STUDIES IN FRUIT GROWTH AND IN VERNALIZATION

Thesis by

Leonard Leroy Jansen

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1953

ACKNOWLEDGMENTS

The writer wishes to take this opportunity to express his thanks and gratitude to all who have helped make this work possible. To Dr. James Bonner goes special thanks for initially stimulating interest in the *in vitro* method and in the culture of fruits particularly. His many constructive criticisms and suggestions have been invaluable throughout the course of all of these investigations. The writer is also deeply indebted to Dr. Anton Lang for expert guidance through the course of the vernalization studies and also for his critical reading of all parts of this thesis during their preparation.

Without the enlightening discussions of many phases of plant growth and development held with Dr. F. W. Went, many significant points might have been overlooked, particularly the significance of spiral reversals. All work in the Phytotron has been greatly enhanced through his help. Many others, also have aided greatly during other phases of the investigations. Drs. G. Laties, A. W. Galston, R. Bandurski, R. McColloch, and B. Axelrod, in particular, have been ever available for stimulating discussions.

Dr. Guy Ch. Camus, now returned to the Sorbonne, and Dr. J. H. M. Henderson, of the Tuskegee Institute, offered many valuable suggestions in connection with the *in vitro* studies. Dr. Camus also was a source of constant encouragement during the phases of the research when the lack of results due to smog damage was becoming very downheartening. Miss Mary E. Durrell has also been extremely helpful in all matters pertaining to tissue culture and has assisted in many ways in the technical aspects of the work.

There is no way adequate to express the most sincerely felt thanks which are due to all of those persons who have made the experiments possible through the excellent care which has been given to the plants used as the source of experimental material. Messrs. C. Newman, and J. Kelleher at the Orlando greenhouse, and P. Keyes, J. Cunningham, P. Thompson, and others of the Phytotron staff are due special thanks for their technical assistance and also for many friendly chats which have "made life worth living."

The writer is also deeply indebted to Mr. and Mrs. Arthur McCallum, who have made possible the continuance of this work during two summers through the financial support received from the McCallum Summer Fellowships in Biology.

Deepest and most heartfelt gratitude must finally go to my wife, Patricia Jansen. Her constant encouragement, prodding, and always generous understanding, as well as her able assistance in typing this thesis and her ever-willingness to assist wherever possible, have greatly facilitated the completion of this work.

ABSTRACT

Growth and development of flowers and fruits of red currant tomato (Lycopersicon pimpinellifolium) have been studied both on the intact plant and with the excised flower cultivated in vitro. A new interpretation of development of the inflorescence has been presented. Two types of growth response of ovaries of pollinated flowers have been identified both on the plant and in culture. In one type growth is linear and in the other it is exponential. The latter type, on the plant, produces seeded fruits, and size of fruits is correlated with number of seeds. Development on the plant is apparently optimal at a night temperature of 17° C. Smog is detrimental to development.

Growth of the pollinated ovary in vitro can be supported on a minimal medium of mineral salts and sucrose. The two response types differ in carbohydrate requirements. A pollination-fertilization factor is postulated as the primary difference between them. Growth of the ovary in vitro can be influenced by temperature, auxin, casein hydrolysate, and concentration of sucrose. The temperature optimum for development in culture differs from that for development on the plant. Mechanisms of the responses have been discussed.

The cold-requiring processes which promote flower initiation in Petkus winter rye have been analyzed more critically for their oxygen and sugar requirements by vernalizing the excised embryos under atmospheres of air or nitrogen on media with or without sugar. The first partial process of vernalization requires sugar but does not require oxygen. The second process does not require sugar but is dependent upon oxygen. Separation of the partial processes now becomes possible.

TABLE OF CONTENTS

INTRODUCTION

The formation of flowers, fruits, and seeds is one of the most important activities of the higher plant. For the continued existence of the plant as a species and for the evolution of new species, reproduction by sexual means is all-important. Through its intimate relation to all phases of agriculture, it also obtains a position of prominence in our own economic welfare.

During the past fifty years considerable progress has been made in the understanding of the physiology and biochemistry of the phenomena of flower and fruit production. Our knowledge, however, is limited to a relatively small number of plants, and detailed information is available for only a few of the more critical phases of the reproductive processes. Even for these phases the mechanisms are incompletely elucidated. It is toward the complementation of our present knowledge of several of these matters, then, that this work is dedicated. In the experiments which follow, a number of the factors which govern the growth and development of the pollinated ovary into a fruit have been demonstrated by growing the excised flower in vitro. Investigation of normal floral ontogeny on the intact plant has also led to a new morphological interpretation of the initiation of reproductive development of one species, the red currant tomato. Finally, certain flower-promoting processes in the winter cereals have been more critically analyzed.

Before entering into the problems at hand, let us first examine in a general way the status of our knowledge of this basic phenomenon.

Strictly speaking, sexual reproduction in most plants is limited to the gametophytic generation, which bears the sexual organs and sex cells proper. In the seed-producing plants, however, the gametophytes are parasitic on the parent sporophyte and the structures which actually carry out sexual reproduction to completion are the seeds. In consequence, the gametophytic generation is considered separately only in comparative morphological work; and when we speak of sexual reproduction in the flowering plants in physiology, our reference is to those changes and processes in the plant which result in or accompany the formation of seeds, in other words, to flower and fruit formation.

