from tolidine as indicated by color changes in the colorimeter tubes, but this appeared to occur subsequent to the peroxidase readings.

For quantitative determination of squash peroxidase, 50 µl of extract were added to a reaction medium consisting of 5×10^{-3} N H_2O_2 and 2×10^{-3} M o-tolidine in a 0.1 M sodium acetate buffer, pH 5.0. Because of the fast reaction rate, the concentration of o-tolidine was reduced to 10^{-4} M for the watermelon and muskmelon determinations. The percent transmission was recorded at 5 to 15 second intervals at 635 mµ using a Spectronic 20 (Bauch & Lomb) colorimeter. Peroxidase activity was expressed as the change in optical density (635 mµ) per unit of protein per minute.

The reaction medium used for the quantitative squash determinations was employed for the detection of peroxidases on the gel columns. Because of the slow oxidation of tolidine by peroxidase in the high pH anodic gels, the gels were left in the reaction medium for 45 minutes before photographing them and recording the results. The cathodic gels were placed in the reaction medium for 15 minutes in the case of squash and 10 minutes in the case of watermelon and muskmelon.

Summary of Procedures

The main procedures used in the quantitative and electrophoretic peroxidase experiments to follow are summarized below:

1. Plants of the same age were harvested at the two to five internode stage of growth. The leaves and apices were removed from the plants, and the remaining stems were stored in polyethylene bags at a -20° C until they were analyzed.

2. The stem material was extracted with a mortar and pestle in a 1 M $CaCl_2$ solution (1:1 w/v). The slurry was then squeezed through 3 layers of cheesecloth, and the resulting extract was centrifuged at 14,350 x g for 25 minutes to remove the larger particles. The resulting supernatant, termed the crude extract, was used for the colorimetric determinations and for electrophoresis.

3. The quantitative measurements were based on total protein as determined by the Folin-Ciocalteau method. The extracts used for the protein test were prepared as follows: (1) 4 ml of acetone were added to 1 ml of crude extract to precipitate the proteins; (2) the proteins were pelleted by centrifugation at 6,620 x g for 20 minutes; (3) the proteins were taken up in 10 ml of 0.5 M NaOH with the aid of a Tri-R, Model S63 homogenizer. The resulting extract was used for the protein determinations employing the Folin-Ciocalteau method (Lowry et al., 1951).

4. A reaction medium of 2×10^{-3} M o-tolidine and 5×10^{-3} N hydrogen peroxide in a sodium acetate buffer, pH 5.0, was used for the quantitative determination of peroxidase activity in the squash extracts. In the case of the muskmelon and watermelon extracts, the concentration of

o-tolidine was reduced to 10^{-4} M. Peroxidase activity was expressed as the change in optical density (635 mμ) per unit of protein per minute using a Spectronic 20 colorimeter.

5. The cathodic and anodic isoperoxidases were separated using polyacrylamide-gel, disc electrophoresis. The reaction medium for peroxidase detection was the same as that employed for the quantitative determination of peroxidase activity in the squash extracts.

RESULTS AND DISCUSSION

Histochemical Localization of Peroxidases

General Morphology

The general features of a Cucurbita stem seen in a cross section are shown diagrammatically in Figure 6. The vascular system is well described by Hayward (1938):

> "The vascular bundles of the stem are arranged in two rings. The smaller ones of the outer ring are located at the angles of the stem, and the larger bundles of the inner ring are alternate with those of the outer. The basic number of bundles is ten, each cycle consisting of five although occasionally additional smaller ones may be present. The bi-cyclic character of the dictyostele is related to the mode of insertion and downward divergence of the leaf traces. Each vascular strand extends downward through the stem for an average of two internodes before anastomosing with another bundle."

The morphology of muskmelon (Cucumis melo) stems differs slightly from that of Cucurbita species. It is more difficult to delineate separate rings of vascular bundles, and the medullary cavity is either absent or not as pronounced as that in Cucurbita. In addition, the pericyclic fibers appear to be more internal in Cucumis melo than in Cucurbita (Figures 7 and 8).

Peroxidase Localization

Figures 7 and 8 show the localization of peroxidase in squash and muskmelon stems respectively. The tissues containing the strongest peroxidase activity were the epidermis (including trichomes), the perivascular fibers, and the xylem. There was also a discrete localization of high peroxidase activity in the phloem tissue of squash and, to a lesser extent, muskmelon. There was a

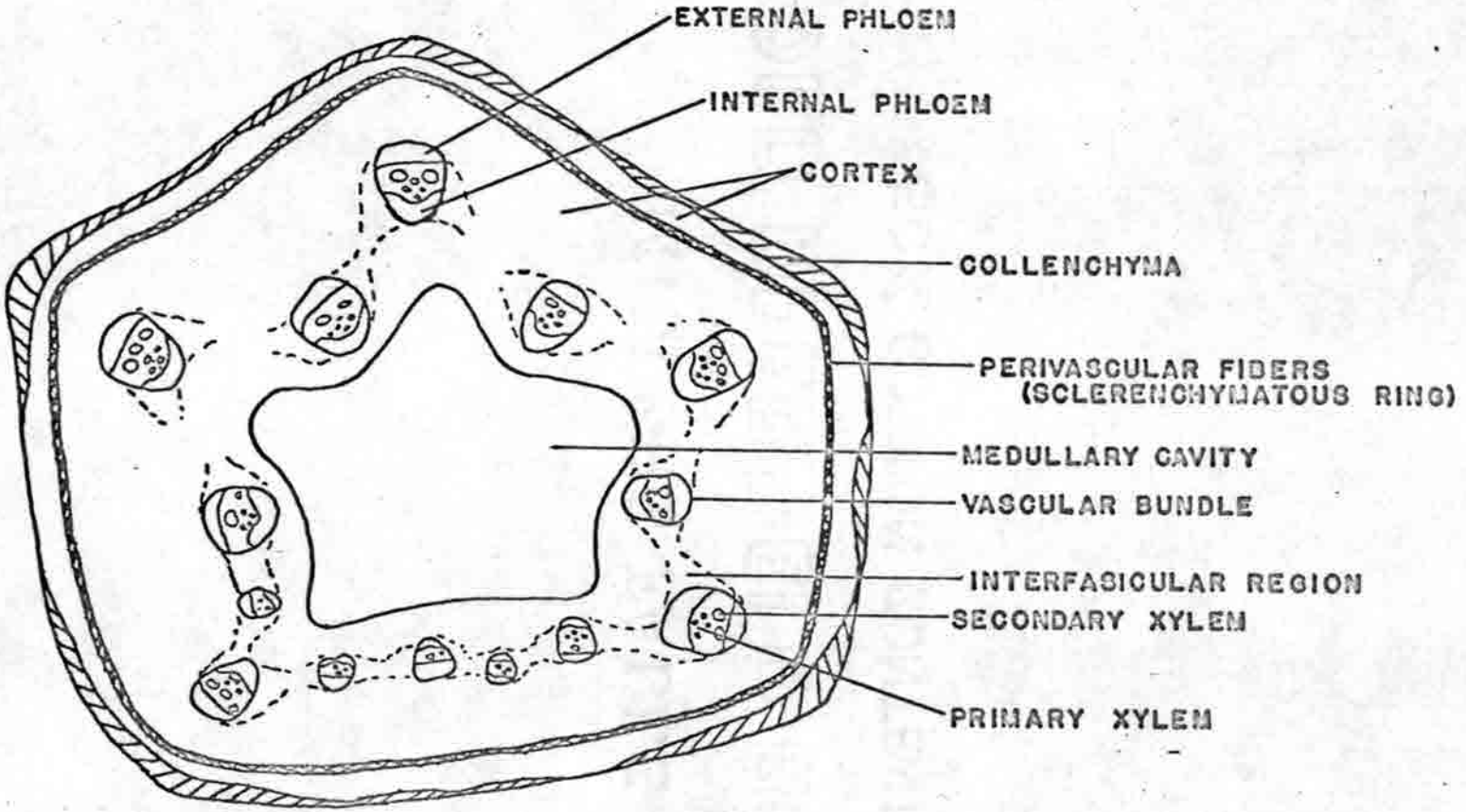


Figure 6. Diagrammatic cross section of a Cucurbita stem.

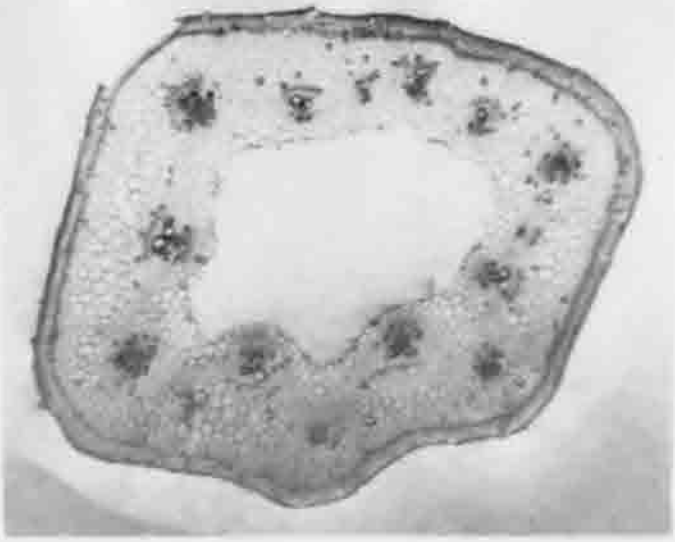


Figure 7. The distribution of peroxidase in the stem of vine Cucurbita pepo, line Cornell 51-26-7 (S-1). Guaiacol used as the hydrogen donor.

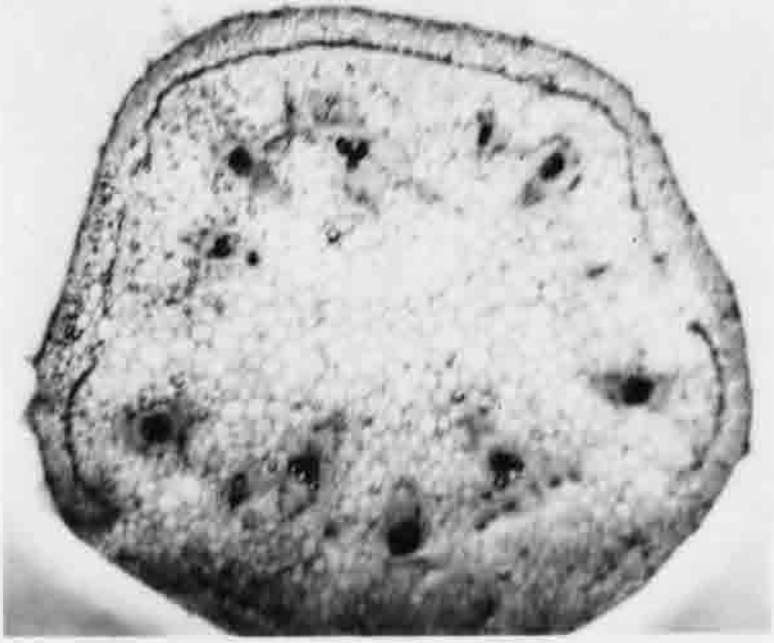


Figure 8. The distribution of peroxidase in the stem of vine muskmelon, variety Rocky Ford (M-6). Ortho-anisidine used as the hydrogen donor.

rather uniform distribution of moderate peroxidase activity throughout the pith and cortex.

The heavy localization of peroxidase in the epidermal and vascular tissues coincides with results of similar investigations on other species of plants (Hussein and Cruess, 1940; Rosoff and Cruess, 1949; Van Fleet, 1947). Van Fleet (1947, 1959) emphasized the strong and continuous localization of peroxidase in the phloem, and gave only slight attention to peroxidase activity localized in the xylem. Apropos of the cucurbits studies here, the results demonstrate that peroxidase activity is much higher in the xylem than in the phloem. This is in accord with the results of Siegel (1953) who found the highest peroxidase activity in the fibro-vascular tissue of Red Kidney bean.

Van Fleet (1959) associated peroxidase loss or inhibition with cessation of cell division. Although the zone of cell division was not analyzed in this study, overall peroxidase activity appeared to rise progressing basipetally down the stem. In the first distinguishable internodes analyzed in squash and muskmelon, peroxidase activity was present in the xylem and epidermis, but it was not observed in the perivascular fibers. In the second or third internodes from the apex peroxidase activity was evident in the perivascular fibers.

Comparison of Bush and Vine Types

Because higher peroxidase activity in dwarf as compared to normal plants had been reported in the literature (Van Overbeek, 1935; Kamerbeek, 1956; McCune and Galston, 1959), it was thought

that any such differences in cucurbits might possibly be reflected in differential localization of peroxidase between bush and vine plants. The greatest interest in this experiment was the comparison of bush and vine muskmelon lines. The bush muskmelon lines exhibited a brittle and twisted characteristic which suggested that "precocious lignification" might be involved in the dwarfing mechanism.

The isogenic bush and vine lines of squash exhibited identical peroxidase patterns in tissue sections from both expanding and mature internodes. The distribution of peroxidase in all squash sections is well represented in Figure 7. These results do not mean that there could not have been differences in peroxidase activity between bush and vine squash, but only that no noticeable qualitative differences were observed.

No apparent differences in peroxidase localization could be detected between the bush and vine muskmelon lines (Figures 9 and 10); however, anatomical differences were readily observable. The vascular bundles of all three dwarf lines were arranged in one continuous ring interconnected by small celled, interfasicular tissue. Although this condition was evident to some extent in the first few expanding internodes, it was especially noticeable in the more mature internodes (four to six internodes from the apex). It is not known whether this condition contributes to the brittle and twisted characteristic of bush muskmelon plants. The brittle characteristic seems to be manifested in all the stem tissue.

Lignin distribution. Figures 11 and 12 show sections from a squash stem which demonstrate a very good correlation between the lignified areas and regions of high peroxidase activity. The same situation exists in the muskmelon-stem sections (Figures 13 and 14). While strong peroxidase activity was observed in xylem tissue of the youngest internodes, lignification was slight or absent until the second or third internode from the apex. Peroxidase activity in the perivascular fibers was usually first detectable in the second internode down; whereas, perivascular lignin was not observed until the third or fourth internode. Thus peroxidase activity always coincides with or precedes lignin formation in the sclerenchymatous ring and in the xylem.

There were no apparent differences in the amount of lignification between bush and vine muskmelon or squash (Figures 15 and 16). Since stem brittleness in bush muskmelon is manifested immediately below the apex, and since lignification does not occur to any appreciable extent at this stage of development, the "precocious lignification" hypothesis appears untenable in this instance.

Peroxidase and Synthetic Lignin

If peroxidase activity was high in epidermal tissue and present in low amounts even in the parenchyma cells, then why were these cells not lignified? There are several possible explanations: lignin precursors or hydrogen peroxide may be limiting in these tissues; the peroxidases in these tissues may not be bound to the cell walls in the right manner to participate in lignification; or

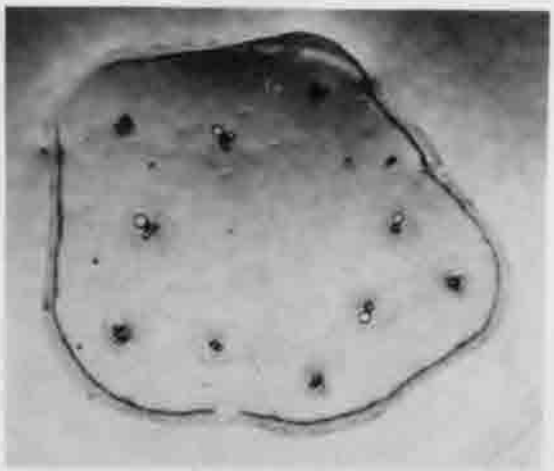


Figure 11. The distribution of lignin in the stem of Cucurbita pepo, line Cornell 51-26-7, as indicated by the phloroglucinol-HCl test.

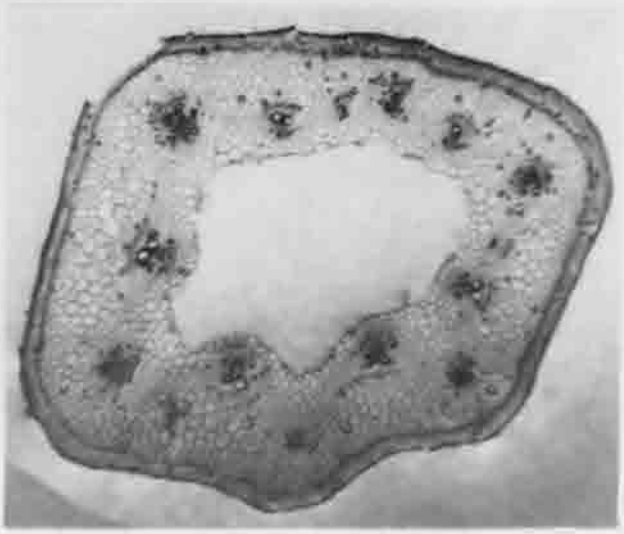


Figure 12. The distribution of peroxidase in the stem of Cucurbita pepo, line Cornell 51-26-7, as indicated by the peroxidation of ortho-anisidine.

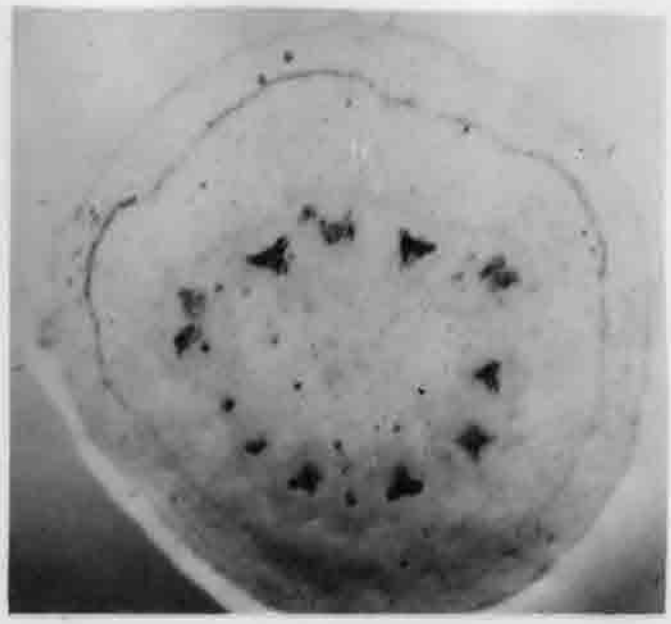


Figure 13. The distribution of lignin in the first internode of bush muskmelon, line #30 (M-3), as indicated by the phloroglucinol-HCl test.

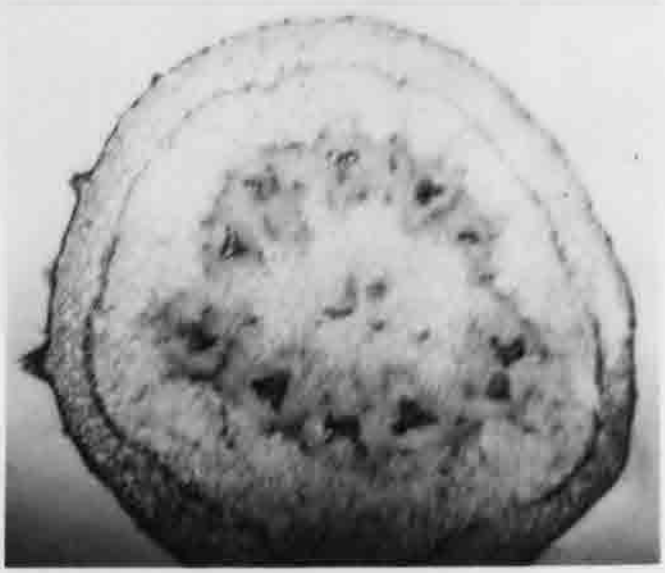


Figure 14. The distribution of peroxidase in the first internode of bush muskmelon, line #30 (M-3), as indicated by the peroxidation of ortho-anisidine.