We must bear in mind, however, that the first physiological changes, which result in the onset of reproductive development (flowering), obviously take place prior to the actual appearance of the reproductive organs (flowers). We know that at least part of these changes take place in vegetative parts of the plants (leaves and vegetative meristems). Therefore, we must include the vegetative stage of the plant in any consideration of its overall reproductive development. The following major stages are distinguished which concern the reproduction of the flowering plants:

1). The vegetative stage, which precedes the onset of reproduction proper (the initiation of flowers);

2). The flowering stages, which constitute the growth and development of the initiated flowers and which result in the production of the ripe sexual organs; and

3). The stages of fruit and seed development, which culminate reproduction in the formation of ripe seeds and fruits from the products and changes established through pollination and fertilization.

The purpose of physiology is to disclose those factors which govern the initiation of the individual stages and which control their progress.

1. The Vegetative Stage.

The fact that a period of vegetative growth foreruns the formation of flowers was well established by the beginning of the nineteenth century (1). Over a hundred years elapsed, however, before its significance in reproduction was appreciated by the German physiologist Georg Klebs (2). Klebs came to the conclusion that before flowering is possible the plant must attain a certain state of physiological maturity, which he termed "ripeness-to-flower" (Blühreife). In his classical experiments with the houseleek (Sempervivum) from 1904 to 1918 he showed that this condition of ripeness-to-flower can be reached only after a period of exposure to low temperature, that it is recognizable only by the ability of the plant to flower on subsequent exposure to periods of light longer than a certain minimum, and that it can be either preserved or destroyed under certain other conditions of light and temperature.

In general, most of our knowledge concerning the vegetative stage has been revealed through the control of flower initiation by light and by low temperature. Garner and Allard (3) showed that flowering in numerous plants depends on daylength and established the existence of two major response types with respect to this factor: short-day plants and long-day plants, which under natural conditions flower only when the length of day is shorter or longer than a certain critical number of hours. The critical daylength differs for various species. As to low

temperature we also know two groups of plants which show a "cold requirement" with regard to flower formation. These are the winter annuals and the biennials. Although Gassner in Germany and Maximov in Russia early demonstrated the importance of low temperature for the flowering of the winter cereals, it is to Gregory and Purvis in England that we owe most of the credit for elucidation of the physiology of vernalization -- the acceleration of flowering by low temperature.

In the compendial and critical reviews (4, 5, 6) of the evidence which has accumulated in recent years on the physiology of photoperiodism and vernalization, it becomes increasingly apparent that the importance of the vegetative stage preceding actual reproduction lies in the generation of a floral stimulus. Application of the necessary photoinductive treatment to a single leaf or portion of a leaf results in flowering in parts of the plant kept under conditions not conducive to flower initiation, nor does the exposure of the plant to the necessary treatment have to be continuous until the actual initiation of flowers. Also, a plant which has received photoinductive treatment can produce flower initiation in non-induced plants by means of grafting. In plants which are photoperiodically sensitive the generation of the stimulus occurs in the leaves through a series of processes involving high and low intensity light of different spectral qualities, carbohydrate metabolism, oxidative mechanisms, and auxin relationships. The floral stimulus, which is formed as the outcome of exposure of a plant to appropriate photoperiods, has been called "florigen" (7). In the plants which require vernalization a series of temperature dependent processes have been distinguished which are concerned with the formation of apparently another substance, "vernalin" (8). Although both florigen and vernalin have been shown to have an intimate relationship

to each other, the nature of this relationship is not yet clear (6).

All attempts to isolate the flowering stimuli have thus far been unsuccessful, but through use of interspecific grafts their identity in different species of the same plant type has been made likely.

2. The Flowering Stages.

The physiology of flower development from the time of initiation, under the influence of the flowering stimulus, until attainment of maturity at anthesis has been but little investigated. Since growth processes are involved, however, one may anticipate that factors which influence either cell divisions or cell elongations will also affect flower development. The growth scheme proposed by Went (9) may be expected to apply. These meristematic and physiological stages -- transformation, initiation, determination, elongation, and maturation -- may all take place simultaneously but at different loci (10). One must therefore distinguish between these stages for each of the different parts of the flower, particularly for those structures more directly concerned with reproduction (the stamens and pistils). Some of these stages, especially the meristematic, are difficult to influence by external conditions. Therefore, in most cases, the flower, once initiated, continues to develop uninterruptedly. General effects of temperature (11) and photoperiod (12) on the flowering stages have been found. Specific influences of temperature and photoperiod on development of the stamens and pistils have also been shown (13, 14, 15, 16). Other instances of environmental control have been reviewed by Lang (6) and Loehwing (11). In the later stages of flowering, growth correlations with auxin contents have been established (17, 18, 19, 20). We have no

knowledge of natural sex hormones, but if they exist, they are not transmitted by grafting (21). In general, experimental evidence on flower development is either lacking or is inconclusive. Critical information is available for only isolated cases, many of which appear to be exceptions.

3. Stages of Fruit and Seed Development.

The production of mature flowers with ripe, fertile pollen and ovaries does not necessarily mean that fruit and seed production will automatically follow. Under natural conditions transfer of the pollen to the stigma (pollination) is necessary to bring about both the initiation of fruit development (fruit set) and the initiation of seed development (fertilization). It is now well recognized that fruit set in some plants depends upon an adequate supply of auxin and that auxin is also important in later stages. But other, though maybe not so specific, factors may also be involved in all stages.