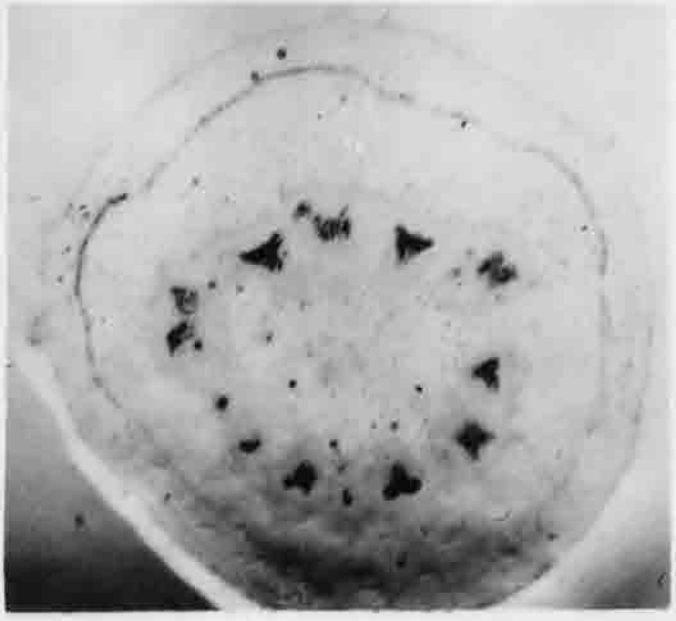


Figure 15. The distribution of lignin in the first internode of bush muskmelon, line #30 (M-3), as indicated by the phloroglucinol-HCl test.

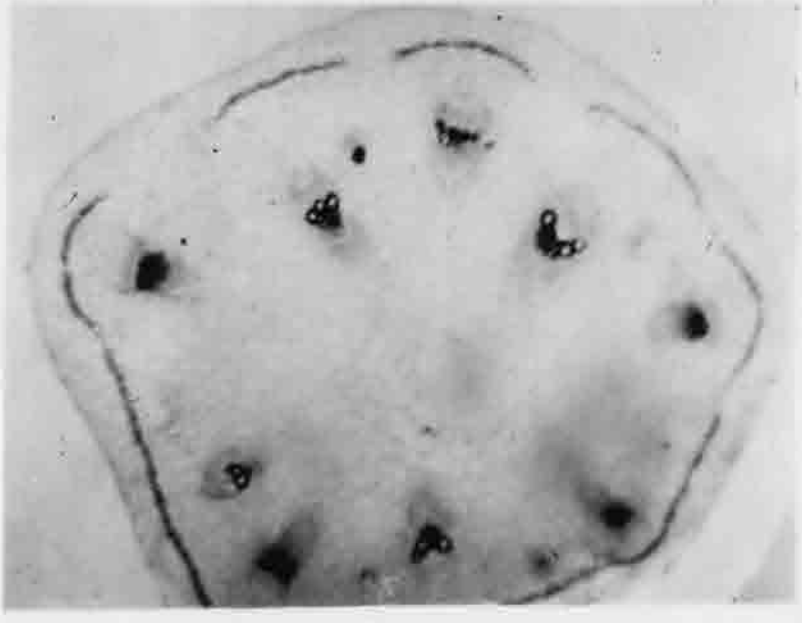


Figure 16. The distribution of lignin in the first internode of vine muskmelon, variety Texas No. 1 (M-7), as indicated by the phloroglucinol-HCl test.

the peroxidases in these tissues may be different than those in the lignified areas, and not specific for the lignin precursors.

In vivo lignification. According to Siegel (1953, 1954, 1955), eugenol can be used as a lignin precursor for producing a synthetic lignin in plant tissue containing peroxidase. To determine whether lignin precursors might be the limiting factor in the lack of lignification in epidermal and other tissues containing peroxidase, stem sections were submersed in a solution of eugenol (6×10^{-3} M) and hydrogen peroxide (5×10^{-3} N) for various periods of time. Following submersion in eugenol, the sections were placed on glass slides and stained for lignin with phloroglucinol-HCl.

Much of the eugenol was converted into a brown pigment, rendering it difficult to resolve the red staining reaction indicative of lignin (Figure 17). However, by observing the sections under 36X magnification, phloroglucinol-positive reactions could be easily observed in epidermal cell walls, in the corners of collenchyma cell walls and in slight amounts on the cell walls of parenchyma tissue. Although the phloroglucinol-HCl test is not entirely specific for lignin, it is a commonly employed histochemical test for lignification (Jensen, 1962). The fact that lignin staining was positive in the epidermis of treated, but not untreated, sections, indicates that a lignin-like polymer was probably formed. Hence, peroxidase in tissues other than the xylem and perivascular fibers can participate in lignin biosynthesis in the presence of the proper substrates. Stafford (1964) found this to be so in Elodea stems which are normally not lignified, but which can synthesize lignin in the presence of H_2O_2 and lignin precursors. Bland (1961) used normally non-lignified,

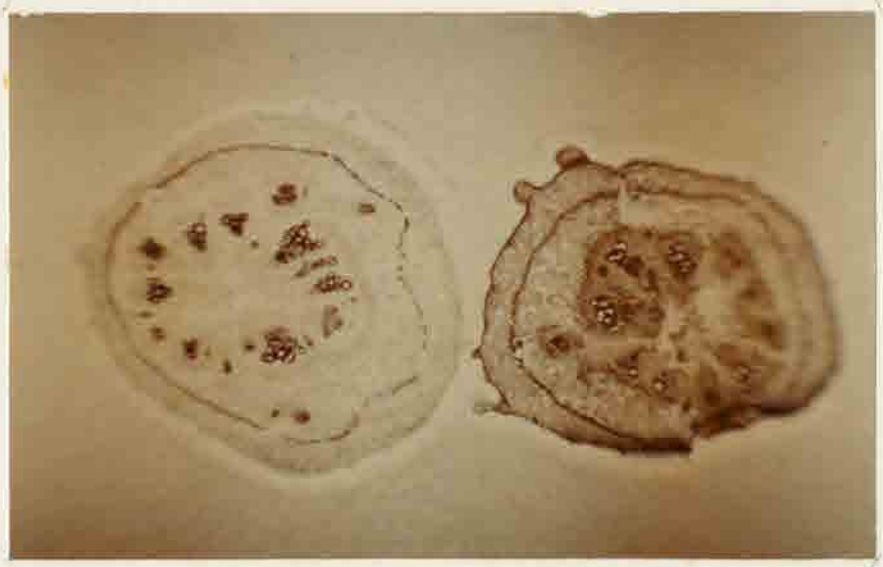


Figure 17. The synthesis of lignin-like polymers in muskmelon-stem sections as indicated by the red-violet, phloroglucinol-positive reactions. The section on the left is a control placed in water for three hours; the section on the right was submersed for three hours in a solution containing 6.2×10^{-3} M eugenol and 5×10^{-3} N hydrogen peroxide.

potato parenchyma to produce an artificial lignin from eugenol and H_2O_2. Siegel (1956a) demonstrated that cellulose or a similar macromolecular matrix is a prerequisite for lignin formation. The work of Jansen et al. (1961) indicated that the "bound" peroxidase in cell walls may be attached to pectins. At any rate, the peroxidase participating in lignification in various tissues is probably localized in the cell walls.

In vitro lignin. Because of the possibility that only certain isoperoxidases may be able to catalyze dehydrogenation reactions in lignin biosynthesis, an experiment was set up to determine if such specificities exist in muskmelon peroxidases. It was thought that the electrophoretic gel matrix might be able to substitute for the macromolecular matrix listed by Siegel (1956a) as a necessity for in vitro lignin synthesis. Anodic and cathodic electrophoretic runs were performed on stem extracts from 84 day-old muskmelon stems. The gels were placed in solutions of eugenol (6.2×10^{-3}M) and hydrogen peroxide (5×10^{-3} N), with guaiacol used as a control hydrogen donor.

The same peroxidase bands were apparent with eugenol as with guaiacol (Figure 18), except that they were opaque and more difficult to detect than the reddish-brown bands formed with guaiacol. After the gels were allowed to remain in the eugenol solution overnight, they were placed in phloroglucinol-HCl solutions. All of the cathodic and anodic bands stained a pink to a dim red color depending on the original intensity of the bands, indicating that lignin-like polymers were probably produced. It appears that there is no absolute specificity of lignin precursors for certain peroxidases, and the

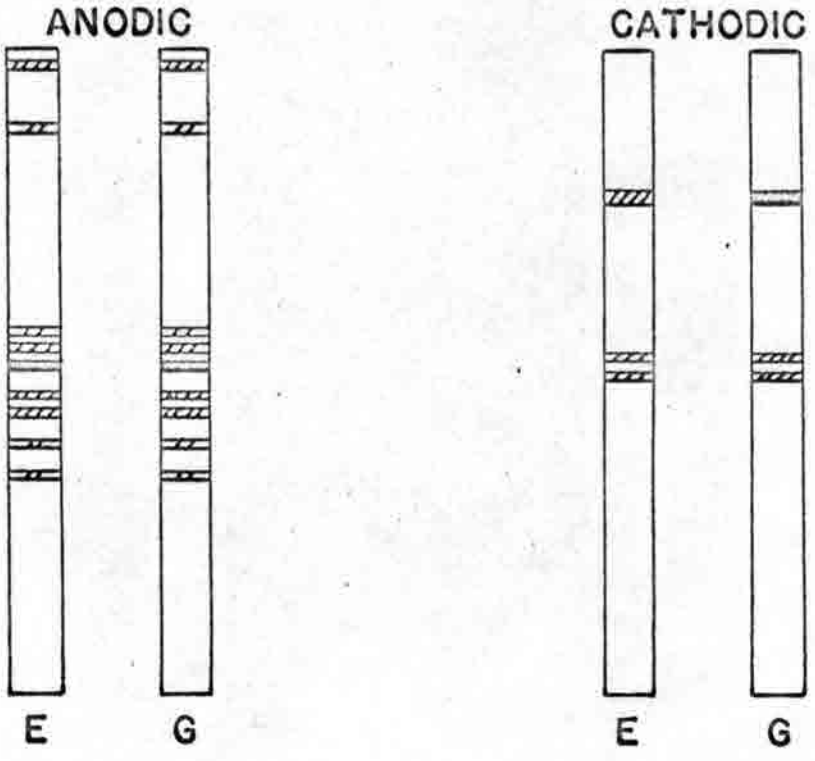


Figure 18. The comparison of the hydrogen donor specificity of eugenol (E) and guaiacol (G) for muskmelon isoperoxidases. All of the peroxidase bands detected with eugenol stained a faint, red color with phloroglucinol-HCl after 12 hours, indicative of small yields of a lignin-like polymer.

results support the concept that differential lignification in the tissues studied is probably regulated at a step(s) prior to the peroxidase catalyzed reaction.

Differentiation of Peroxidase in Tissues

Because of the large number of isoperoxidases found in cucurbit stems in preliminary experiments, and because of the differential localization of peroxidase activity in stem tissues, it was presumed that the peroxidase patterns would probably differ according to the tissues analyzed. An experiment was set up for an electrophoretic analysis of the peroxidases in the different stem tissues of muskmelon. Using a razor blade, stems of a bush muskmelon (#30) were separated into the epidermal tissue (E); an outer ring (OR) of tissue including collenchyma cells, the sclerenchymatous ring, and part of the cortex; and an inner core (IC) of tissue containing part of the cortex, the pith, and the vascular bundles. An attempt was made to dissect out the vascular bundles, but not enough vascular tissue was obtained for an adequate sample. This experiment was designed only to detect any gross qualitative differences among the tissues, and serve as a basis for future experimentation.

The results of the experiment are shown in Figure 19. The isoperoxidase patterns were remarkably similar among the tissues. Isoperoxidases A_6, A_7, and A_8 were clearly present in the epidermis, but were barely detectable in the other tissues. The faint band, A_2 was practically absent from the inner core (IC) while band A_1 was absent from the epidermis. There were no distinct qualitative differences in the cathodic isoperoxidases, but bands C_2 and C_3 were

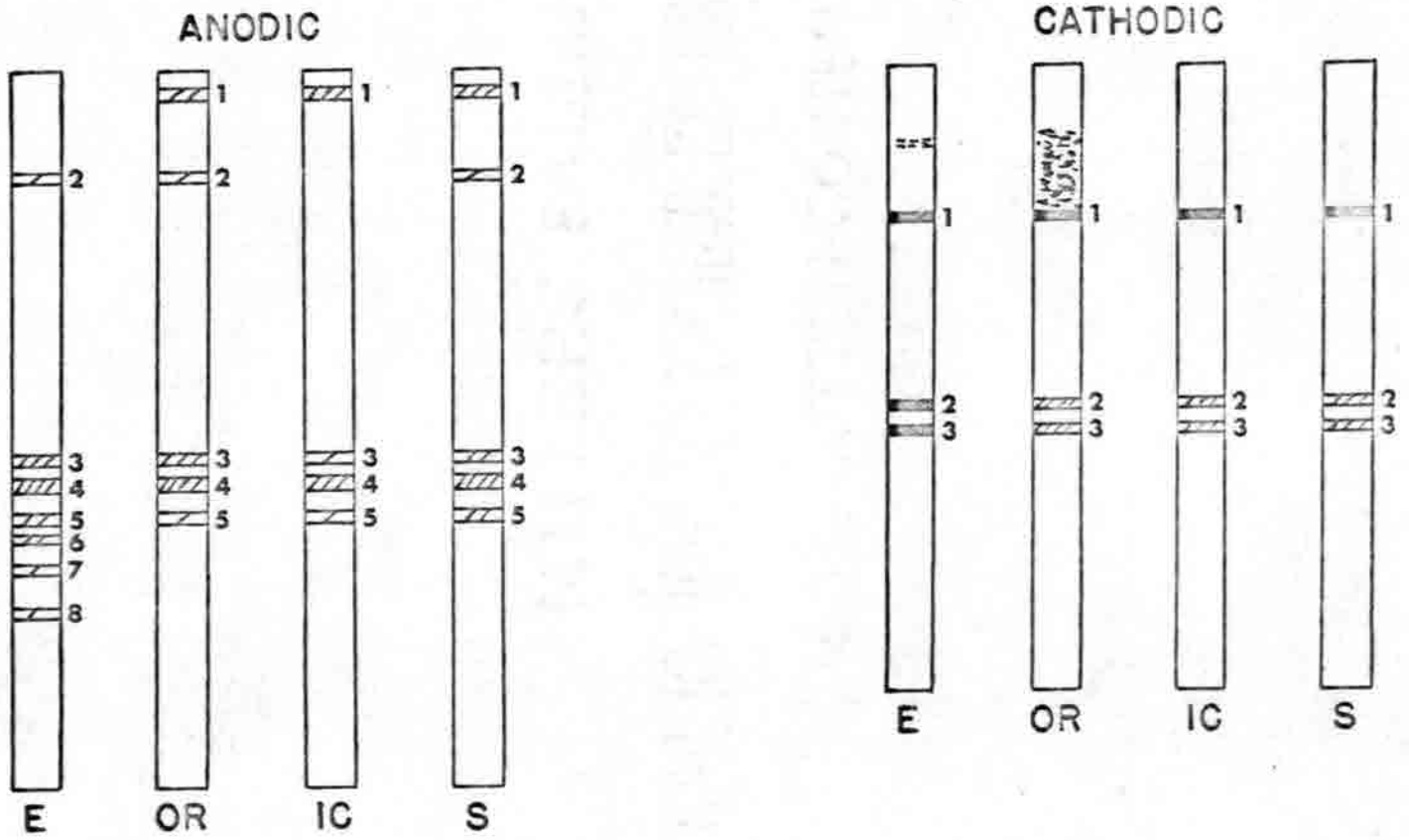


Figure 19. The cathodic and anodic isoperoxidases in stem tissues of muskmelon. Symbols: E = epidermis, OR = outer ring, IC = inner core, and S = whole stem. The reaction medium consisted of 4.2×10^{-2} M guaiacol and 5×10^{-3} N hydrogen peroxide in a sodium acetate buffer, pH 5.0.

darker in the epidermal run than in the others. About the only conclusion that can be reached from the limited results is that quantitative rather than qualitative peroxidase differences seem to predominate among the stem tissues.

Isoperoxidase A_1 is very unusual in that it remains near the top of the separating gel, and does not migrate appreciably even after long electrophoretic runs. Because this protein moves through the large-pore sample and spacer gels during short electrophoretic runs, but remains at the top of the smaller pore separating gel, its lack of migration may be due to its large size. Siegel and Galston (1967) found that the slow-moving, anodic bands (A_r-1 and A_s-1) in pea roots and shoots could be stained with acetocarmine. They postulated that such bands may be intact cellular organelles, thus accounting for their slow electrophoretic migration. Plesnicar, Bonner, and Storey (1967) have recently reported that a small portion of the peroxidase from Mung bean hypocotyls is tightly bound to a particulate fraction. It is possible that the slow moving anodic peroxidase in the muskmelon extracts may represent peroxidase still bound to some organelle.

Quantitative Peroxidase Study

Effect of Growth Regulators on Stem Elongation

It was hoped that the growth retardant treatments would sufficiently inhibit growth in the vine lines so their habit of growth would resemble the bush phenotypes. The results of the growth retardant treatments were somewhat disappointing. Figure 20 illustrates the effects of the growth retardants on the squash lines. Only the growth retardant B-995 appreciably inhibited the growth of the squash lines. The reduced growth was mainly limited to the normally faster growing squash lines -- Blue Hubbard (S-9), Japanese Pie (S-18), and Connecticut Field (S-10). Zucchini (S-13), a bush pepo, appeared to be stimulated by B-995. The effect of the growth retardants on the growth of the watermelon lines is shown in Figure 21. Only B-995 inhibited growth, and the inhibition was not pronounced. As shown in Figure 22, none of the growth retardants inhibited growth of the muskmelon lines. Phosphon-D appeared to stimulate growth in all of the squash lines and in the bush muskmelon lines.