The necessity of the application of pollen to the stigma before the development of fruit and seeds can occur was realized by Camerarius around 1700 (22). While this observation formed the basis for the development of the concept of sexuality in the higher plants, it was there that our knowledge of fruit development stood for nearly 150 years. Gaertner in 1849 reported the development of seedless cucurbits after placing spores of Lycopodium on the stigmas of unpollinated flowers (23). In 1934 Yasuda (24) investigated these seedless, or parthenocarpic, fruits much more in detail and found that all that was necessary for their formation was that the pollen tubes of pollen from either the same or another species penetrate deeply enough into the style. The injection of water extracts of pollen into the ovary

would accomplish the same result. Hence, pollination without fertilization is sufficient to bring about the growth and development of some fruits and separates the stages of fruit development from those of seed development.

In 1936 Gustafson (25) demonstrated that substances active in the Avena test (26) were also effective in inducing parthenocarpy in tomato. Since that time, most investigations of fruit physiology, at least of the stage of initiation (fruit set), have centered around auxin relationships. Since the presence of auxin in pollen was detected, it was first thought that the explanation of the role of pollination in fruit set had been found. Later, by calculations, it was found that the amount of auxin in the pollen required for a normal pollination could not account for the amount detectable in the pollinated pistil, and we now know that in at least one plant, tobacco, pollination results in the establishment of an auxin-producing mechanism which moves down the style at approximately the rate of pollen tube extension (27, 28, 29). After fertilization there is a sharp increase in the amount of diffusible auxin moving out of the cut pedicel of the tobacco flower. While a known enzyme system, the tryptophan-converting enzyme, has been identified in the pollen and pollinated ovary of tobacco (30), it is not this system which is established through pollination, since this same enzyme is also found in the unpollinated pistil in a form which does not require activation. Whether or not pollination may activate or supply some system for the production of tryptophan has not yet been established (29).

The auxin relationships during later stages of fruit and seed development have also been investigated. Gustafson (31) has found

that developing seeds and adjacent placental tissues are apparently centers of auxin production or at least of auxin content and that a gradient exists to the pericarp. In the strawberry, removal of developing achenes causes a cessation of growth of the subjacent parts of the receptacle. The achenes have been found to contain auxin at all stages (32). Spraying of poorly pollinated tomatoes with synthetic growth substances increases the final size (33). Investigation of the auxin content of the endosperm of developing cereal grains has revealed a sequence of auxin conversions (19, 34, 35). Precursor, free, active-complex, and inactive-complex forms have been identified (35). Two auxins, indole-3-acetic acid and its ethyl ester, have been isolated from corn kernels (36, 37). The ester is much less active in the Avena test but is more effective in inducing parthenocarpy in tomatoes.

Evidence is gradually accumulating that materials other than auxin are necessary for fruit initiation and may also be required during later stages of fruit and seed development. Went (38) has observed that fruit set in tomato may be almost completely abolished by exposure of plants to additional light under otherwise optimal growing conditions, and recently Leopold and Scott (39) have demonstrated a relationship between the number of leaves per shoot and the ability of cross-pollinated and auxin-sprayed self-sterile tomato flowers to set fruit. This effect is restricted to the mature leaves and is promoted by light. With excised and sprayed flowers a wide variety of organic substances, and also inorganic nitrogen compounds are capable of achieving the same action. We may say, then, that there are both a) specific factors, pollination or auxin, and b) non-specific factors, obtained from leaves, which are required for fruit set. The role of

light in fruit set of tomato requires critical investigation.

In addition to these comparatively simple substances we have to take into account complex correlative effects, particularly during the later stages of fruit and seed development. The complication is added through the simultaneous development of two diploid tissue species and a haploid and triploid as well. Morphological evidence exists which demonstrates considerable interplay between these tissues during development (40, 41, 42). Analysis of the growth curves of developing fruits has been used to correlate fruit development both with seed development (43) and with the cell divisions and elongations which determine the size and shape of fruits (44,45). Relationships between fruit size, leaf area, and food supply have also been shown (46, 47, 48).

In the final analysis, although our knowledge of reproduction is considerable, there are many gaps in its continuity. By various and sometimes devious means, however, we have been able to elucidate a few of the more critical processes of a complex phenomenon and to implicate others. In a few instances we have identified a stepwise series of reactions. Room exists for exploratory investigations as well as much critical experimentation.

In PART ONE of this thesis, we will discuss some of the morphological, physiological, and biochemical factors which govern the growth

of the pollinated ovary of the red currant tomato.

In PART TWO we will separate certain partial processes which take place during the vernalization of winter rye.

PART ONE

STUDIES IN FRUIT GROWTH

A. Purpose and Introductory Remarks.

At the time of the initiation of these investigations, no definitive knowledge was available on the nutritional requirements for the growth of fruits. During the past twenty years, however, through the development of the technique of culturing isolated tissues and organs in vitro, many of the biochemical and physiological processes of growth and development in general have been elucidated. The purpose of these investigations from the outset, therefore, has been to reveal at least some of the growth factor requirements of the isolated ovary, particularly of the pollinated and fertilized ovary.

After a number of unsuccessful attempts to obtain growth of the isolated ovaries and flowers of several species of dicotyledons, the excised pollinated flowers of red currant tomato, Lycopersicon pimpinellifolium, responded very gratifyingly, and the ovaries grew and ripened into fruits on a number of media. Development of these fruits on completely synthetic media was preliminarily reported by Jansen and Bonner in 1949 (49) a short time after Nitsch (50, 51), also working in these laboratories, reported similar success with excised pollinated flowers of the common tomato (L. esculentum). The experiments which constitute the bulk of this section consequently represent the more detailed follow-up investigations on the red currant tomato.