The failure of the growth retardants to appreciably inhibit growth can be ascribed either to a lack of specificity of the growth retardants for inhibiting growth in the cucurbit species treated, or to an application of insufficient concentrations of the growth retardants necessary to inhibit growth. Denna (1962) reported that 10^{-3} M CCC (soil drench or foliar) was an effective inhibitor of squash and cucumber growth, but was relatively ineffective on muskmelon. Preston and Link (1958) found that cucumber growth was retarded by Phosphon-D. These reports do not give much insight into

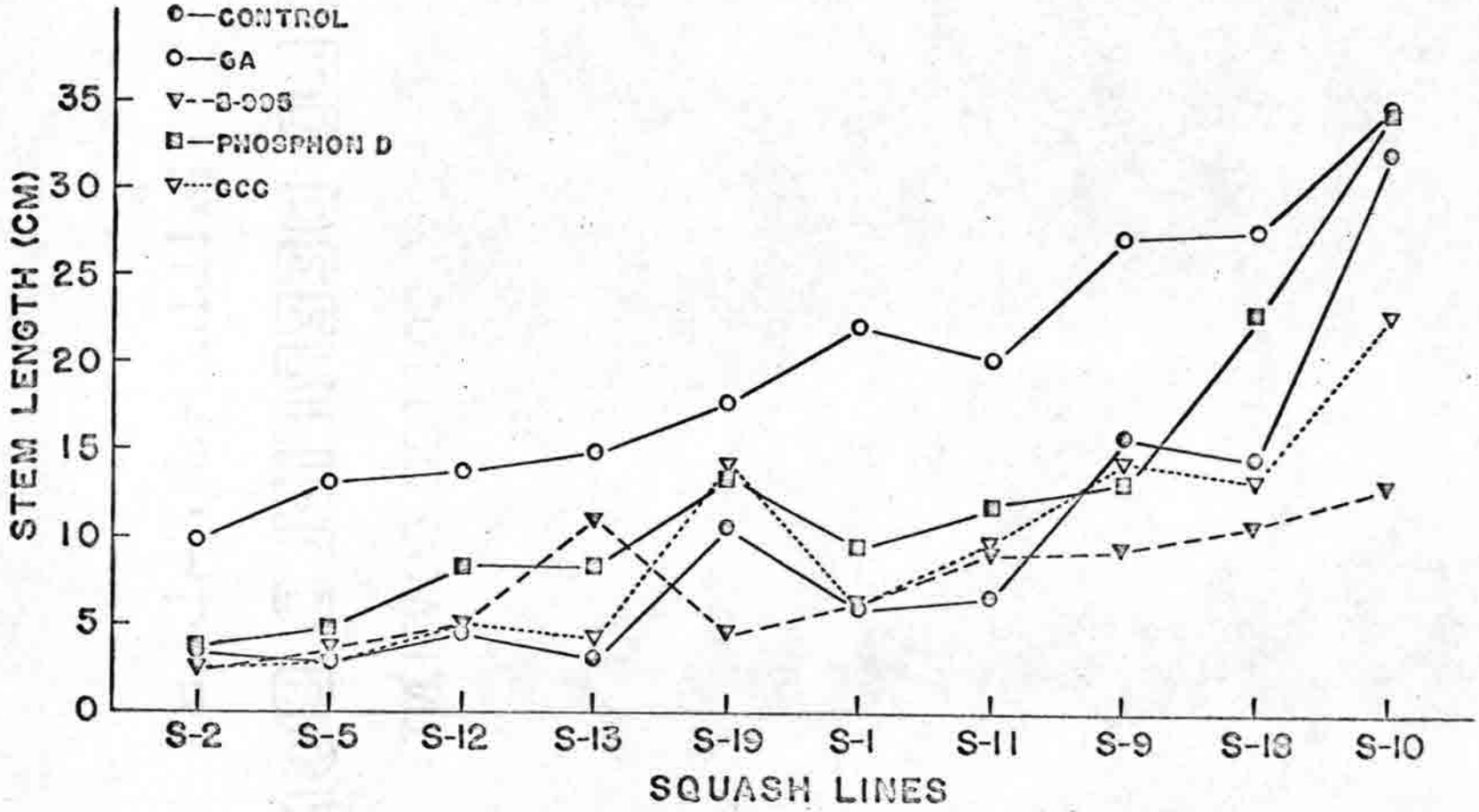


Figure 20. The effects of B-995, Phosphon-D, CCC, and GA on the stem elongation of squash lines Cornell 60-4 (S-2), Minnesota 503.58 (S-5), Cornell 59-166-9 (S-12), Zucchini (S-13), Kentucky Field (S-19), Cornell 51-26-7 (S-1), Lagenaria (S-11), Blue Hubbard (S-9), Japanese Pie (S-18), and Connecticut Field (S-10). The plants were harvested 29 days after seeding. The growth regulator concentrations were: GA - 10^{-3} M, daily spray; B-995 - 5×10^{-3} M, one spray; Phosphon-D - 10^{-4} M, soil drench (5 liters/cu.ft.); and CCC - 5×10^{-4} M, soil drench (5 liters/cu.ft.).

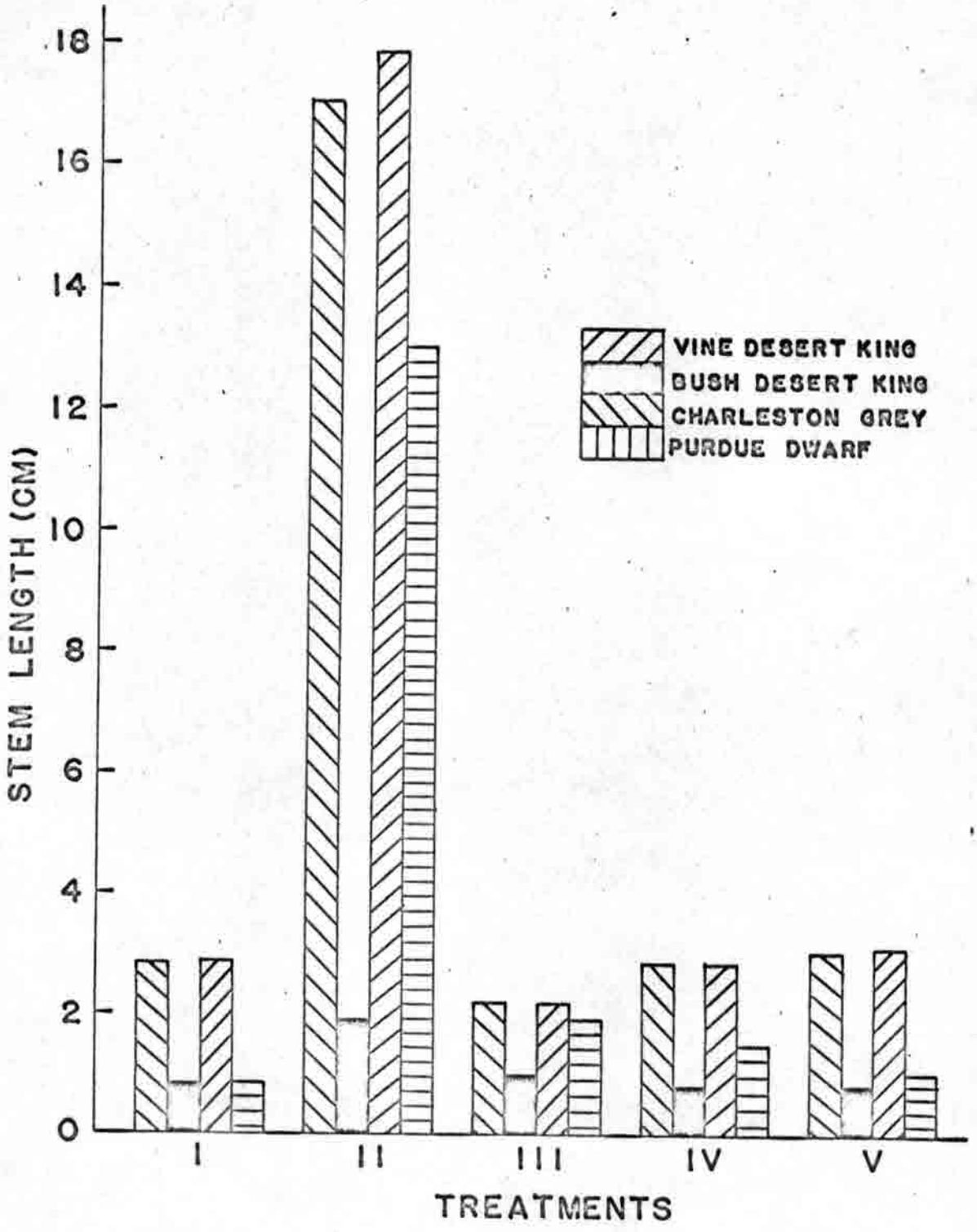


Figure 21. The effects of B-995, Phosphon-D, CCC, and GA on stem elongation of the watermelon lines. The plants were harvested 35 days after seeding. Symbols: I = Control; II = GA treatment; III = B-995 treatment; IV = Phosphon-D treatment; V = CCC treatment. The growth regulator concentrations were: GA - 10^{-3} M, daily spray; B-995 - 5×10^{-3} M, one spray; Phosphon-D - 10^{-4} M, soil drench (5 liters/cu.ft.); and CCC - 5×10^{-4} M, soil drench (5 liters/cu.ft.).

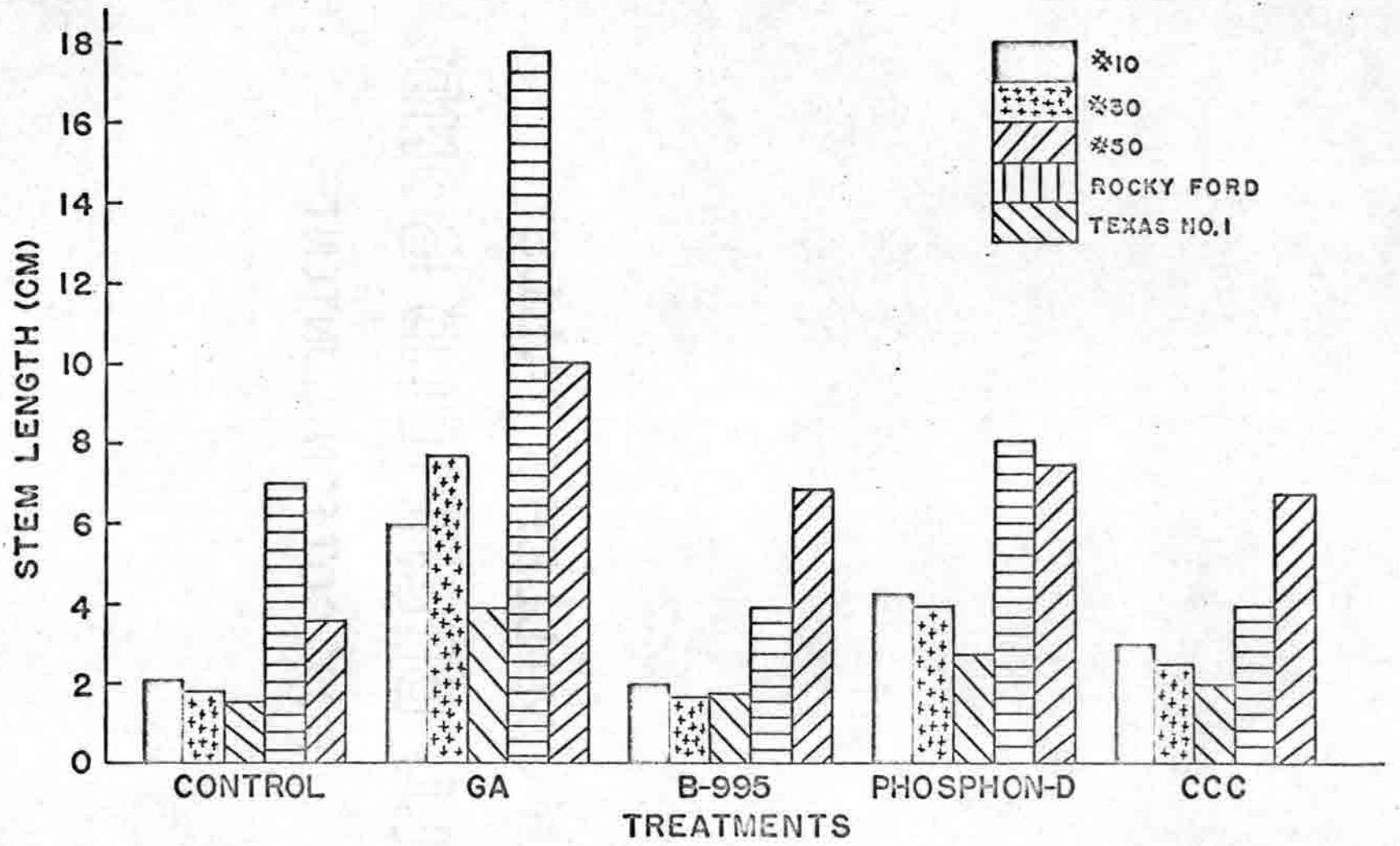


Figure 22. The effects of B-995, Phosphon-D, CCC, and GA on stem elongation of the muskmelon lines. The plants were harvested 34 days after seeding. The growth regulator concentrations were: GA - 10^{-3} M, daily spray; B-995 - 5×10^{-3} M, one spray; Phosphon-D - 10^{-4} M, soil drench (5 liters /cu.ft.); and CCC - 5×10^{-4} M, soil drench (5 liters/cu.ft.).

what results to expect with the growth retardants used. The failure of CCC 10^{-4} M CCC (soil drench) to retard the growth of the squash lines under the conditions of this experiment suggests that CCC was applied in too low a concentration. The apparent stimulation of growth in squash and muskmelon lines by Phosphon-D is not an unprecedented phenomenon. Cathey and Stuart (1961) reported that Phosphon-D stimulated growth of 4 out of 54 treated plant species.

Gibberellin treatments. Denna (1963) found that isogenic bush and vine lines of Cucurbita pepo grown in the greenhouse during the winter exhibited identical growth curves when both were treated every other day with 10^{-3} M gibberellin starting at the first true-leaf stage. Under similar conditions, Denna (1962) found that muskmelon #30 treated with gibberellin in the same way as the squash above, exhibited a growth rate similar to two untreated vine muskmelon lines. It was expected that the gibberellin treatment would at least enhance the growth of the bush muskmelon lines, and might result in similar growth rates for the bush and vine squash lines.

As shown in Figure 20, a 10^{-3} M daily foliar spray with gibberellin enhanced growth in all of the squash lines. The increase in growth rate over the controls was as great or greater for the vine lines as for the bush lines. The one exception was Connecticut Field (S-10), an extremely rapid growing C. pepo variety, which responded little to the gibberellin treatment.

The results of the GA treatment of the watermelon lines is given in Figure 21. The two bush watermelon lines responded differently; Bush Desert King barely responded to the GA treatment,

while the growth of Purdue Dwarf was nearly as great as that of the two vine watermelon lines. Both of the vine watermelon varieties, Vine Desert King and Charleston Grey, were highly responsive to gibberellin.

Figure 22 illustrates the effect of GA on the muskmelon lines. The bush muskmelon lines treated with 10^{-3} M gibberellin grew as tall as the non-treated vine lines. The vine muskmelon varieties, Rocky Ford and Texas No. 1, responded as much or more than the bush lines to gibberellin treatment.

The large growth increase in both the vine and bush cucurbits treated with gibberellin-A_3 suggests that the vine as well as the bush lines contained suboptimal concentrations of endogenous gibberellin needed for stem elongation. Bush Desert King watermelon exhibited the lowest growth response to gibberellin of all the bush cucurbit lines, while Purdue Dwarf watermelon displayed the largest response.

Comparison of Peroxidase Activity in Bush and Vine Cucurbit Lines

The hypotheses that peroxidase is involved in dwarfing mechanisms, through either precocious lignification or increased IAA inactivation, assumes that dwarf varieties have higher peroxidase activity than non-dwarfs. To account for higher peroxidase activity in dwarf plants which appear from their response to exogenous GA to be low in endogenous gibberellin, it can be supposed that gibberellin represses peroxidase synthesis. Perhaps some genetic dwarfs could be the result of mutations resulting in constitutive peroxidase synthesis and hence, greater IAA inactivation or precocious

lignification. The control of peroxidase synthesis through the existence of regulatory genes, such as those proposed by Jacob and Monod (1961) for bacteria, could account for the above scheme. The absence or inactivation of a hypothetical peroxidase repressor protein due to a regulator-gene mutation could result in constitutive synthesis of peroxidases normally under the control of the mutated regulator gene.

Several bush and vine lines of cucurbits were used for the peroxidase investigations. This was done to reduce the chance that varietal differences would bias the comparisons between the bush and vine lines, and to provide a more comprehensive survey of the peroxidases in cucurbit species. Among the lines examined were isogenic bush and vine lines of C. pepo, Cornell 60-4 and Cornell 51-26-7 respectively, and two lines of watermelon, Bush and Vine Desert King, which were probably isogenic.

In Figure 23 the squash lines are listed in order of increasing peroxidase activity from left to right. The means of the control, B-995-treatment, and the GA-treatment were pooled in Figure 23 since there were no significant interactions among the treatments (Appendix C). The bush lines Cornell 59-166-9 (S-12, C. maxima), Minnesota 503.58 (S-5, C. maxima), and Cornell 60-4 (S-2, C. pepo) were at the low end of the peroxidase scale, while Zucchini (S-13, C. pepo) had relatively high peroxidase activity. Considering the vine types, Cornell 51-26-7 (S-1), a pepo, exhibited low activity; Connecticut Field (S-10), a pepo, had high activity; and the rest of the lines fell in the intermediate range of peroxidase activity.

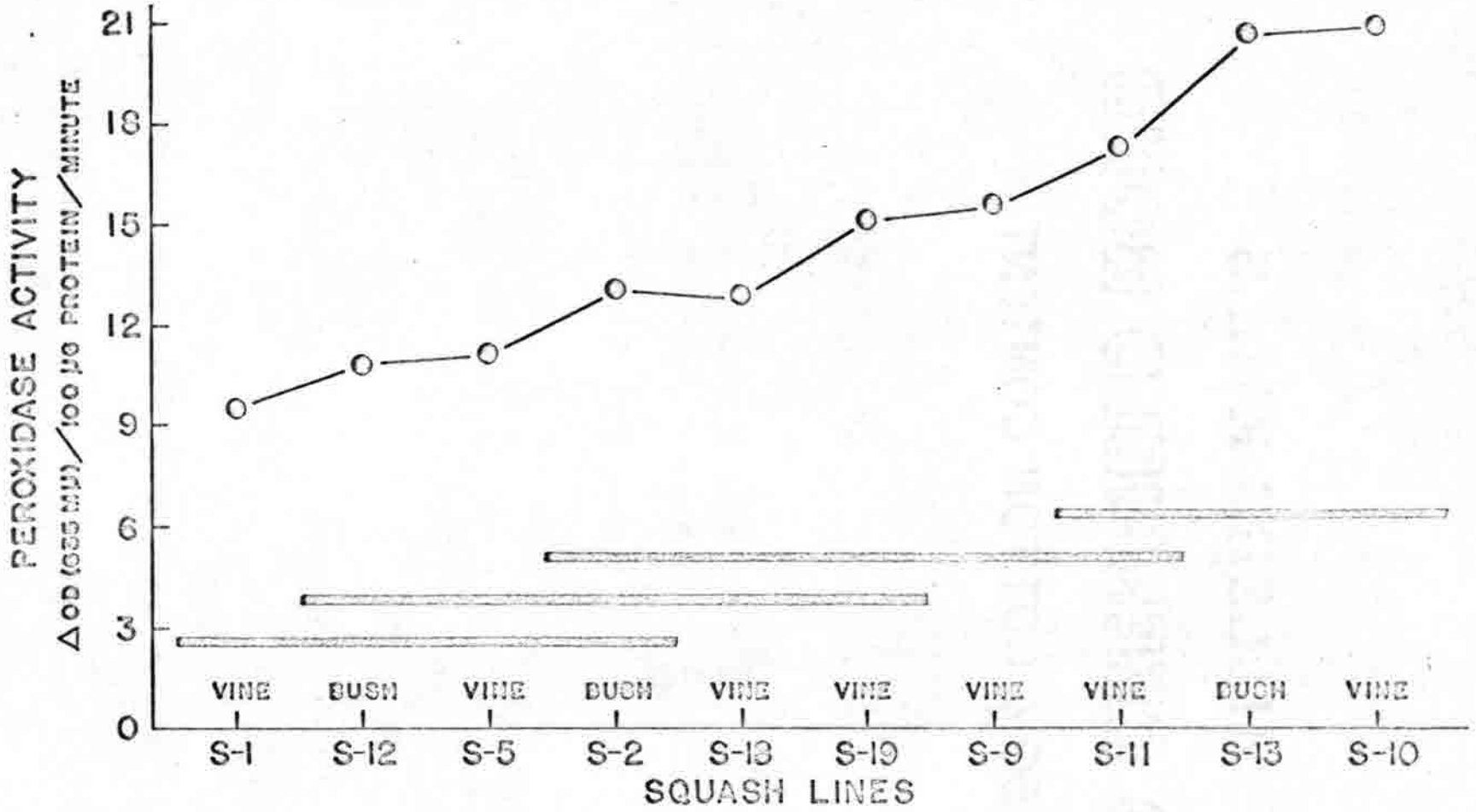


Figure 23. Peroxidase activity in the squash lines Cornell 51-26-7 (S-1), Cornell 59-166-9 (S-12), Minnesota 503.58 (S-5), Cornell 60-4 (S-2), Japanese Pie (S-18), Kentucky Field (S-19), Blue Hubbard (S-9), Lagenaria (S-11), Zucchini (S-13), and Connecticut Field (S-10). The reaction medium consisted of 2×10^{-3} M o-tolidine and 5×10^{-3} N hydrogen peroxide in a sodium acetate buffer, pH 5.0. The bars represent the grouping of the lines according to the New Multiple Range Test. Any two squash lines not underscored by the same bar are significantly different at the 5% level.

Using the New Multiple Range Test (Li, 1964) the squash lines could be placed into groups significantly different from each other (Figure 23 and Appendix D). The grouping in no way reflects an association of high peroxidase activity with the bush phenotype. This is more strongly illustrated in Figure 24 where stem length is plotted against peroxidase activity. The peroxidase levels among the squash lines appear to be randomly distributed with respect to stem elongation. It is readily apparent (Figure 23), for example, that if only Zucchini (a bush line with high peroxidase activity) and Cornell 51-26-7 (a vine line with low peroxidase activity) had been selected for the peroxidase study, incorrect conclusions might have been reached concerning the relationship of peroxidase to dwarfism.

The peroxidase levels of the isogenic bush (Cornell 60-4) and vine (Cornell 51-26-7) squash lines were not significantly different from each other in the above experiment. More comparisons were conducted to further substantiate this since this material was the most suitable for comparing peroxidase levels between the bush and the vine phenotypes. As shown in Table 9, there were no significant differences in peroxidase activity between Cornell 60-4 and Cornell 51-26-7. At least in squash, it appears that peroxidase activity can vary among species and varieties, but that no consistent differences exist between bush and vine plants.

The quantitative determinations of peroxidase activity in watermelon and muskmelon lines were incomplete due to some sample sizes which were too small to analyze. Although no consistent differences

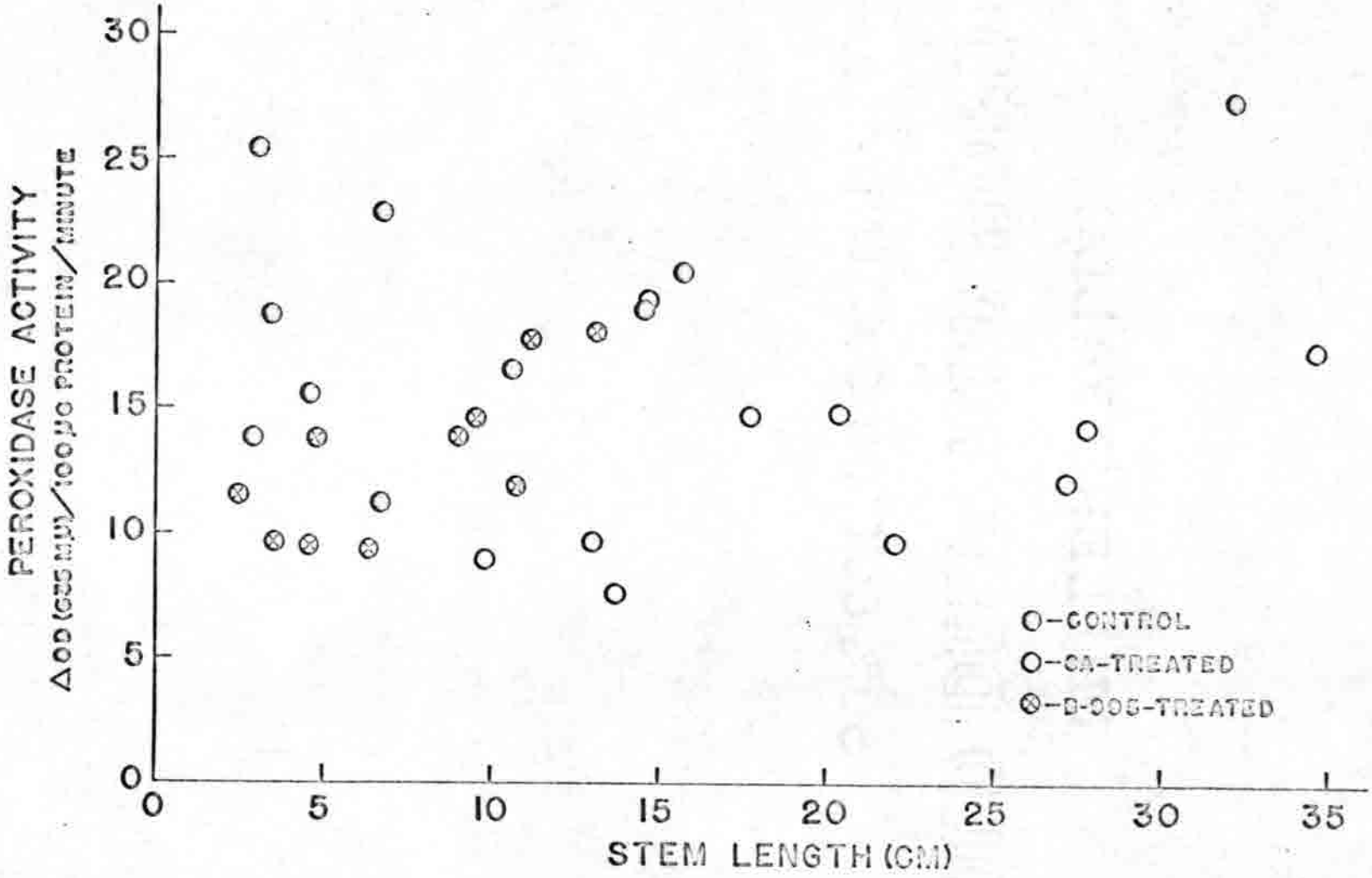


Figure 24. Peroxidase activity of the squash lines plotted against stem length. The reaction medium consisted of 2×10^{-3} M o-tolidine and 5×10^{-3} N hydrogen peroxide in a sodium acetate buffer, pH 5.0.

Table 9. Peroxidase activity of the isogenic bush and vine squash lines, Cornell 60-4 and Cornell 51-26-7. The differences are not statistically significant at the 5% level (Appendix E). Ortho-tolidine (10^{-4} M) was used as the hydrogen donor.

		Peroxidase Activity Δ OD(635 mμ) per mg protein per minute	
		Cornell 51-26-7	Cornell 60-4
Replications (4 stems each)	1	12.32	13.56
	2	15.25	15.11
	3	14.28	13.99

in peroxidase activity were noted in comparisons of bush and vine lines of watermelon and muskmelon, the data will not be presented here because of its questionable validity.

Effect of Growth Regulators on Peroxidase Activity. The growth regulator treatments were set up to provide further information on the peroxidase relationships between bush and vine plants. McCune (1961) reported that gibberellin stimulated the growth of three dwarf corn mutants, and reversed the effect of dwarfism on the isoperoxidase activities. It was conceived that any differences in peroxidase levels associated with the bush phenotype in GA responsive cucurbit lines might be overcome by gibberellin treatment. Although none of the bush cucurbit lines were completely restored to the vine phenotype with gibberellin, the growth differences between the non-treated and the GA-treated lines were large enough so that any effects of gibberellin on peroxidase activity should have been readily apparent. If the growth inhibition due to the growth retardants were analogous to the retarded growth of genetic dwarfs, then any changes in peroxidase levels associated with genetic dwarfing might also be apparent in the chemically induced dwarfs. Furthermore, since gibberellin and growth retardants are antagonistic in their effects on growth, they might be expected to have opposite effects on the peroxidase activity of plants. Halevy (1963) reported an inverse relationship of GA and Amo-1618 on peroxidase activity of cucumber hypocotyl tips and cotyledons; GA inhibited and Amo-1618 stimulated peroxidase activity. The stimulation of peroxidase as well as IAA-oxidase activity was also obtained with CCC, B-995, Phosphon, and Carvadan.

As shown in Figure 25, the GA-treated and B-995 treated squash lines had lower peroxidase activity than the non-treated lines. It will be recalled that the GA-treated squash plants had to be harvested four days earlier than the other treatments. According to earlier results (Figure 1), peroxidase increases with age of the squash stems. It is therefore probable that the peroxidase activity of the GA-treated lines would have been at least as great as the controls if they both had been harvested at the same time. According to the "IAA-inactivation" hypothesis of dwarfing, gibberellin should decrease, and growth retardants should increase the peroxidase levels in plants. The above data do not support such a hypothesis. Brian, Hemming, and Lowe (1958) reported that gibberellin accelerated the maturation of pea internodes. If gibberellin has a similar effect on squash lines, then peroxidase activity might be expected to rise in GA-treated plants along with the increased physiological age of such plants. There has been little information reported apropos of the effects of growth retardants on maturation (Cathey, 1964). Since gibberellin and growth retardants have opposite effects on stem elongation, growth retardants might be expected to delay maturation, and to decrease peroxidase levels in plants. The effect of the B-995 treatment on maturation of the squash plants was not determined, so that such an hypothesis cannot be supported in this experiment.

Enough samples of the watermelon lines Vine Desert King (W-1) and Charleston Grey (W-1) were available to make a limited peroxidase comparison between the control, the B-995 and the GA

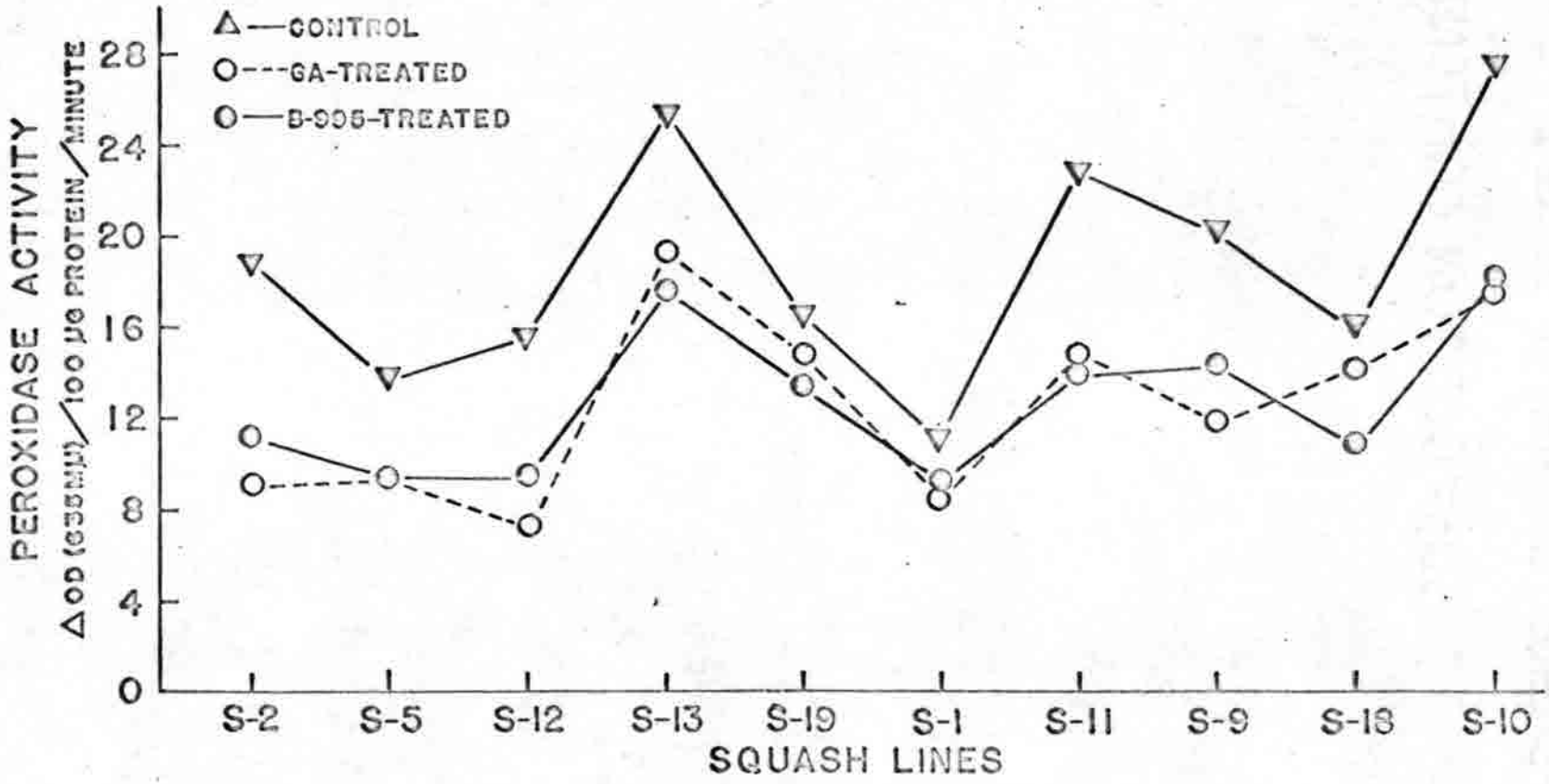


Figure 25. A comparison of the peroxidase activities among the control, B-995, and GA treatments in the squash lines Cornell 60-4 (S-2), Minnesota 503.58 (S-5), Cornell 59-166-9 (S-12), Zucchini (S-13), Kentucky Field (S-19), Cornell 51-26-7 (S-1), Lagenaria (S-11), Blue Hubbard (S-9), Japanese Pie (S-18), and Connecticut Field (S-10). Each point represents the mean of two peroxidase determinations. The reaction medium consisted of 2×10^{-3} M o-tolidine and 5×10^{-3} N hydrogen peroxide in a sodium acetate buffer, pH 5.0.

treatments. The results are given in Table 10. No significant differences were found between the treatments or among the varieties (Appendix F); however, the number of replications (two) was not sufficient to conclude beyond a doubt that there were no differences in peroxidase activity among the watermelon lines.

Electrophoretic Peroxidase Study

Polyacrylamide-gel, disc electrophoresis was performed at both pH 9.0 and pH 4.3. In the experiments to follow the peroxidases migrating during electrophoresis at pH 9.0 will be termed the anodic isoperoxidases, whereas those migrating during electrophoresis at pH 4.3 will be called the cathodic isoperoxidases. Peroxidases possessing isoelectric points at near-neutral pH values could, of course, migrate during electrophoresis at both pH 9.0 and 4.3. It must be kept in mind that the total number of cucurbit isoperoxidases is probably less than the total number detected in both the anodic and the cathodic electrophoretic runs. This problem will be discussed further in a later section.

Isoperoxidases of Bush and Vine Squash

Although no consistent quantitative differences were found among the bush and the vine squash lines, significant differences could still exist in the electrophoretic peroxidase patterns. If an *in vivo* functioning IAA-oxidase is a peroxidase, it seems unlikely that all the multimolecular forms of peroxidase in a cell could be involved in such a function. If constitutive peroxidase synthesis is involved in the dwarfing mechanism, only certain isoperoxidases would be expected to be involved. Increased synthesis of only a

Table 10. Peroxidase activity of Vine Desert King and Charleston Grey in relation to the control and the B-995 and GA treatments. The differences among the lines and between the treatments were not significant at the 5% level (Appendix F). Ortho-tolidine (10^{-4} M) was the hydrogen donor.

	Peroxidase Activity △OD (635 mμ) per 100 μg protein per minute			
Treatments	Vine Desert King		Charleston Grey	
Control	4.000	3.241	3.054	3.822
GA	3.302	2.980	3.237	2.965
B-995	3.437	3.403	4.144	4.038

few of the isoperoxidases in bush cucurbit lines would not necessarily be reflected in significant increases in the total quantitative peroxidase determinations since such isoperoxidases might represent only a small fraction of the total plant peroxidase.

It was impossible to assign different numbers to all of the different anodic isoperoxidases in the squash lines. Certain of the peroxidase bands having similar mobilities, and thought to be homologous in a phylogenetic sense, were numbered the same. This numbering problem was not encountered with the anodic peroxidases of the watermelon and muskmelon lines nor with any of the cathodic peroxidases; in these latter cases the isoperoxidases within a species could be identified as being analogous. Because of slight variations in lengths of the electrophoretic runs, some differences in enzyme mobility may be exaggerated, and this complicated the peroxidase comparisons among the squash lines.

Figure 26 shows the anodic peroxidase patterns in the non-treated squash lines; the lines are arranged in order of increasing stem length from left to right. The most striking feature is the large amount of variability in the enzyme patterns among the squash lines. The isoperoxidases A_1, A_2, A_3, A_6, A_7, A_{10}, A_{13}, A_{14}, and A_{15} are common to most of the lines although the intensities of the bands vary. The peroxidases A_{13}, A_{14}, and A_{15} were generally too diffuse to be distinguished as three separate peroxidases. There appear to be no trends in the isoperoxidase patterns when progressing from the shorter internode to the longer internode squash lines. There are no isoperoxidases unique to the bush lines of squash

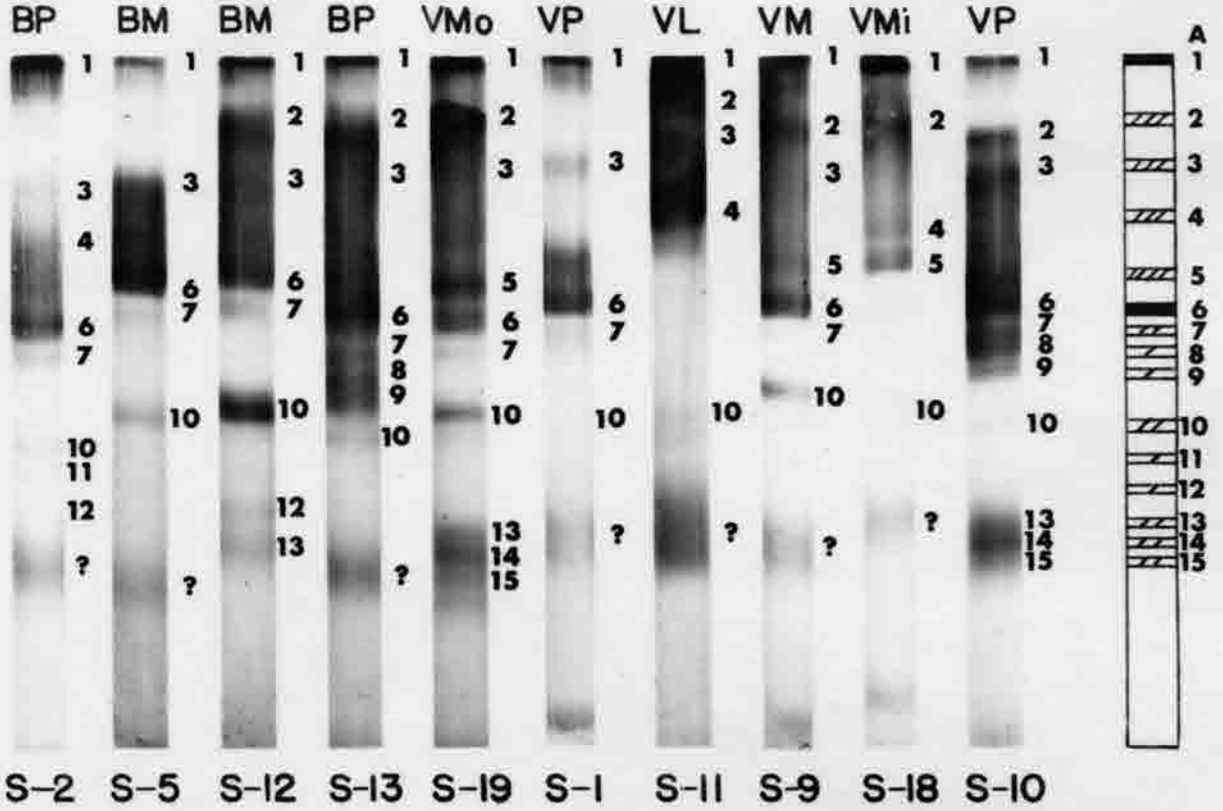


Figure 26. The anodic isoperoxidases of the squash lines as revealed by polyacrylamide-gel, disc electrophoresis. A reaction medium of 2 x 10^{-3} M o-tolidine and 5 x 10^{-3} N hydrogen peroxide in a sodium acetate buffer, pH 5.0, was used for peroxidase detection. Symbols: BP = bush pepo, BM = bush maxima, VP = vine pepo, VM = vine maxima, VMo = vine moschata, VMi = vine mixta, VL = vine Lagenaria, S-2 = Cornell 60-4, S-5 = Minnesota 503.58, S-12 = Cornell 59-166-9, S-13 = Zucchini, S-19 = Kentucky Field, S-1 = Cornell 51-26-7, S-11 = Lagenaria, S-9 = Blue Hubbard, S-18 = Japanese Pie, and S-10 = Connecticut Field.

although isoperoxidase A_{10} shows up as an extremely dark band in Cornell 59-166-9 (S-12), a bush maxima.

The cathodic peroxidases are more distinct and easier to compare than the anodic enzymes (Figure 27). Most of the isoperoxidases are common to all of the lines. The only major differences are the absence of C_4 in Lagenaria (S-11), the absence of C_9 in Blue Hubbard (S-9), and the lack of bands C_8 and C_9 in Minnesota 503.58 (S-5) and Cornell 59-166-9 (S-12). Most of the lines contained three of the four (6, 7, 8, and 9) fast moving cathodic peroxidases. Distinguishing which of these four bands were present in the different lines was difficult and somewhat arbitrary. There were no apparent consistent differences between the bush and vine squash lines.