B. Literature.

The tissue culture technique is now widely employed in growth studies of the higher plants. Reviews of the subject have been given repeatedly in the past, the latest being those of White (52) and Gautheret (53). Both of these authors have also written manuals pertaining to the techniques employed (54, 55). Plant materials which have been effectively studied in this manner have been vegetative organs (roots, stems, and leaves) of flowering plants, gymnosperms, and ferns; excised, immature embryos; isolated pieces of cambial and other meristematic (or potentially meristematic) tissues; and plant tumors and other diseased tissues. Many of the various growth substances which have been shown necessary for the growth of these tissues and organs have also been reviewed (56, 57, 58).

In actuality, growth and development of mature, seeded fruits from pollinated ovaries of detached flowers is not a new discovery. As early as 1577 Conrad Gessner found that the white lily (now known as Lilium candidum) will easily form capsules and seeds if the flowering stalk is cut off and placed in water. Němec (59) has shown that this plant is generally self-incompatible and that even the few individuals which are self-compatible do not produce seeds and fruits when attached to the bulb. When detached and placed in water, however, even the former plants not only readily produce fruits when cross-pollinated but also do so when selfed, providing the stigma is first moistened with 5% fructose solution. Gaertner also, in 1844, stated that many other monocotyledons will form fruits when the inflorescence is separated from the root. Dahl et al (60) have recently made use of this phenomenon in saving valuable hand-pollinated Iris specimens which

were accidently broken in the field. The seeds procured in this manner germinate well.

This ability of excised flowers to develop both seeds and fruits in the absence of added organic substrates is not shared by any of the dicotyledons yet investigated. La Rue (61) observed that ovaries developed into small fruits without seeds in an investigation of the rooting of excised flowers. An agar medium containing mineral salts, 2% sucrose, and indoleacetic acid in a concentration of 1:100,000 was employed. Of the 92 species studied, 80 of which were dicotyledons, growth of the ovaries of excised flowers was observed in only 4 species -- tomato, Kalanchoë, Forsythia suspensa, and Caltha. No further investigation of these observations has been reported by La Rue.

Ovules, portions of ovules, and immature embryos have all been successfully cultured (60, 62, 63, 64, 65, 66). As a general rule it may be stated that these structures must have passed a certain stage of development prior to excision before growth to maturity in vitro is possible. Also, the younger the embryo the more complex are its nutrient requirements. Specific differences in growth requirements have been found (64). Datura embryos (62) require only sugar and mineral salts when nearly mature. At younger stages, however, they must also be supplied B-vitamins and amino acids; and at a still earlier stage, certain unidentified factors of coconut milk are necessary. Proembryos do not grow normally even with coconut milk. Even during the later stages of embryo development, for which further growth is supported by only sucrose and salts, such factors as casein hydrolysate and mannitol can control differentiation, apparently through an osmotic action (65). The interpretation of the latter findings is that these

substances are substituting for the normal environment of the developing embryo.

The bulk of our knowledge of fruit growth factors is to be found in the continued investigations of Nitsch (20, 67). On a medium containing mineral salts, 2% sucrose, thiamine, cysteine, and 50% tomato juice a five-fold increase in ovary diameter has been obtained after seven weeks of culture. On a simpler medium, containing only sucrose (5%) and mineral salts, pollinated ovaries have been found to give an increase in diameter of 133% after three weeks of culture. Unpollinated ovaries increase no more than 38% during the same period, unless they are also supplied with an auxin in the medium. Unpollinated gherkin ovaries, however, do not grow on this basal medium even when supplied with auxin. Auxin application to the cut style is also ineffective. Pollinated gherkin ovaries, on the other hand, not only grow on the basal medium but produce a few viable seeds as well. This shows quite clearly that, at least for the unpollinated ovaries, growth is limited by different factors in the different species. Since no seeds are observed in fruits developed from the pollinated tomato ovaries, we must also conclude either that the growth factor requirements for seed development in tomato differ from those for gherkin or that there are fundamental differences between the ovaries in their ability to supply these factors.

Although the organ culture approach to the problem of the growth of fruits and seeds has provided us with valuable information concerning the growth factors involved, too few species have been studied to permit drawing general conclusions. We must also demonstrate the comparability of the results obtained <u>in vitro</u> with normal development

of the attached organ. In the work which follows, which involves studies in the growth and nutrition of the fruit of another species, Lycopersicon pimpinellifolium, these comparisons will be made wherever possible.

C. Materials and Basic Techniques.

1. Plant Materials.

The red currant tomato (Lycopersicon pimpinellifolium Mill.) is the plant used in these investigations. Seeds of this species, representing accession #2 of the source of spotted wilt resistance, were kindly supplied by Dr. Paul G. Smith of the California Agricultural Experiment Station, University of California, Davis, California.

Those plants used as a source of flowers for the preliminary studies of fruit growth in vitro were grown from seeds sown in sand flats in the Dolk Greenhouse on January 19, 1949. After three weeks, the seedings were transplanted to soil in half-gallon crocks. All plants were watered daily and supplemental nutrient (Hoagland's) was applied once a week. Flowering commenced on April 19. On June 1, 1949, all plants were transferred to the outdoors.

In all later work use has been made of the facilities of the Earhart Plant Research Laboratories (68). Plants used as flower sources have been grown at 20° C day temperature, 14° C night temperature, under natural daylength. These plants, which were sown on August 18, 1949, began flowering on October 21. They have continued to flower uninterruptedly under these temperature conditions. Because of rapid growth of the weak stems, plants require staking and periodic pruning about every two months. No detrimental effects on subsequent flowering have been observed even when plants are reduced to stumps.