Additional replications of the isoperoxidases of the isogenic bush and vine squash lines are illustrated in Figure 28. The isozyme patterns are essentially identical. The peroxidases A_2, A_{13}, A_{14}, and A_{15} appear slightly stronger in the bush line (S-2), while C_1 appears stronger in the vine line (S-1). These slight differences were not consistently evident in the various comparisons of these two lines (Figures 26, 28, 29, and 30), and it is thus unlikely that they are related to the dwarfing phenomenon. Although Cornell 51-26-7 (S-1) and Cornell 60-4 (S-2) are considered to be isogenic, they are not absolutely identical. The bush line (S-2) was produced through six backcrosses to Cornell 51-26-7; hence, some differences in enzyme patterns might be expected.

Since the investigation of the isoperoxidases of the muskmelon and the watermelon lines was more limited than that of the squash lines, their discussion will be included in the next section.

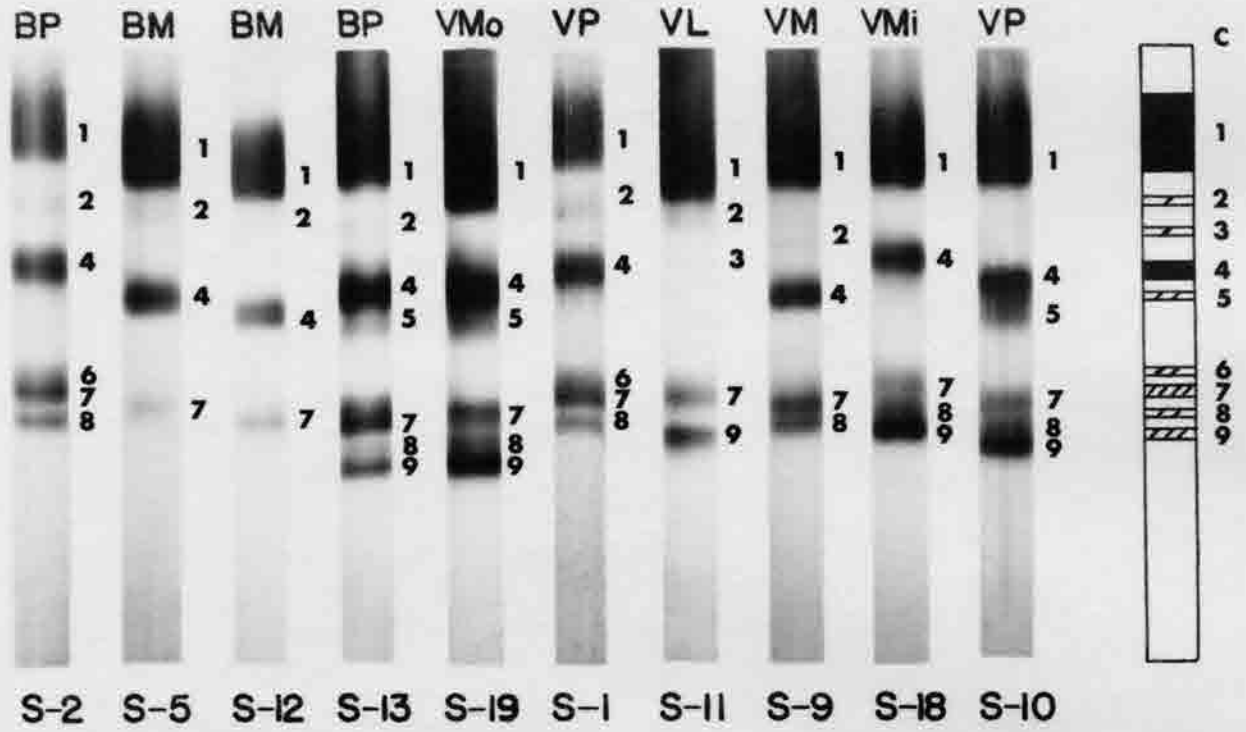


Figure 27. The cathodic isoperoxidases of the squash lines as revealed by polyacrylamide-gel, disc electrophoresis. A reaction medium of 2 x 10^{-3} M o-tolidine and 5 x 10^{-3} N hydrogen peroxide in a sodium acetate buffer, pH 5.0, was used for peroxidase detection. Symbols: BP = bush pepo, BM = bush maxima, VP = vine pepo, VM = vine maxima, VMo = vine moschata, VMi = vine mixta, VL = vine Lagenaria, S-2 = Cornell 60-4, S-5 = Minnesota 503.58, S-12 = Cornell 59-166-9, S-13 = Zucchini, S-19 = Kentucky Field, S-1 = Cornell 51-26-7, S-11 = Lagenaria, S-9 = Blue Hubbard, S-18 = Japanese Pie, and S-10 = Connecticut Field.

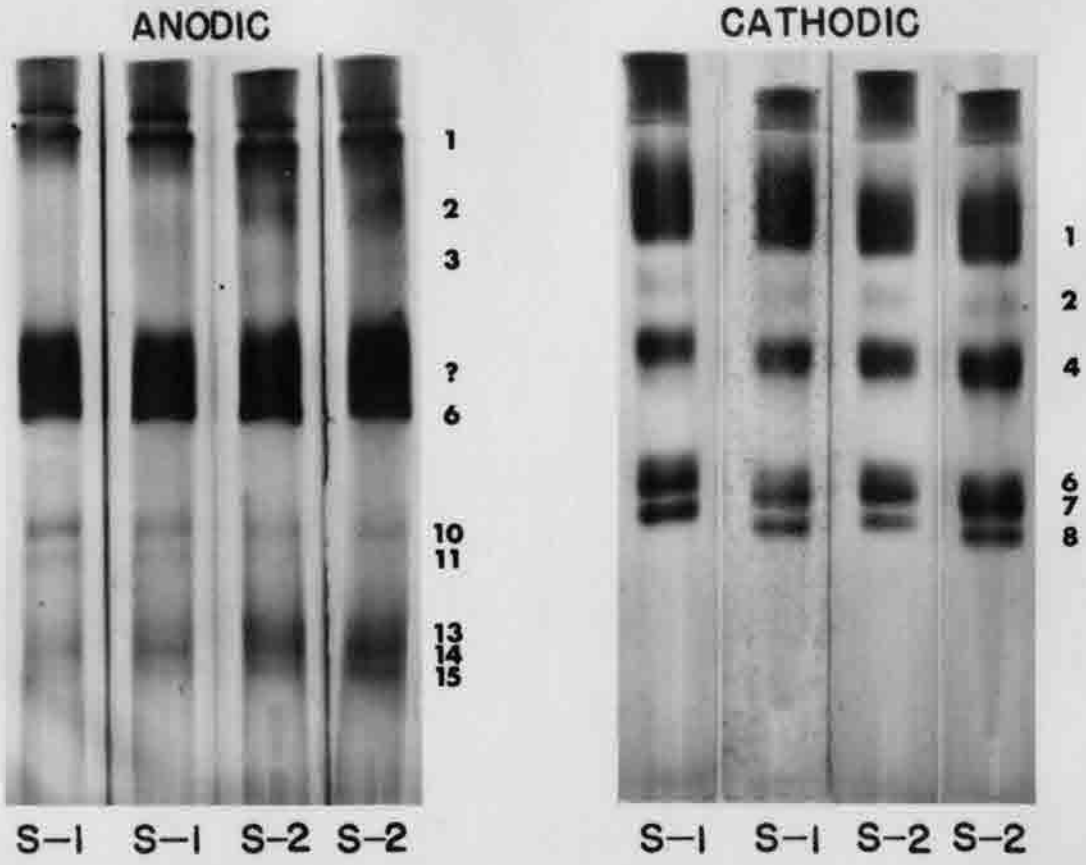


Figure 28. The cathodic and anodic isoperoxidases of the isogenic bush (Cornell 60-4) and vine (51-26-7) squash lines as revealed by polyacrylamide-gel, disc electrophoresis. A reaction medium of 2×10^{-3} M o-tolidine and 5×10^{-3} N hydrogen peroxide in a sodium acetate buffer, pH 5.0, was used for peroxidase detection.

Effect of the Growth Regulator Treatments on the Isoperoxidases of Bush and Vine Cucurbits

This experiment was undertaken with the idea that the reversal of the bush and vine phenotypes with gibberellin and B-995, respectively, might also affect the isoperoxidase patterns of the plants. This might give some further insight into the regulation of peroxidases by endogenous gibberellins, and elucidate the role, if any, of peroxidase in genetic dwarfing.

Squash. As shown in Figures 29 and 30, the various treatments resulted in relatively few changes in the anodic isoperoxidase patterns of the squash lines. Lines S-1, S-2, S-10, and S-13 varied to some extent among the treatments, particularly in the intensities of some of the bands. Peroxidase A_{10} in Cornell 59-166-9 (S-12) was of interest because of its high intensity. Visual comparison among the treatments suggests that the GA-treatment may have resulted in lower activity of this peroxidase. Line S-19 exhibited unusually faint peroxidase bands in the B-995 treatment (III). Since this low peroxidase activity was not revealed in the quantitative study, it was probably due to an experimental error. The replicate sample was lost, and so this cannot be confirmed. In treatment III (B-995) of line S-18 one of the replicates showed low activity for the slow moving anodic peroxidases while the other replication exhibited a strong reaction for these peroxidases. An unknown experimental error was probably again involved since the sample gave high peroxidase activity by quantitative determination.

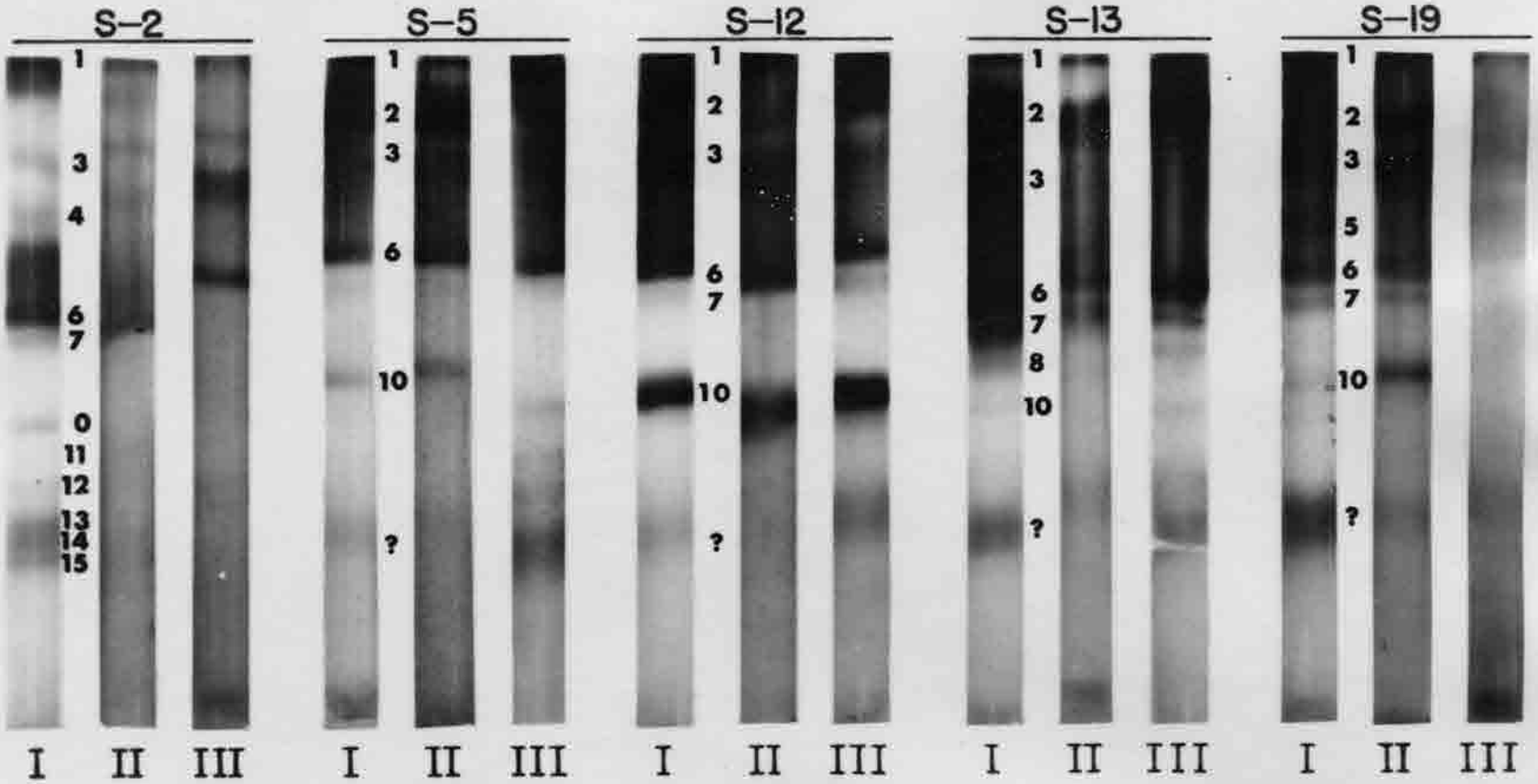


Figure 29. A comparison of the anodic isoperoxidases of the control (I), GA-treated (II), and B-995-treated (III) squash lines—Cornell 60-4 (S-2), Minnesota 503.58 (S-5), Cornell 59-166-9 (S-12), Zucchini (S-13), and Kentucky Field (S-19). A reaction medium of 2×10^{-3} M o-tolidine and 5×10^{-3} N hydrogen peroxide in a sodium acetate buffer, pH 5.0, was used for peroxidase detection.

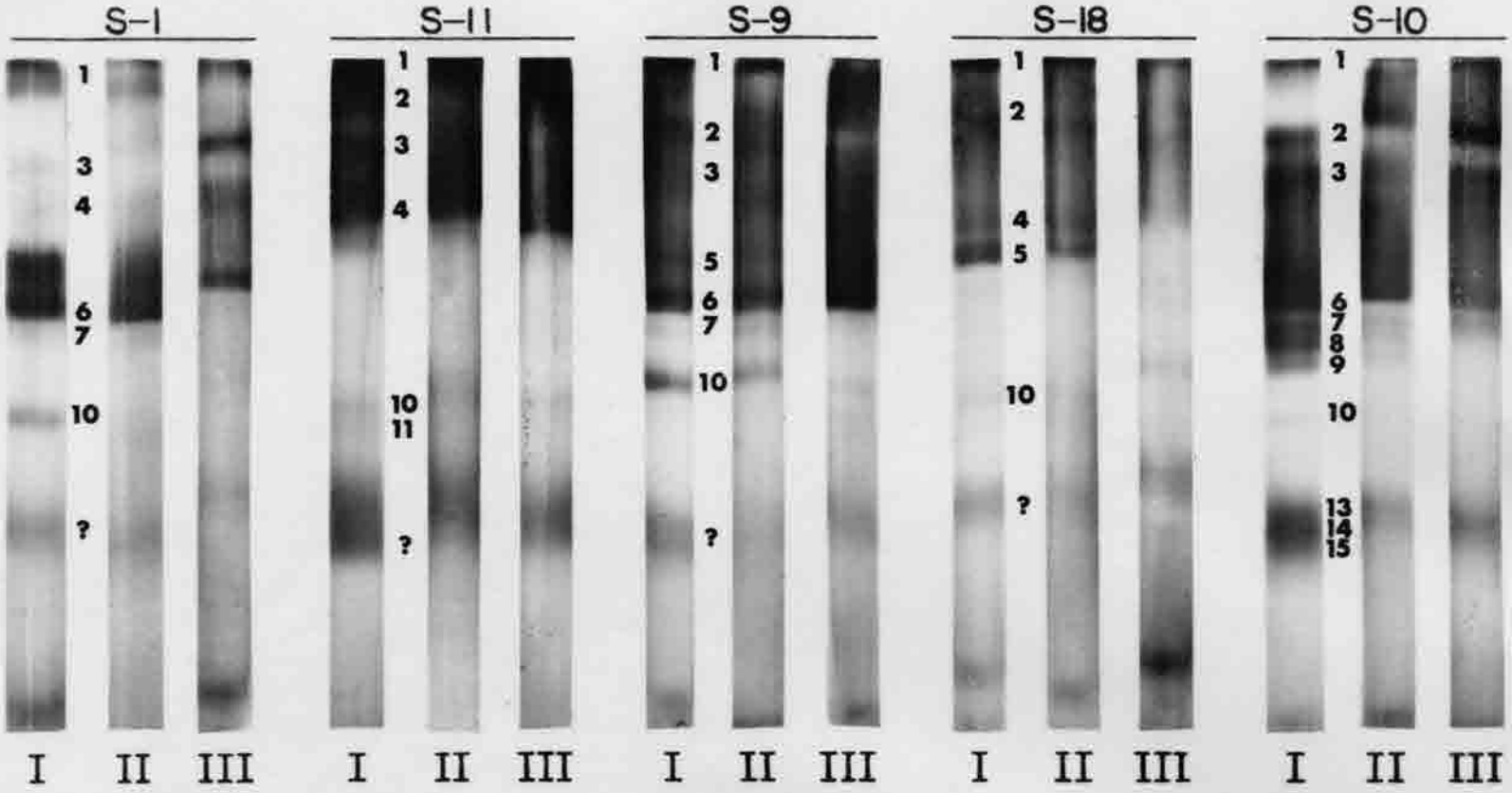


Figure 30. A comparison of the anodic isoperoxidases of the control (I), GA-treated (II), and B-995-treated (III) squash lines—Cornell 51-26-7 (S-1), Lagenaria (S-11), Blue Hubbard (S-9), Japanese Pie (S-18), and Connecticut Field (S-10). A reaction medium of 2 x 10^{-3} M o-tolidine and 5 x 10^{-3} N hydrogen peroxide in a sodium acetate buffer, pH 5.0, was used for peroxidase detection.

The effects of the GA and the B-995 treatments on the cathodic isoperoxidase patterns are shown in Figures 31 and 32. Extremely uniform patterns were characteristic among the three treatments. The only readily noticeable difference was the absence of C_8 in the GA and B-995 treatments of line S-9 (Blue Hubbard). It also appeared that C_8 was present in the control and absent in the GA and B-995 treatments of Connecticut Field (S-10).

McCune (1961) analyzed six electrophoretically separable peroxidases in the leaf-sheaths of four corn dwarfs and their normal counterparts. The activities of the six isozymes differed between the dwarf and normal segregates. Gibberellic acid stimulated growth in three of the dwarfs, and reversed the effect of dwarfism on the isoperoxidase activities. Although gibberellin more than doubled the growth of the squash lines, the gibberellin treatment had no striking effects on the isoperoxidase patterns. Some differences were observed, and can possibly be ascribed to slight changes in the developmental stages of plant growth due to the GA treatment. The isogenic bush and vine squash lines, Cornell 60-4 and Cornell 51-26-7, responded almost identically in terms of their peroxidase patterns to the different treatments. This, together with the lack of consistent peroxidase differences among the non-isogenic bush and vine squash lines, is incongruent with the hypothesis that peroxidases are directly involved in the dwarfing mechanism. The presence of high peroxidase activity in A_{10} of Cornell Bush (S-12) warrants further investigation.

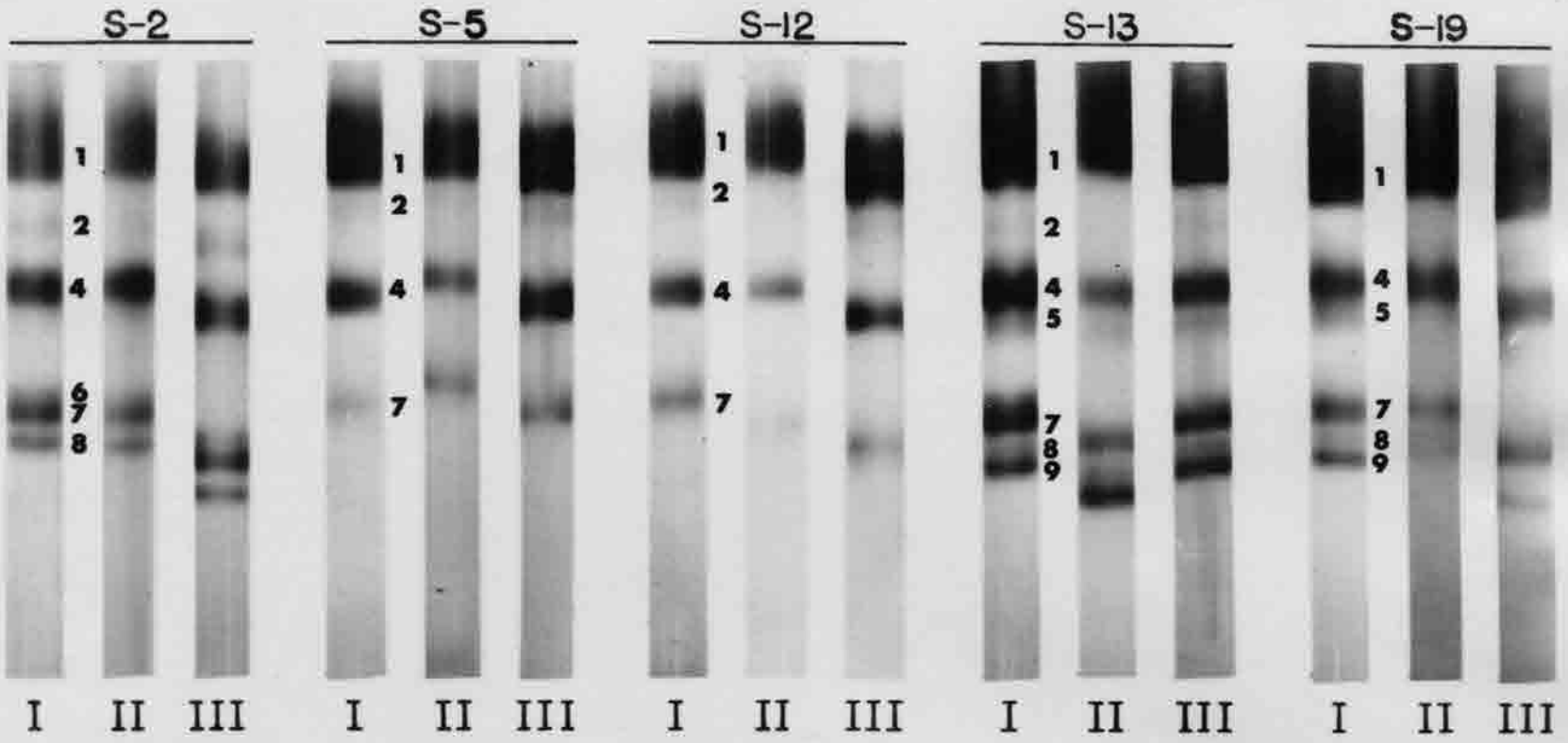


Figure 31. A comparison of the cathodic isoperoxidases of the control (I), GA-treated (II), and B-995-treated (III) squash lines—Cornell 60-4 (S-2), Minnesota 503.58 (S-5), Cornell 59-166-9 (S-12), Zucchini (S-13), and Kentucky Field (S-19). A reaction medium of 2×10^{-3} M o-tolidine and 5×10^{-3} N hydrogen peroxide in a sodium acetate buffer, pH 5.0, was used for peroxidase detection.

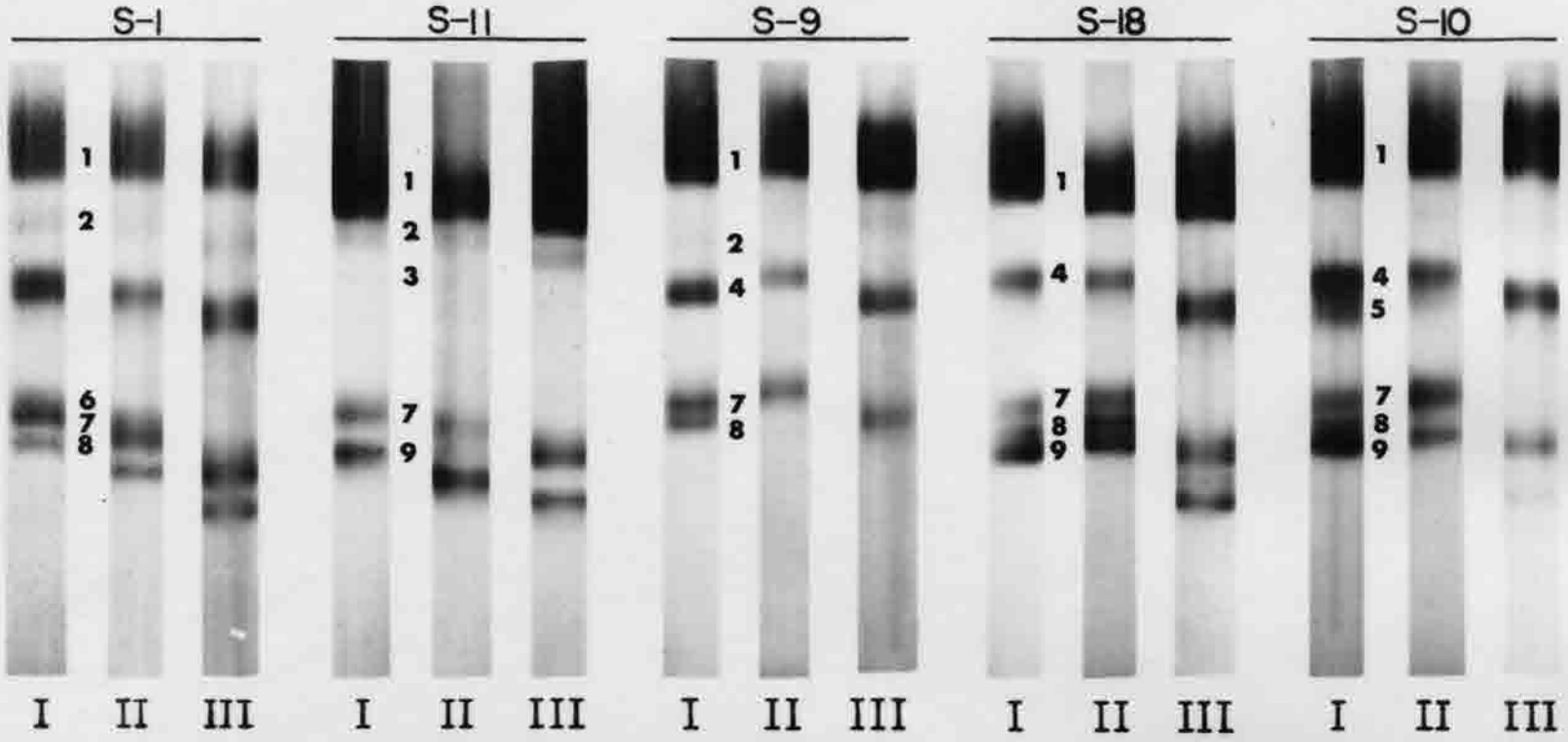


Figure 32. A comparison of the cathodic isoperoxidases of the control (I), GA-treated (II), and B-995-treated (III) squash lines—Cornell 51-26-7 (S-1), Lagenaria (S-11), Blue Hubbard (S-9), Japanese Pie (S-18), and Connecticut Field (S-10). A reaction medium of 2×10^{-3} M o-tolidine and 5×10^{-3} N hydrogen peroxide in a sodium acetate buffer, pH 5.0, was used for peroxidase detection.

Watermelon. The watermelon lines provided excellent material for examining the relationship of peroxidase to genetic dwarfing. Two of the lines were probably isogenic (Bush and Vine Desert King), and one of the bush lines was highly responsive to GA, (Purdue Dwarf) while the other bush line (Bush Desert King) was unresponsive.

In comparing the peroxidase patterns among the lines practically no differences were evident (Figures 33 and 34). Isoperoxidases A_{10} and A_{11} appeared slightly darker in the bush as compared to the vine lines in the GA treatment (Figure 33). There were no qualitative differences between the bush and vine lines.

There were some noticeable differences in peroxidase patterns between the controls and the GA-treated watermelon lines. Peroxidases A_4, A_5, and probably A_6 were detected in the GA-treated lines, but were either absent or barely discernible in the non-treated lines.

The cathodic peroxidases were similar among the treatments. Although the peroxidases in the GA-treated lines appear darker than the other treatments (Figure 34), the wide peroxidase bands in this treatment indicate that the gels may have been left too long in the peroxidase reaction medium. Peroxidase C_2 was faint and barely detectable in the GA-treated lines, but was quite evident in the controls and the B-995 treated lines. Furthermore, C_3 appeared to increase concomitantly with the decrease of C_2 in the GA-treated lines. Further investigations are needed to confirm the above results.

Although the growth responses of Bush Desert King and Purdue Dwarf to gibberellin were different, their peroxidase patterns were the same. The peroxidase patterns for all of the watermelon lines

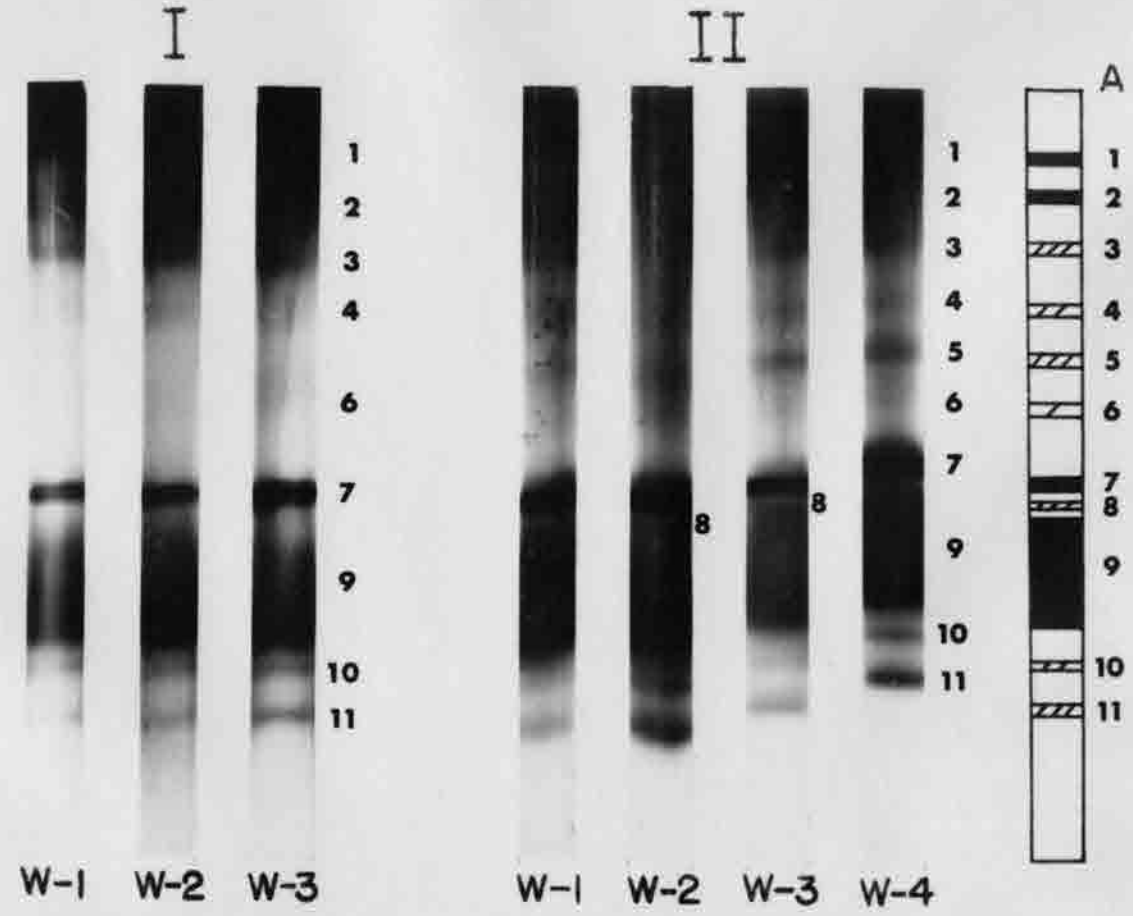


Figure 33. The anodic isoperoxidases of the control (I) and GA-treated (II) watermelon lines—Vine Desert King (W-1), Bush Desert King (W-2), Charleston Grey (W-3), and Purdue Dwarf (W-4). A reaction medium of 2 x 10^{-3} M o-tolidine and 5 x 10^{-3} N hydrogen peroxide in a sodium acetate buffer, pH 5.0, was used for peroxidase detection.

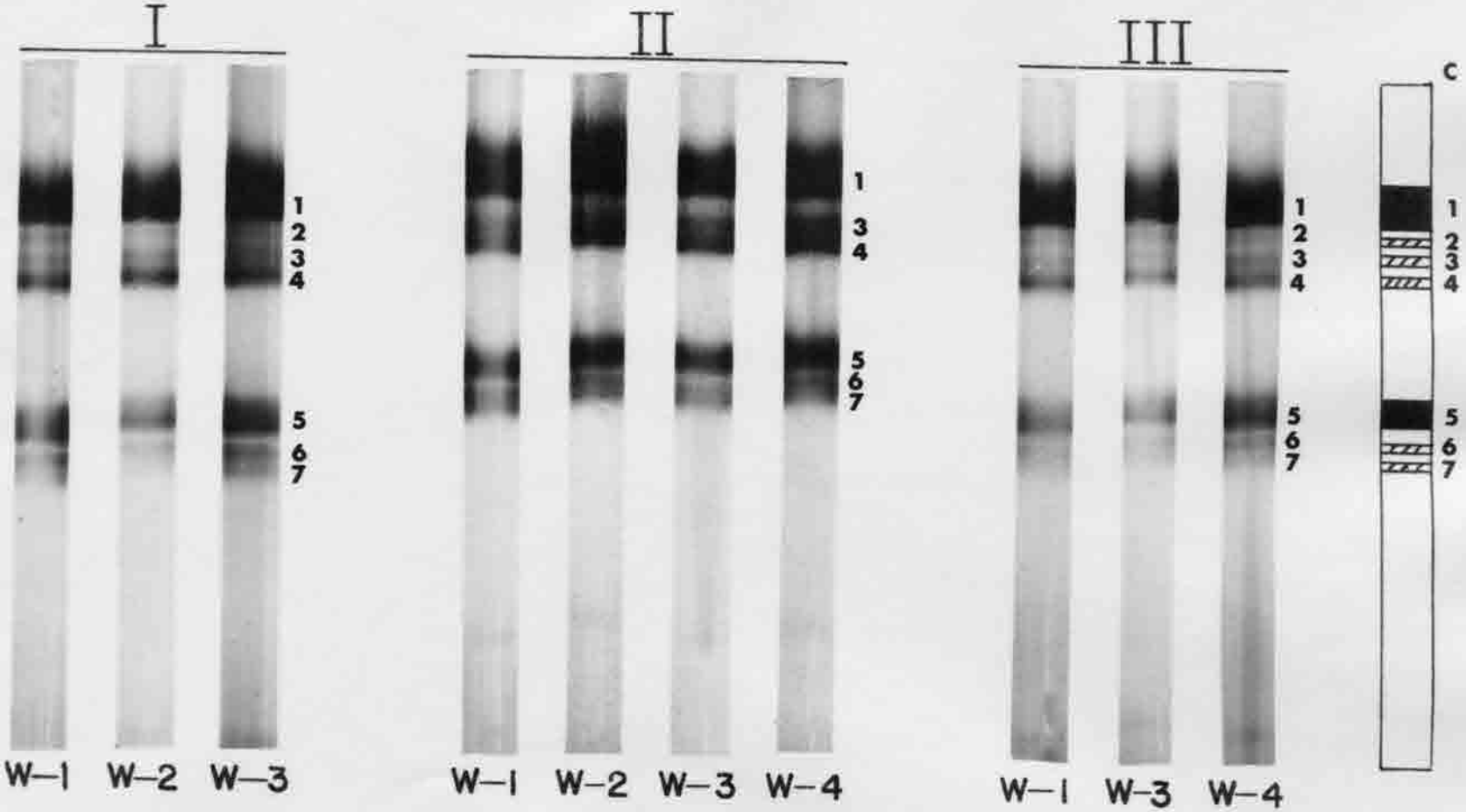


Figure 34. The cathodic isoperoxidase of the control (I), GA-treated (II), and B-995-treated (III) watermelon lines—Vine Desert King (W-1), Bush Desert King (W-2), Charleston Grey (W-3), and Purdue Dwarf (W-4). A reaction medium of 2 x 10^{-3} M o-tolidine and 5 x 10^{-3} N hydrogen peroxide in a sodium acetate buffer, pH 5.0, was used for peroxidase detection.

were nearly identical for each of the treatments. This is strong evidence that the watermelon isoperoxidases are not involved in the dwarfing mechanism.

Muskmelon. The bush muskmelon lines were originally selected for this investigation because of their brittle and twisted stems which suggested that they might fit the "precocious lignification" hypothesis of inhibition of stem elongation. The histochemical study of bush and vine muskmelon lines did not support this view. The "IAA-inactivation" hypothesis of dwarfism still remained a possibility. A study of the muskmelon isoperoxidases and their relationship to the isoperoxidases of squash and watermelon was alone a worthwhile project.

The analysis of the muskmelon peroxidases was rather incomplete, including only one replication of the control or non-treated lines and two replications of the GA-treated lines. The results, as illustrated in Figures 35 and 36, do show some interesting differences between the bush and vine lines in the two treatments. Most of the anodic bands in the control treatment were darker in the vine (M-6 and M-7) than in the bush lines (M-1, M-3, and M-5) (Figure 35). This was especially true for the slow migrating anodic peroxidases. There were also some differences in peroxidase patterns between the two vine lines, Rocky Ford (M-6) and Texas No. 1 (M-7). There were some distinct qualitative differences in the cathodic peroxidase patterns of the bush and vine lines (Figure 36). Isoperoxidases C_5 and C_6 were absent in all three of the bush lines (#10, #30, and #50), but were present in Rocky Ford and Texas No. 1.