2. Culture Media.

The mineral nutrients employed in the present work are those commonly used by various workers for the culture of roots, calluses, and embryos. Both Gautheret's (53) and Bonner's (69) concentrations were tried in preliminary experiments but no differences could be detected in their ability to support growth of ovaries. A slightly modified Bonner's mineral salt solution, previously shown satisfactory for the culture of excised tomato roots, has consequently been adopted for the fruit work. The concentrations of the major salts per liter of nutrient are as follows:

$Ca(NO_3)_2.4H_2O$	285 mg
KNO_3	85 mg
KCl	61 mg
$MgSO_4.7H_2O$	21 mg
KH_2PO_4	20 mg

Iron and trace elements are added separately as 1 ml of ferric tartrate solution (1.5 mg/ml) and 1 ml of a solution containing in 1 liter 40 mg $MnSO_4$, 20 mg $ZnSO_4$, 2 mg H_3BO_4, 1 mg each of $NiCl_2$, and $CuSO_4$, and 0.75 mg KI. With the exception of the last salt, this is the trace element solution recommended by Gautheret (53) for the culture of stem tissues. Only glass distilled water has been used, and all salts are of reagent grade.

The above salt solution containing 4% sucrose constitutes the minimal medium referred to in the in vitro experiments. In other supplemented media mixtures of amino acids are added as vitamin-free, acid-hydrolyzed casein. B-vitamins are supplied by a mixture which is currently employed in Neurospora research but is modified according to

demonstrated requirements for the growth of some roots. The composition of this mixture (added as 1 ml/l of nutrient) is

Thiamine-HCl	0.1 mg/ml
Riboflavine	0.05
Pyridoxine	0.1
p-Aminobenzoic acid	0.05
Pantothenic acid	0.8
Nicotinic acid	0.5
Inositol	0.5
Choline	0.1
Folic acid	0.1
Biotin	0.005.

Other organic substances and mixtures are added in the concentrations stated in the experiments.

Adjustments of pH, when necessary, are made by addition of KOH or HCl. Both Universal indicator papers and a Beckman Model-G pH-meter have been used for determining pH.

3. Maintenance of Sterility.

All media, glassware, and paper materials are sterilized by autoclaving at 15 pounds pressure for 15 minutes. Liquid endosperm of ripe coconuts (coconut milk) is obtained free of microorganisms by careful shaving and flaming of the "eye"-end of selected coconuts showing no surface cracks. Two eyes are punctured with a flamed cork borer and a sterilized piece of Pyrex tubing, previously bent to proper siphon dimensions, is inserted in one hole. A short piece of glass tubing, plugged with cotton and sterilized, is inserted in the air hole. The milk is then collected in a suitable sterile container and stored in the refrigerator. It is added to media by sterile pipette either before or after autoclaving.

While the common aseptic techniques employed by microbiologists have been used throughout these investigations, danger of contaminations during the longer handling of larger-sized material has been considerably lessened through utilization of an especially designed "sterile" room. Provision has been made for reducing air currents to a minimum and for preliminary sterilization of the air and all exposed working surfaces by weak ultraviolet light. All-tile working surfaces facilitate additional sterilization with suitable antiseptic and bacteriocidal solutions. Instruments used for handling plant materials are dipped in alcohol, flamed, and cooled between sterile paper towels.

D. Experiments with Red Currant Tomato

The original intent of these experiments has already been stated -- to reveal, through *in vitro* control, the growth factor requirements of the developing ovary. It has also been pointed out that the value of information gained in this manner resides in the demonstration of comparable development *in situ*. During the early course of the *in vitro* investigations, however, considerable lack of uniformity in cultural response of the excised flowers was encountered. Consequently, before the originally intended investigations could be satisfactorily carried out, it became necessary to either eliminate or isolate the sources of the variability through use of as uniform starting material as possible and through standardization of the culture techniques.

The experiments reported in this chapter have consequently been separated into three sections. Section 1 deals with the studies on the growth and development of the attached flowers and fruits. The stages of floral ontogeny have been delineated for purposes of supplying uniform culture material and the growth of the fruits characterized in order that comparisons may be made with cultured fruits. Section 2 involves the development of standard procedures and investigation of possible influencing factors arising from the *in vitro* methods. Finally, in Section 3 are given the experiments which deal with the growth factor requirements of the developing ovary and characterization of *in vitro* development.

1. Characterization and Description of Flower and Fruit Development *in situ*.

The selection of uniform experimental material depends upon a fairly accurate delineation of stages of development. While this information is available for the tomato of commerce (*L. esculentum* Mill.), a survey of the literature revealed little of comparable nature with regard to the red currant tomato. A morphological description of the ontogeny of the flower and inflorescence consequently seemed in order, particularly to ascertain the comparability of the final stages of floral development after anthesis commences.

All studies on the floral morphology of *Lycopersicon pimpinellifolium* which have been previously carried out have been along genetic or taxonomic lines. Murray (70) has investigated the anatomy of the ovary with regard to other members of the Solanaceae. The species has been included in monographs of the genus (71) and has also been utilized in genetic studies because of the ease with which it can be crossed with *L. esculentum* (72, 73, 74). Hybrids exhibit intergrading floral characteristics. Lindstrom (75) has demonstrated direct effects of genetical constitution on the floral structure of *L. pimpinellifolium* through the development of a tetraploid hybrid. The hybrid is self-fertile in itself but cross-sterile with the parent diploid plant. The tetraploid produces flowers, fruits, and seeds approximately twice the size of those of the diploid, although the vegetative growth and number of seeds per fruit are reduced. Gustafson (76) found that the stem tips of the tetraploids contain only about 75% as much extractable auxin as do those of the diploids. Cooper and Brink (77) have observed that only two

thirds of the seeds of the tetraploid are sound; the rest are shriveled. Anatomical analysis of various crosses has led Cooper and Brink to assign a special function to the endosperm in embryo development. Mention should also be made that one genetic strain of this species, and the source of the material used in this work, has been shown by Smith (74) to be resistant to the spotted wilt disease. This strain has subsequently proven of value in breeding programs for disease resistance in L. esculentum.

Development and Ontogenetic Nature of the Inflorescence.

The flowers of Lycopersicon pimpinellifolium are borne in an inflorescence initiated from the terminal growing point of stems. There is a variance of opinion, however, as to the nature of this inflorescence in relation to the vegetative organs and to the manner of development of the flowers in the inflorescence. Hayward (78, p. 554) says of the genus Lycopersicon, "the inflorescence is usually a two-ranked raceme or a branched racemose-cyme, the former type occurring only in the currant tomato." Bailey (73, 79) also says that in L. pimpinellifolium the inflorescence is an elongating, distichous raceme. Most of the older literature on the genus, however, assigns a sympodial type of growth to the tomato. The normal inflorescence shown in Figure 1, while appearing to be a raceme, could as easily be called a scorpioid cyme, as depicted by Goebel (80, p. 408). Went (9) has explained all of the inflorescence variations found in tomato (L. esculentum) by such a sympodial scheme. Therefore each flower would be considered to be terminal in position and the continuation of the floral axis beyond as an axillary branch from a subtending (though often reduced or totally

absent) leaf or bract.

The growth of the vegetative axis of the Solanaceae in general has also been described as being sympodial, the inflorescences representing the terminal differentiation of each of a succession of lateral shoots. Caldwell (81) and Went (9) have both described true dichotomy of the vegetative axis. Venning (82) is of the opinion that dichotomy is the normal situation in formation of the inflorescence in L. esculentum. Certainly the anatomical evidence of vascularization in the vegetative dichotomy observed by Caldwell and that used by Venning is quite similar, consisting of a splitting of the entire vascular ring of the axis, whereas lateral (axillary) shoots, as pointed out by Caldwell, derive their vascular supply from the traces of the subtending leaf. A preliminary dissection of the stem of L. pimpinellifolium has revealed a similar situation at the region of departure of the floral axis. Below the branching a ring of vascular bundles exists. Near the point of divergence the ring flattens, the individual vascular bundles branch, thereby increasing the total number. Two rings are thus formed, one of which passes into the inflorescence and the other continues on upward as the vegetative axis. No evidence for any extrastelar traces, which would indicate leaves or bracts, could be found. Inasmuch as true dichotomy occasionally occurs in the inflorescence, Figure 2, it is possible that the entire structure of the floral axis of L. pimpinellifolium can be explained as dichotomous rather than either sympodial or racemic. The presence of well developed axillary buds in the leaves both above and below the inflorescence (Figure 3), even in the terminal bud, would also indicate this to be true. The appearance of leafy structures in the inflorescence is likewise always in the fork and not

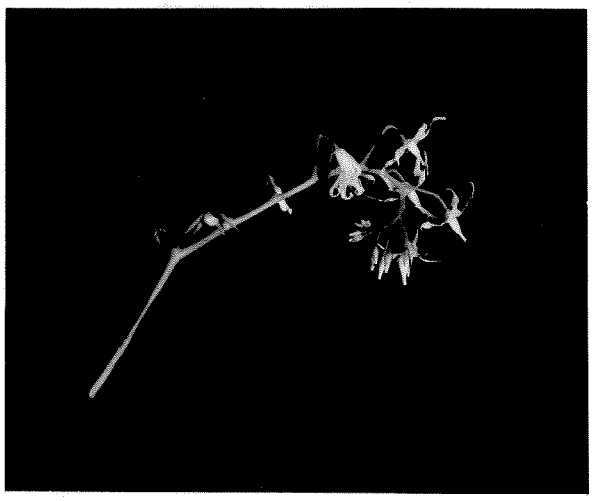


Figure 1. Normal inflorescence of Lycopersicon pimpinellifolium.

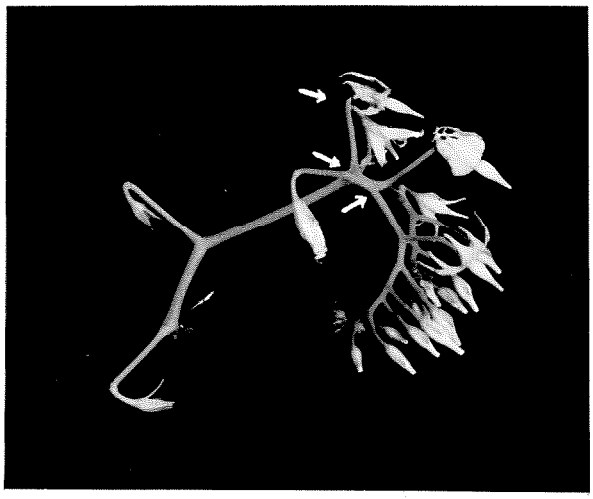


Figure 2. Dichotomy in the inflorescence of Lycopersicon pimpinellifolium. Arrows indicate points of unusual branching. Upper arrow: twinning of flowers. Center arrow: a point of trichotomy -- two flowers and also continuation of the inflorescence axis. Lower arrow: branching of the inflorescence axis.

in a position such as to denote axial origin of either of the branches. In L. esculentum the situation is less clear cut since the leaf above, or frequently immediately opposite, the inflorescence generally lacks an axillary bud except when quite old and usually at times when extra-axillary, adventitious buds appear on the petioles of other leaves. Venning (82), however, states that nodal sections of the questionable leaf reveal an axillary bud with common bundle (leaf trace) connections, even though the bud may be dormant.