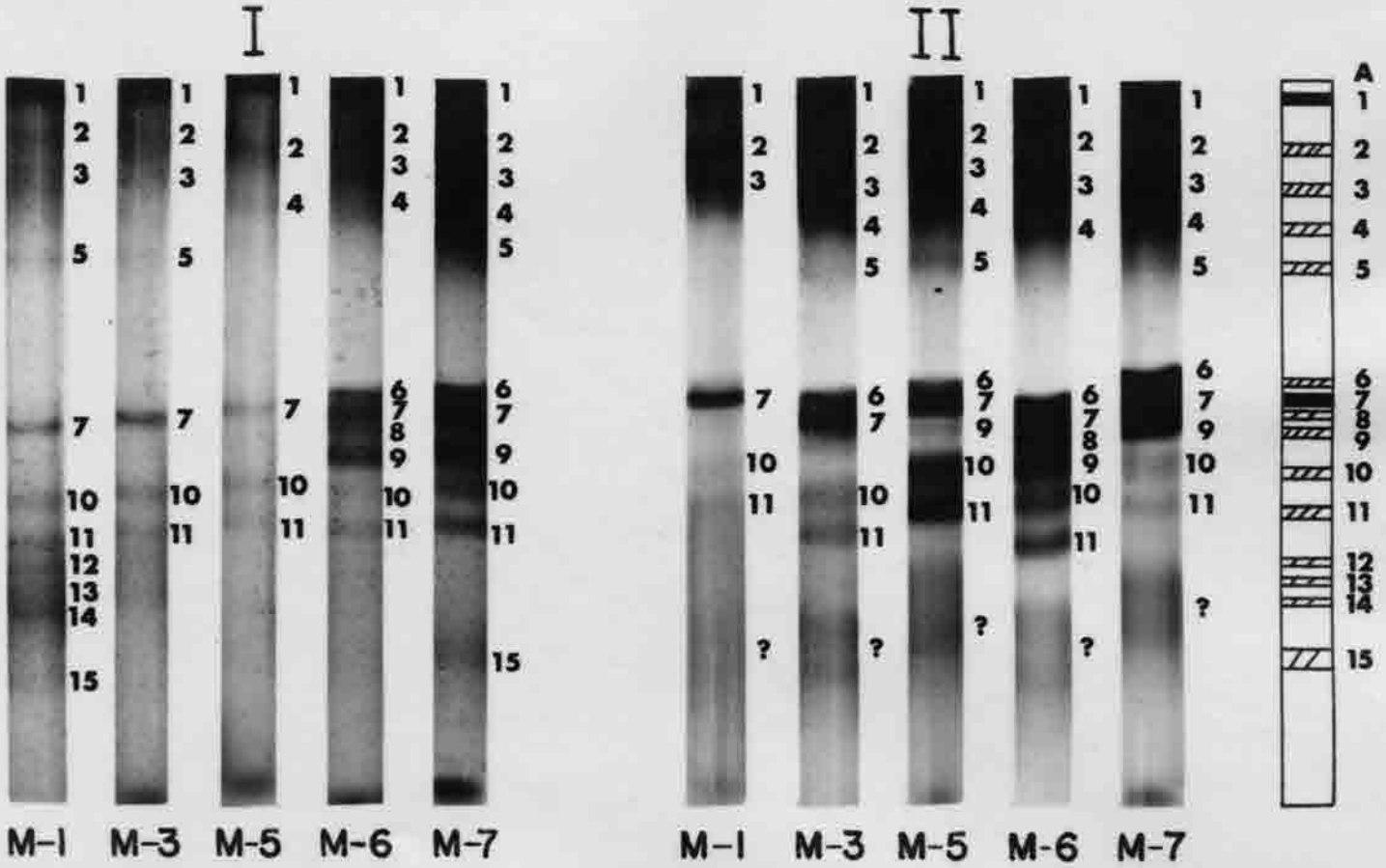


Figure 35. The anodic isoperoxidases of the control (I) and GA-treated (II) muskmelon lines—#10 (M-1), #30 (M-3), #50 (M-5), Rocky Ford (M-6), and Texas No. 1 (M-7). A reaction medium of 2 x 10^{-3} M o-tolidine and 5 x 10^{-3} N hydrogen peroxide in a sodium acetate buffer, pH 5.0, was used for peroxidase detection.

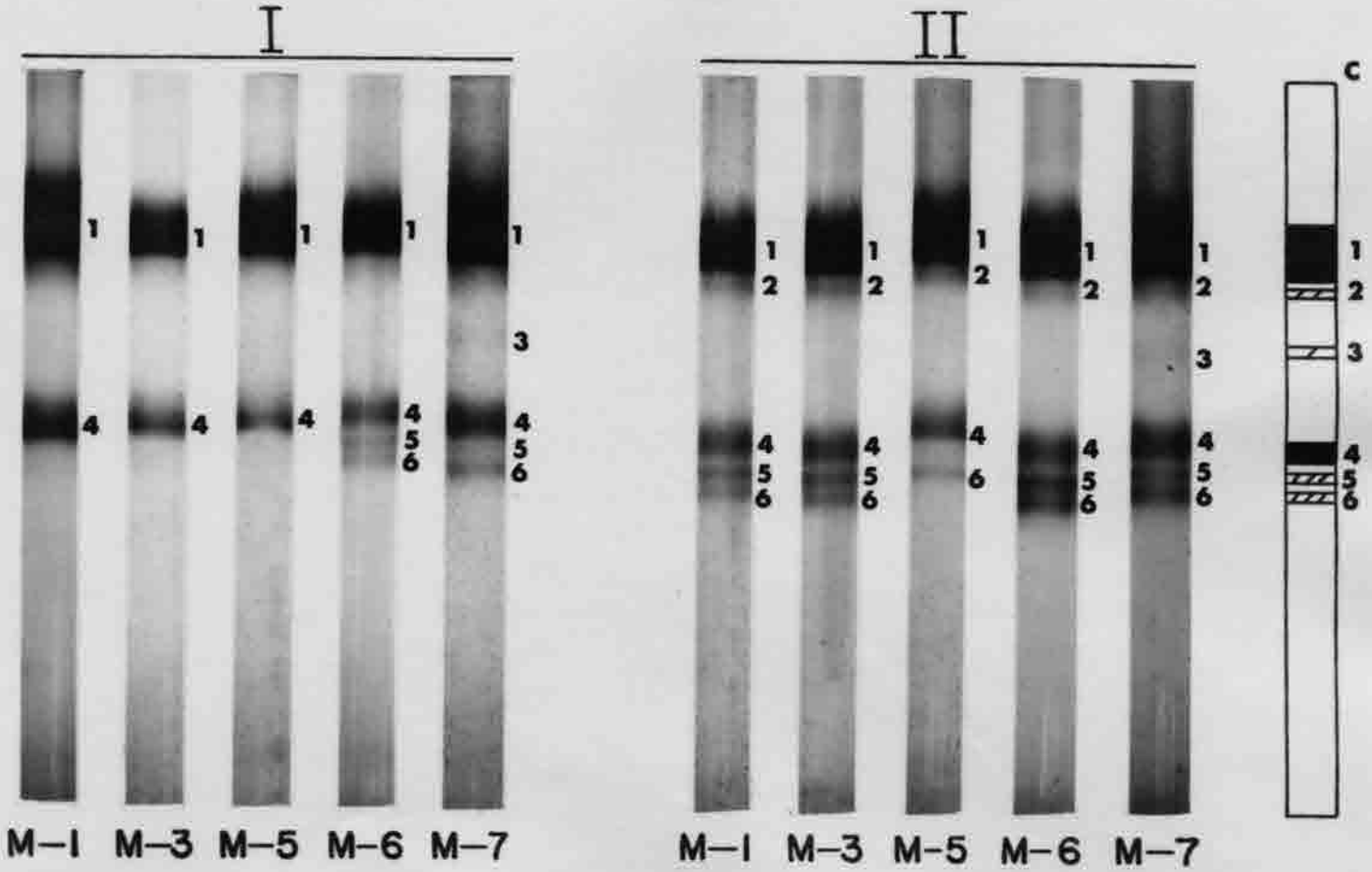


Figure 36. The cathodic isoperoxidases of the control (I) and GA-treated (II) muskmelon lines—#10 (M-1), #30 (M-3), #50 (M-5), Rocky Ford (M-6), and Texas No. 1 (M-7). A reaction medium of 2×10^{-3} M o-tolidine and 5×10^{-3} N hydrogen peroxide in a sodium acetate buffer, pH 5.0, was used for peroxidase detection.

The gibberellin treatment resulted in an apparent increase in activity of the anodic peroxidases in the muskmelon lines (Figure 35). This was particularly evident in the bush lines, and as such, the dissimilarities between the bush and vine lines were reduced. Apropos of the cathodic runs, the GA treatment resulted in the appearance of peroxidases C_5 and C_6 in the bush muskmelon lines (Figure 36). These two bands were also more intense in the vine lines. The absence or presence of C_5 and C_6 in the bush lines was not absolute. If the gels were allowed to remain in the peroxidase substrate solutions for longer periods of time, these two bands became faintly visible. In addition to the above mentioned differences, it appeared that the GA treatment increased the intensity of band C_2 in all of the lines.

The results support neither a hypothesis of constitutive peroxidase synthesis in dwarfs, nor the hypothesis that dwarfs contain higher peroxidase levels than normal plants. On the contrary, the vine muskmelon lines seemed to have greater peroxidase activity than the bush lines in many of the anodic and cathodic bands. The growth of the GA-treated bush muskmelon lines equalled that of the non-treated vine lines, and the GA treatment also appeared to alleviate some of the qualitative isoperoxidase differences between the bush and vine lines. There was not always a direct correspondence between the amount of stem growth and the intensity of a particular peroxidase band.

It should be kept in mind that visual observation of the peroxidase bands is not very precise; it is extremely difficult to detect

peroxidase differences in the intensely staining bands since the transmittance of light through such bands reaches zero rather quickly. In bands of lighter intensity only pronounced differences could be observed visually. This probably accounts for the lack of consistent correlations between the quantitative measurements and the intensities of the isoperoxidase patterns among the cucurbit lines. Differences in isoperoxidase patterns which seem insignificant or are not detectable by visual observation, may be of quantitative importance in the plant. Moreover, the molecular activity of peroxidases toward *in vivo* substrates may be considerably different than that observed for *in vitro* hydrogen donors such as o-tolidine.

The Taxonomic Significance of Cucurbit Isoperoxidases

The large number of isoperoxidases detected and the variability of their patterns among the various Cucurbitaceae genera suggested that they might serve as a useful tool in taxonomy. The exact phylogenetic relationships among the *Cucurbita* species is vague (Whitaker and Davis, 1962), and thus, enzyme comparisons among the species might provide a better insight into their taxonomic relationships. The peroxidase patterns might also reveal more precise taxonomic relationships among genera in Cucurbitaceae. This family is somewhat unique in that certain genera originated in the old world while others originated in the new world. *Cucurbita* species (squash) are indigenous to the Americas while *Cucumis* and *Citrullus* are apparently indigenous to Africa (Whitaker and Davis, 1962). The genus *Lagenaria* has been distributed throughout the world for several thousand years,

and its place of origin is obscure (Whitaker and Davis, 1962). There is some evidence that the early bihemispheric distribution could be explained by the drifting of gourds from tropical Africa to the coast of Brazil (Whitaker and Carter, 1954).

Isoperoxidase Differences among the Cucurbita Species

Figure 37 shows the anodic isoperoxidase patterns of the squash lines grouped according to species. The pepo lines S-19 and S-13 are very similar, but have different peroxidase patterns than the isogenic bush (S-2) and vine (S-1) lines of C. pepo. The maxima lines S-5 and S-12 differed only in band A_{10}, but both of these lines differed from the maxima line S-9 in the mobility of isoperoxidases A_6 and A_7. Line S-19, a moschata, had an anodic peroxidase pattern similar to that of the pepo lines, S-13 and S-10. The mixta line (S-18) appeared to be unique among the squash lines because it lacked peroxidase A_6.

Although the cathodic peroxidase patterns were more uniform than the anodic patterns (Figure 38), the relationships among the species were the same as for the anodic isoperoxidases. The pepo lines S-10 and S-13 were similar, but different from the isogenic pepo lines S-1 and S-2. The maxima lines S-5 and S-12 were identical, but lacked peroxidase C_8 which was present in S-9, a maxima. Again, line S-19, a moschata, was similar to the pepo lines S-10 and S-13. Line S-18, a mixta, differed from the rest of the lines in the slow migration of peroxidase C_4.

Because of the dissimilarities of the peroxidase patterns among the varieties within a species, as well as among the species, it is

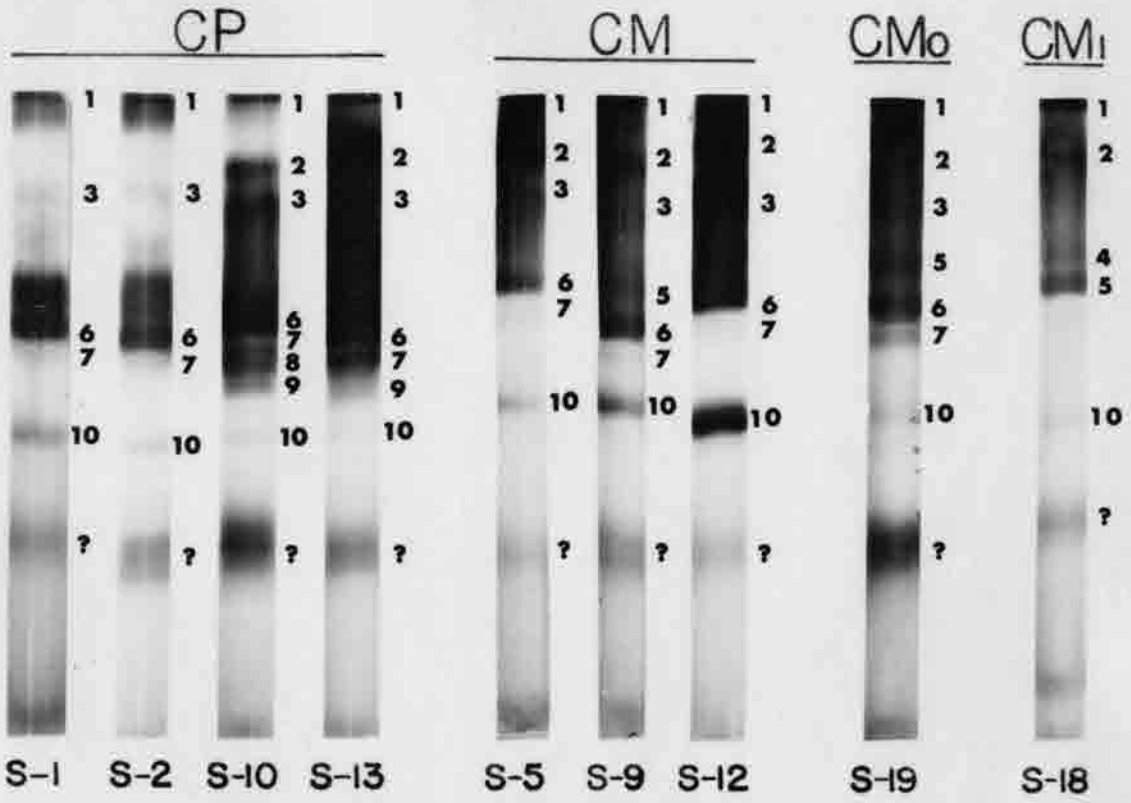


Figure 37. A comparison of the anodic isoperoxidase patterns among the Cucurbita species using polyacrylamide-gel, disc electrophoresis. A reaction medium of 2 x 10^{-3} M o-tolidine and 5 x 10^{-3} N hydrogen peroxide in a sodium acetate buffer, pH 5.0, was used for peroxidase detection. Symbols: CP = Cucurbita pepo, CM = Cucurbita maxima, CMo = Cucurbita moschata, CMi = Cucurbita mixta, S-1 = Cornell 51-26-7, S-2 = Cornell 60-4, S-10 = Connecticut Field, S-13 = Zucchini, S-5 = Minnesota 503.58, S-9 = Blue Hubbard, S-12 = Cornell 59-166-9, S-19 = Kentucky Field, and S-18 = Japanese Pie.

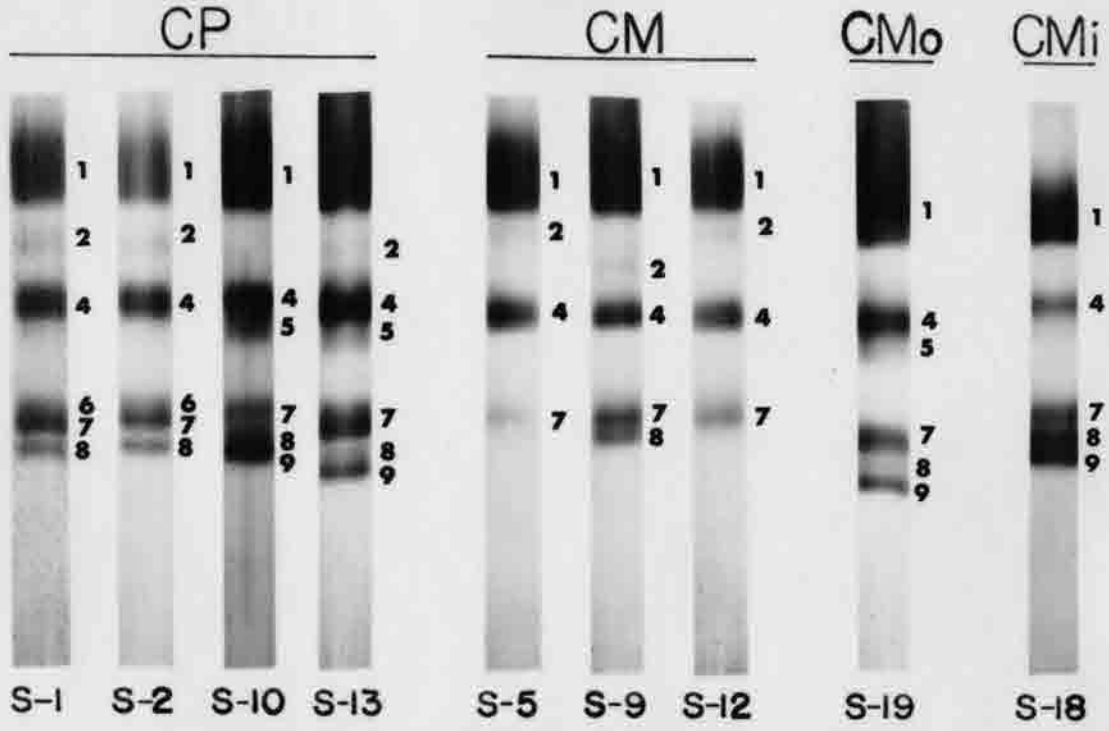


Figure 38. A comparison of the cathodic isoperoxidase patterns among the Cucurbita species using polyacrylamide-gel, disc electrophoresis. A reaction medium of 2 x 10^{-3} M o-tolidine and 5 x 10^{-3} N hydrogen peroxide in a sodium acetate buffer, pH 5.0, was used for peroxidase detection. Symbols: CP = Cucurbita pepo, CM = Cucurbita maxima, CMo = Cucurbita moschata, CMi = Cucurbita mixta, S-1 = Cornell 51-26-7, S-2 = Cornell 60-4, S-10 = Connecticut Field, S-13 = Zucchini, S-5 = Minnesota 503.58, S-9 = Blue Hubbard, S-12 = Cornell 59-166-9, S-19 = Kentucky Field, and S-18 = Japanese Pie.

difficult to generalize about any taxonomic relationships as indicated by their peroxidase patterns. It appears from this study that C. moschata has a closer affinity to C. pepo than to the other squash species, while C. mixta has diverged slightly from the other species. According to Whitaker and Davis (1962), studies of species hybridization have suggested the following relationships among the species: (1) C. maxima and C. pepo are more closely related to C. moschata than to one another; (2) C. mixta has a closer affinity to C. pepo than to either C. moschata or C. maxima; and (3) C. moschata appears to occupy a central position apropos of relationships among these four species. The peroxidase results neither confirm nor disprove the relationships suggested above. It should be kept in mind that some of the differences in peroxidase patterns among the squash lines may reflect developmental differences rather than absolute enzyme differences. The analysis of the isoperoxidases in seeds which are at a static stage of development might alleviate such a problem if it exists.

Isoperoxidase Differences Among Cucurbit Genera

Four genera, Lagenaria, Cucurbita, Cucumis, and Citrullus were representatives of Cucurbitaceae used in this comparison. Since both muskmelon (Cucumis melo) and watermelon (Citrullus vulgaris) probably originated in Africa (Whitaker and Davis, 1962), it was expected that these two genera would show more isoperoxidase similarities between each other than either would to Cucurbita (squash) which is indigenous to the Americas. Lagenaria appears to occupy a central position with respect to the cucurbit genera. There is dispute as to

whether it is indigenous to the new or the old world. Lagenaria has a chromosome number of 11 which is the same as that of Citrullus vulgaris (Whitaker, 1930), while Cucumis melo has 12 chromosomes (Whitaker and Bohn, 1950). Cucurbita differs considerably from these genera, having a chromosome number of 20 (Whitaker, 1933). Lagenaria is usually placed in the tribe Cucumerinae along with Cucumis and Citrullus, while Cucurbita is included under the tribe Cucurbitineae (Whitaker and Davis, 1962).