The phyllotaxis generally ascribed to Lycopersicon is 2/5 or 3/5, depending upon the direction of determination. This, however, is true only before the initiation of the first inflorescence. Went (9) has described occasional reversal of the spiral during this early period of growth, which is strictly vegetative, but after the first inflorescence such reversals have been frequently noted. In L. pimpinellifolium the latter reversals are of regular occurrence after each inflorescence and, with but few exceptions, a new inflorescence is formed after every third leaf.

These regular reversals in the leaf spiral offer additional evidence to the theory of dichotomous branching. The following statement concerning the leaf spiral has been made by Velenovsky (83, p. 573):

> "The leaf-spiral proceeds in a course on the axis of one and the same plant always in the same direction; on the shoots however, which arise in the axils of the leaves of this axis, this spiral proceeds either also in the same direction (homodrom) or in the opposite direction (antidrom)." (in German).

With the exception of the observations of Went (9) on the common tomato, no contradiction of this rule has been found in the literature. The expected ratio for axillary reversals is 1:1. If, then, the axis of tomato is monopodial and the inflorescence lateral as described by

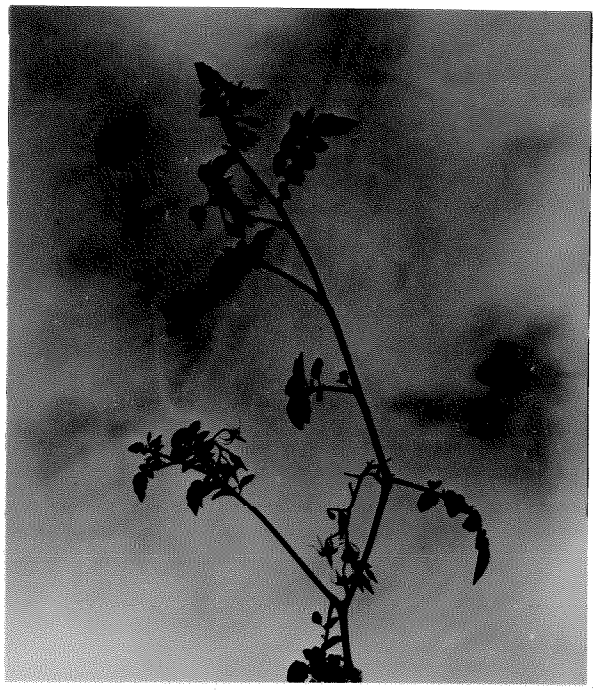


Figure 3. Flowering stem of Lycopersicon pimpinellifolium, showing last mature growth cycle. The third leaf of this cycle, i.e. the third above the lowest inflorescence, has been removed to show development of the axillary bud. Note, also, axillary bud in the axil of the first leaf of this cycle. X 1/2.

Muller (71), there should be no discontinuity in the direction of the spiral. On the other hand, if this is a sympodial type growth and the continuity of the vegetative axis is maintained only through successive development of axillary shoots, reversals are to be expected for only 50% of the inter-inflorescence axes. The observations on L. pimpinellifolium, however, indicate that these reversals are invariable. The only alternative explanation, then, is that it possesses another type of growth, viz. dichotomy.

In order to check the above hypothesis, a large number of stems have been taken at random and the directions of the leaf spirals have been determined in the following manner. The direction of the spiral on the inter-inflorescence axis is determined first. This, then, provides a basis of reference for the direction of the initial spiral in the axillary buds of each of the leaves which compose the main axial spiral and for the direction of the spiral on the distally adjacent inter-inflorescence axis. The data for these determinations are given in Table 1. The figures are quite conclusive. Regardless of the number of leaves per spiral, reversal occurs in the next axis. However, it should be noted that in the usual state of affairs (3 leaves per spiral) only the bud of the first leaf approaches the 1:1 ratio with regard to reversal. There is a strong tendency for the bud of the second leaf to be homodromic while that of the third leaf is invariably antidromic or reversed.

If one accepts the dichotomous theory of branching, it thus becomes possible to characterize the growth of L. pimpinellifolium as follows. After the production of a certain minimal number of leaves (around 9-12 observed at various times) the first inflorescence is produced by

TABLE 1. Relation of initial direction of leaf-spirals of axillary shoots and of adjacent axes to direction of leaf-spiral of the inter-inflorescence parent axis. (≠), same direction; (-) reversed direction. Leaf number refers to position of the leaf in the parent spiral, determined from base to tip.