The electrophoretic comparisons of the four genera are shown in Figure 39. Line S-19, a moschata, was selected as a representative of the Cucurbita genus. The presence of several fast moving anodic peroxidase bands of high intensity are characteristic of Cucumis melo and Citrullus. On the other hand, most of the anodic peroxidase activity in Lagenaria and Cucurbita was concentrated in the isoperoxidases of slow mobility. The cathodic isoperoxidases were fairly uniform among the four genera, but the genera could be distinguished from one another on the basis of slight differences. The extremely dark band, C_2, in Cucurbita was unique to that genus. Again, Lagenaria appears to be more closely related to the genus Cucurbita than to either Cucumis melo or Citrullus, while the latter genera have very evident similarities in peroxidase patterns. The results are very encouraging as to the possible usefulness of isoperoxidase patterns in establishing closer taxonomic relationships among the Cucurbit genera, as well as finding close ancestors to the genus Cucurbita. From these results it appears that Lagenaria could just as well be placed into the tribe Cucurbitineae along

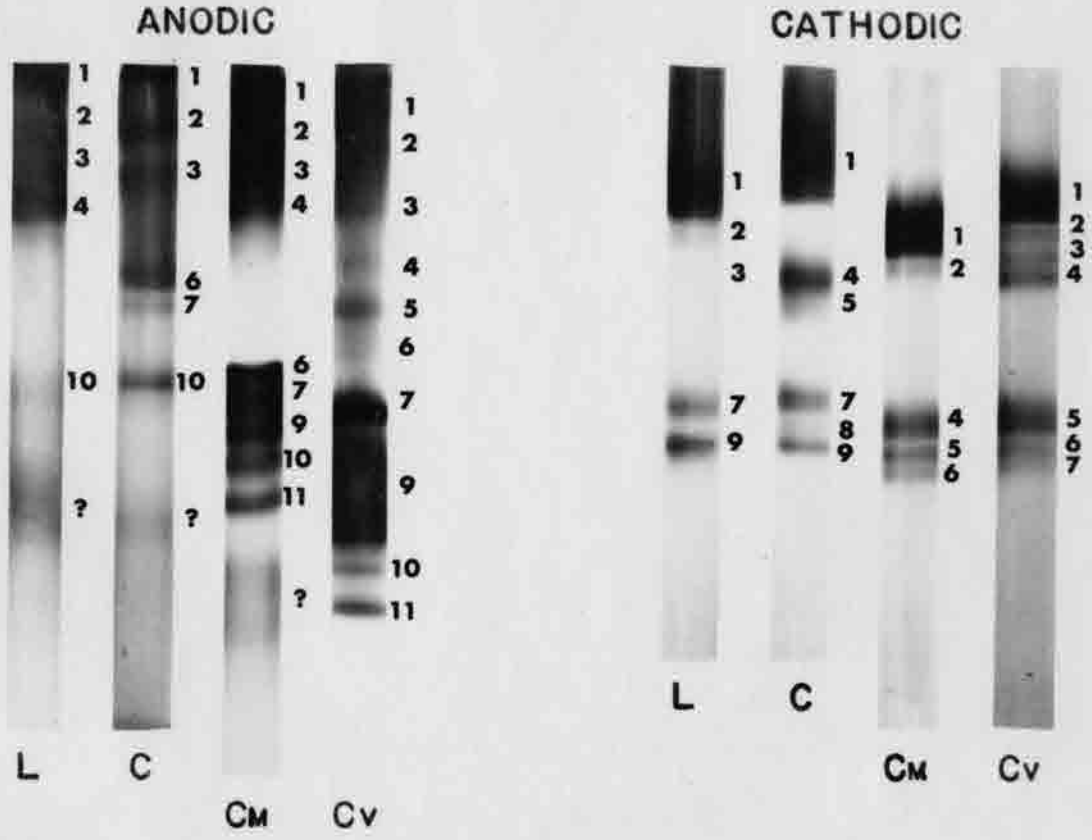


Figure 39. A comparison of the isoperoxidase patterns among the four Cucurbitaceae genera—Lagenaria (L), Cucurbita (C), Cucumis melo (Cm), and Citrullus vulgaris (Cv). A reaction medium of 2×10^{-3} M o-tolidine and 5×10^{-3} N hydrogen peroxide in a sodium acetate buffer, pH 5.0, was used for peroxidase detection.

with Cucurbita as into Cucumerinae, which includes Cucumis and Citrullus. It is possible that Cucurbita could be an allopolyploid originating from hybridization of gourd species which floated from the old to the new world several thousand years ago.

GENERAL DISCUSSION AND CONCLUSIONS

The original objectives of this research were formulated with the idea that changes in peroxidase levels in plants can result in dwarfing. More specifically, the experiments were designed to test the hypothesis that genetically dwarf cucurbits contain high peroxidase levels, and that this could cause dwarfing by (1) increasing IAA inactivation in plant tissue or (2) by resulting in precocious lignification in stems which, by forming a rigid cell matrix, could impede stem elongation. The precocious lignification hypothesis was considered to be a likely dwarfing mechanism in the case of the bush muskmelon lines because of their brittle stems, the brittleness possibly being associated with heavy lignification. The possible explanations for higher peroxidase levels in dwarfs as compared to non-dwarfs were: (1) gibberellin represses peroxidase synthesis, such that plants low in endogenous gibberellin have high peroxidase activity, or (2) a mutation of a regulator gene controlling peroxidase synthesis could occur, and this could result in constitutive peroxidase synthesis.

The first approach to this problem was the histochemical investigation of peroxidase and lignin distribution in stems of bush and vine squash and muskmelon lines. Peroxidase activity was strongly localized in the epidermis, the sclerenchymatous ring, and the xylem in cucurbit stems. Lignin distribution correlated quite well with peroxidase localization in the sclerenchymatous ring and the xylem. No qualitative differences in peroxidase activity and in the amount of lignification were found between the bush and vine

cucurbit lines. In the case of the bush muskmelon lines, the brittle characteristic of the stems was evident before any appreciable lignification was apparent. Thus, the results were not in accordance with the concept of peroxidase involvement in dwarfing mechanisms.

The histochemical study has further implications concerning the idea of peroxidase involvement in IAA inactivation. Namely, for peroxidase to inactivate IAA, it has to be present in tissues which contain appreciable amounts of IAA. Whereas peroxidase activity is strongest in the xylem, the sclerenchymatous ring, and the epidermis, IAA is apparently transported in the phloem and parenchyma tissue (Strafford, 1965). In order to associate high peroxidase activity with increased IAA destruction it would seem logical to examine peroxidase activity in tissues where rapid IAA inactivation might be expected to retard stem growth. It is possible that the localization or site of attachment of peroxidases within cells determines their specific function. The demonstration of IAA inactivation by peroxidases in tissue homogenates is no sound evidence that this is their function in situ.

The muskmelon isoperoxidases were tested for their specificity in catalyzing the dehydrogenation of eugenol, a compound commonly used for producing a synthetic lignin. All of the muskmelon isoperoxidases detected with guaiacol were also capable of oxidizing eugenol. After 12 hours the eugenol product stained a faint red color with phloroglucinol-HCl, indicating that a lignin-like polymer had probably been formed. Muskmelon stem sections immersed in a solution of eugenol and hydrogen peroxide for three or more hours

synthesized a lignin-like polymer in the epidermis as well as in the xylem and the sclerenchymatous ring. This indicates that peroxidase is probably not normally the limiting factor for lignification.

The quantitative determinations of total peroxidase activity in the ten squash lines showed considerable variability among the lines, but no correlation between the rates of stem elongation and the levels of peroxidase activity was found. As pointed out above, the quantitative data on total peroxidase activity in plant extracts do not necessarily provide a valid means of demonstrating the involvement of peroxidase in a dwarfing mechanism. The results of peroxidase comparisons among the squash lines were valuable in that they demonstrated that varietal differences in peroxidase activity can be relatively great. As such, reported peroxidase differences between non-isogenic or non-segregating bush and vine lines (Kamerbeek, 1956; Evans and Alldridge, 1965) may be varietal differences rather than differences related to the particular phenotype.

It seemed likely that not all of the isoperoxidases in plant tissue could be involved in a dwarfing mechanism. For this reason the electrophoretic analysis of the peroxidases in bush and vine cucurbit lines was expected to provide the most convincing evidence for or against a dwarfing mechanism involving peroxidase. The anodic isoperoxidase patterns varied considerably among the Cucurbita lines, but there were no apparent consistent peroxidase patterns characteristic of the bush lines. The cathodic squash isoperoxidases were more uniform than the anodic peroxidases among the squash lines, but there were no consistent differences between the vine and the bush

lines. Both the cathodic and the anodic peroxidase patterns were practically identical among the bush and the vine watermelon lines. This was not true for the muskmelon lines. The intensity of several of the anodic peroxidase bands was greater in the vine than in the bush muskmelon lines. Two of the fast moving cathodic isoperoxidases present in the two vine lines were absent in the three bush muskmelon lines. Gibberellin treatment stimulated growth in both the bush and the vine muskmelon lines, and alleviated some of the isoperoxidase differences between them. Since GA can apparently accelerate stem maturation (Brian, Hemming, and Lowe, 1958), the peroxidase differences between the bush and the vine muskmelon lines might have been due to slight differences in the rate of stem differentiation.

From the above discussion it is obvious that the results of the squash, watermelon, and muskmelon electrophoretic experiments were incongruent with the hypotheses that peroxidases could be involved in dwarfing mechanisms through IAA inactivation or precocious lignification. Since the electrophoretic comparisons were only qualitative, it is possible that some important differences might not have been detected. This appears unlikely because of the large number of cucurbit lines investigated which failed to exhibit any peroxidase patterns unique to the bush phenotype.

The failure to detect consistent differences in total peroxidase activity or in peroxidase patterns between bush and vine cucurbit lines does not detract from the hypothesis of an isoperoxidase or isoperoxidases acting as IAA oxidases <u>in vivo</u>. As discussed in the literature review (pages 33, 34, 35, and 36), there is little direct

evidence that low levels of endogenous IAA are a major cause of dwarfism. The best argument against IAA involvement in dwarfism is the failure of genetic dwarfs to be restored to the normal phenotype with exogenous applications of IAA (Brian and Hemming, 1955; Plummer and Tomes, 1957; Denna, 1962). On the other hand, there are many genetic dwarfs restored to the normal phenotype with gibberellin treatment (Phinney, 1956a; Gorter, 1961; Denna, 1963). The question arises as to why genetic dwarfs are not found which are the result of low levels of endogenous auxin, assuming the endogenous auxin is IAA. Auxin is known to exert a diversity of effects on plant growth and differentiation (Leopold, 1965). It is possible that levels of endogenous auxin low enough to cause dwarfism in plants would result in such severe developmental abnormalities, that the affected seedlings would not survive or would be rogued out of experimental populations.

The investigation of plant isoperoxidases offers several practical and basic applications. As mentioned previously, studies of isoperoxidase patterns may be useful for assessing phylogenetic relationships among genera and possibly among species of plants. This is particularly true for cucurbits because the relationships among the genera and species of this family are obscure. It is suggested that analysis of isoperoxidase patterns in seeds may prove to be more valuable for taxonomic purposes than analyzing other developing plant organs because seeds represent a rather uniform stage of development.

Another possible, but quite speculative, application of isoperoxidase analysis is for the selection of parents for producing highly heterotic hybrids. Hutchins and Croston (1941) compared the productivity of F_1 hybrids of cultivars of C. maxima with their parents. Only the F_1's produced from parents which differed considerably in their visual characters were significantly higher yielding than the higher yielding parent. It seems to be true for most crops that crosses between the phenotypically most dissimilar parents result in the most productive hybrids (Allard, 1960). It is possible that parents used for producing hybrids could be selected on the bases of dissimilarities in their isoperoxidase patterns. This method would assume that variations in enzyme patterns would reflect genetic differences more accurately than the observation of morphological differences. Peroxidases are ideal enzymes for such comparisons because they are easy to detect, to separate electrophoretically, and it appears that there are a large number of them in plants.

A more basic problem for the future is the further isolation and purification of the cucurbit peroxidases. The electrophoretic separation of peroxidases, and their detection by use of hydrogen donors which form colored products, is not absolute proof that they are all different and true peroxidases. Under certain conditions catalase and several haematin compounds can act peroxidatively (Saunders et al., 1964). It is also possible that some of the peroxidases were adsorbed to other molecules, and this could have resulted in the appearance of more peroxidase bands than the actual

number of isoperoxidases present. Such adsorption phenomena might have resulted in the diffuse and often broad anodic bands. The peroxidases separated electrophoretically at pH 4.3 did not seem to fit this picture. The cathodic isoperoxidases were distinct and exhibited high molecular activity as indicated by the rapid formation of the colored products. It seems unlikely that the peroxidases separated at pH 4.3 were artifacts. As mentioned previously, it is difficult to estimate the total number of isoperoxidases in the cucurbit species because the electrophoretic runs were made at widely different pH values. Some of the peroxidases with isoelectric points at near-neutral pH values could have migrated in both the cathodic and the anodic runs. The presence of peroxidase activity in the sample gels after the electrophoretic runs indicates that some of the isoperoxidases were not migrating in both directions. The anodic isoperoxidase patterns of the squash lines (Figure 26) and the GA-treated muskmelon lines (Figure 35) differed considerably among the lines, while the cathodic isoperoxidase patterns in these lines (Figures 27 and 36) varied only slightly. This argues strongly that the majority of the isoperoxidases did not migrate at both pH values. This conclusion agrees with the results of Shannon, Kay, and Lew (1966). The seven isoperoxidases which they isolated from horseradish roots were either strongly basic (4) or strongly acidic (3). Using the same pH systems for disc electrophoresis as were employed in this study, none of the horseradish isoperoxidases moved during both the anodic and the cathodic runs. Since most of the cucurbits exhibited between 7 to 12 anodic peroxidases and between

5 to 7 cathodic peroxidases, it can be estimated with a fair amount of certainty that the cucurbits studied contained at least 10 isoperoxidases.

The most perplexing problems concerning peroxidases are the conclusive demonstration of their functions in plants, and determining why so many of these enzymes are present in plant tissues. The broad specificity of the peroxidases hinders the determination of their function or functions. The results (Figure 18) indicated that all of the muskmelon peroxidases probably have the potential for catalyzing the final enzymatic step in lignification. The work of others (McCune, 1961; Morita et al., 1962; Shin and Kakamura, 1962) indicates that all of the isoperoxidases so far tested can catalyze the oxidation of IAA. No experiments were carried out with respect to the ability of the cucurbit peroxidases to oxidize IAA. It was noted that 5×10^{-3} M IAA severely inhibited the ability of the muskmelon isoperoxidases to oxidase o-tolidine, but it was not determined if this was a competitive type of inhibition.

The demonstration of peroxidase functions <u>in vivo</u> will probably require the correlation between metabolic or morphologically observable defects in plants and the absence of particular isoperoxidases due to mutations. The discovery of such defects and of peroxidase changes is another problem. It was postulated that dwarfism or the bush phenotype in some of the cucurbits might be correlated with or caused by the presence of increased amounts of certain isoperoxidases, but such did not appear to be the case.

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APPENDIX

Appendix A. Peroxidase activity of squash stems harvested at the five internode stage of growth. Peroxidase activity is expressed as change in optical density (470 mu) per mg of protein per minute using guaiacol as the hydrogen donor

Line number	Line or variety	Replications 1	2
S-5	Minnesota 503.58	7.24 (23)*	5.05 (25)
S-7	Butternut 77	6.44 (23)	7.81 (25)
S-11	Lagenaria	2.72 (23)	2.65 (23)
S-12	Cornell 59-166-9	2.37 (20)	2.28 (20)
S-13	Zucchini	1.47 (19)	6.87 (21)
S-14	Early White Bush Scallop	2.78 (20)	3.94 (24)
S-15	Warren Turbon	1.72 (20)	3.26 (22)
S-16	Caserta	2.09 (18)	3.14 (20)
S-17	Dickinson	4.22 (20)	10.54 (24)
S-18	Japanese Pie	1.78 (18)	2.43 (20)
S-19	Kentucky Field	5.70 (22)	7.20 (24)
S-20	Striped Cushaw	2.16 (18)	1.50 (21)
S-22	Mammoth Table Queen	4.97 (21)	4.51 (22)
S-23	Burpee's Bush Table Queen	5.50 (22)	9.25 (24)
S-24	Large Cheese	5.44 (24)	5.26 (25)

*The numbers in parentheses indicate the age of the squash plants from seeding to when they were harvested.

Appendix B. Peroxidase activity of the control, B-995-treated, and GA-treated squash lines expressed as change in optical density (635 mu) per 100 ug protein per minute, using o-tolidine as the hydrogen donor.

Line Number	Line or variety	Treatments					
		Control		GA Treatment		B-995 Treatment	
S-1	Cornell 51-26-7	10.87	11.32	9.38	7.48	9.29	9.35
S-2	Cornell 60-4	19.74	17.76	8.66	9.20	12.16	10.47
S-5	Minnesota 503.58	12.32	15.33	8.18	10.82	9.43	9.66
S-9	Blue Hubbard	16.67	23.80	13.41	10.49	14.39	--
S-10	Connecticut Field	32.30	22.30	19.19	15.97	16.62	19.31
S-11	Lagenaria	21.02	24.68	13.10	16.56	12.73	15.18
S-12	Cornell 59-166-9	13.76	17.26	7.12	7.90	9.31	9.67
S-13	Zucchini	20.05	30.63	23.27	15.30	17.44	18.05
S-18	Japanese Pie	15.52	16.24	10.89	17.55	12.10	11.53
S-19	Kentucky Field	18.04	15.02	12.33	17.18	--	13.63

Appendix C. Analysis of variance for data on peroxidase activity of the control, GA-treated, and B-995 treated squash lines

Source of Variance	Degrees of freedom	Sums of squares	Mean squares	F value
Corrected total	59	1,685.04		
Varieties	9	848.07	92.23	8.77**
Treatments	2	468.31	234.16	22.26**
Replications	1	7.43	7.43	
V x T	18	117.67	6.54	
V x R	9	50.55	5.62	
R x T	2	3.71	1.85	
Error	18	189.30	10.52	

$F_{1, 18}$ (.05) = 4.41
(.01) = 8.28

$F_{2, 18}$ (.05) = 3.55
(.01) = 6.01

$F_{9, 18}$ (.05) = 2.46
(.01) = 3.60

$F_{18, 18}$ (.05) = 2.19
(.01) = 3.08

** Highly significant.

Appendix D. The New Multiple Range Test for difference in peroxidase activity in the stems of the ten squash lines

$S_{\bar{x}}$ = 1.32		P	2	3	4	5	6	7	8	9	10
	SSR	.05	2.97	3.12	3.21	3.27	3.32	3.35	3.37	3.39	3.41
	LSR	.05	3.92	4.12	4.24	4.32	4.38	4.42	4.45	4.47	4.50

P		2	3	4	5	6	7	8	9	10
Squash lines	S-1	S-12	S-5	S-2	S-18	S-19	S-9	S-11	S-13	S-10
Means of lines	9.62	10.84	10.96	13.00	13.99	14.97	15.52	17.21	20.79	20.94
		1.22	1.34	3.38	4.35	5.35	5.90	7.59	11.17	11.32
Difference			0.12	2.16	3.13	4.13	4.68	6.37	9.95	10.10
between				2.04	3.01	4.01	4.56	6.25	9.83	9.98
means					.97	1.97	2.52	4.21	7.79	7.94
among						1.00	1.55	3.24	6.82	6.97
squash							.55	2.24	5.82	5.97
lines								1.69	5.27	5.42
									3.58	3.73
										0.15

Any two means not underscored by the same line are significantly different.

Appendix E. Analysis of variance for data on peroxidase activity of the bush and vine squash lines

Source of variance	Degrees of freedom	Sums of squares	Mean squares	F values
Corrected total	5	5.8461		
Lines	1	0.0090	0.009	0.022
Replications	2	5.0250	2.512	6.187
Error	2	0.8121	0.406	

$F_{1, 2}$ (.01) = 98.50
(.05) = 18.51

$F_{2, 2}$ (.01) = 99.00
(.05) = 19.00

Appendix F. Analysis of variance for peroxidase activity of Vine Desert King and Charleston Grey in relation to the control and the B-995 and GA treatments

Source of variance	Degrees of freedom	Sums of squares	Mean squares	F values
Corrected total	11	1.990		
Varieties	1	0.067	0.067	0.34
Treatments	2	0.827	0.414	2.09
Replications	1	0.044	0.044	0.22
V x T	2	0.419	0.210	1.06
V x R	1	0.188	0.188	0.94
R x T	2	0.049	0.025	0.13
Error	2	0.396	0.198	

$F_{1, 2}$ (.01) = 98.50
(.05) = 18.51

$F_{2, 2}$ (.01) = 99.00
(.05) = 19.00