Number of leaves in reference spiral	Axillary Buds of Leaf										Next Distal Axis	
	1		2		3		4		5			
	≠	-	≠	-	≠	-	≠	-	≠	-	≠	-
3	27	23	45	5	0	100					0	100
2			2	0							0	2
4		1	1			1	1				0	1
5	1			1		1		1		1	0	1
TOTALS	28	24	47	6	0	102	1	1	0	1	0	104

dichotomy of the terminal meristem. In subsequent growth the vegetative axis goes through a cyclic pattern of development consisting of three leaves, dichotomy, three leaves (in a reverse spiral), dichotomy, three leaves, etc. (Figure 3). In addition, the axillary bud of the third leaf of each cycle (the one just below the inflorescence) develops strongly, immediately following the initiation of that leaf in the terminal bud. It is well developed with primordial buttresses by the time the third leaf is the second or third oldest structure cut off by the meristem. The order of formation of organs from this new meristem is typically three or four leaves (in a reversed spiral from the preceding one which includes the subtending leaf), dichotomy, three leaves, dichotomy, etc.

Dissection of terminal buds and notation of the relative positions of the various structures has made possible the formulation of a growth diagram (see Figure 4). It appears, consequently, that the 2/5 phyllotaxy holds for the three leaves of each cycle when referred to an inter-inflorescence monopodial axis (A). Dichotomy at the inflorescence, however, shifts the vegetative axis in a vertical plane approximately 120° from the vertical plane of the third leaf (3a) and to the side of the first leaf (1a), while the inflorescence (Infl.^{a}) is formed in a plane 120° removed from both of these. The first leaf of the new vegetative axis (B) is then formed on the side farthest from the old axis. This situation is maintained and is quite evident so long as the various structures remain in the terminal bud, i.e. above the last macroscopically discernible internode.

As the internodes commence to elongate, the vegetative axes of successive cycles become more or less superimposed, while the inflorescence

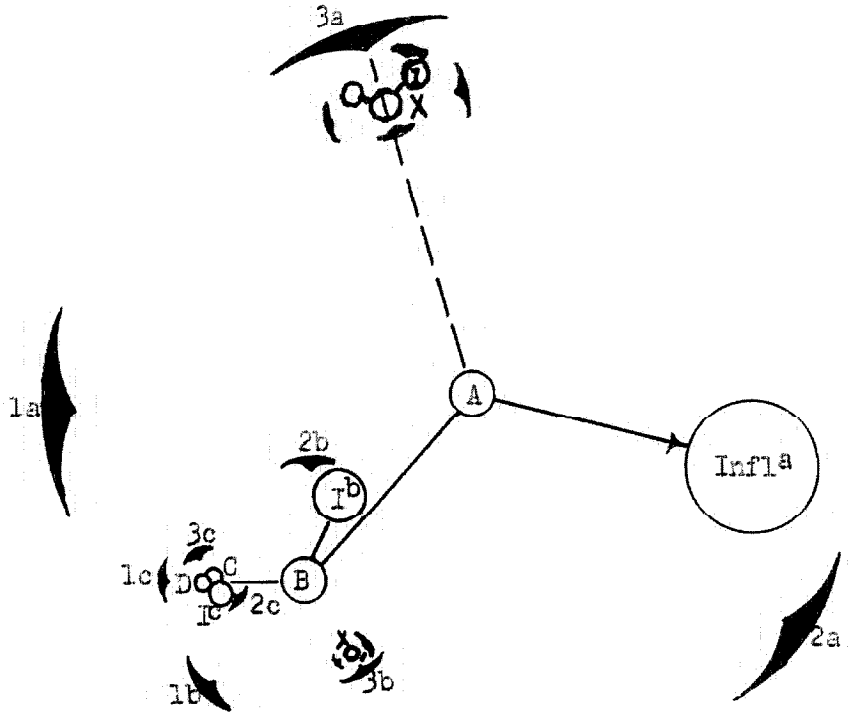


Figure 4. Growth plan of the stem tip of Lycopersicon pimpinellifolium. A, vegetative axis of last mature growth cycle; B, C, and D, successive shifts of vegetative axis by dichotomy; Infl., inflorescences resulting from dichotomy; numbers, order of leaf formation; small letters, parts of growth cycles corresponding to A, B, C, etc. Axillary buds (X) shown for leaves 3a and 3b.

axes assume a lateral position. This situation is represented in Figure 5. Perhaps the most striking feature of this diagram is the occurrence of the inflorescences in a restricted sector to one side of the continuous vegetative axis. Sectoral development of flowers in the inflorescence is also evident. The reproductive meristem apparently undergoes bifurcation in a plane which pivots about the inflorescence axis and alternates to either side of the vertical. The "adaxial" fork of each bifurcation develops into a flower and the abaxial continues the axis. So long as this plane of bifurcation continues to oscillate about the inflorescence axis in such a way that one of the forks falls within the sector F1 (in Figure 5), this fork will develop into a flower. It would appear, however, that if the plane of bifurcation deviates to the extent that neither fork falls within this sector both will result in extensions of the axis. The branched inflorescence shown in Figure 2 may be explained in this way.

The position of the first leaf of each new vegetative axis offers another point of interest. After elongation of the growth cycle to which it belongs (Figure 5) the relationship of this leaf to those of the previous cycle is more apparent, and one can discern that its position is such as to continue the previous spiral. This finds logical explanation in the Hofmeister rule which states that "new lateral members have their origin above the center of the widest gaps on the circumference of the growing point between the insertions of the nearest older members of the same kind" (84, p. 177). The Hofmeister rule does not hold for the position of the second leaf, however. One must consequently turn to the Schwendener pressure hypothesis (85). In this respect, then, the inflorescence also enters into the determination of

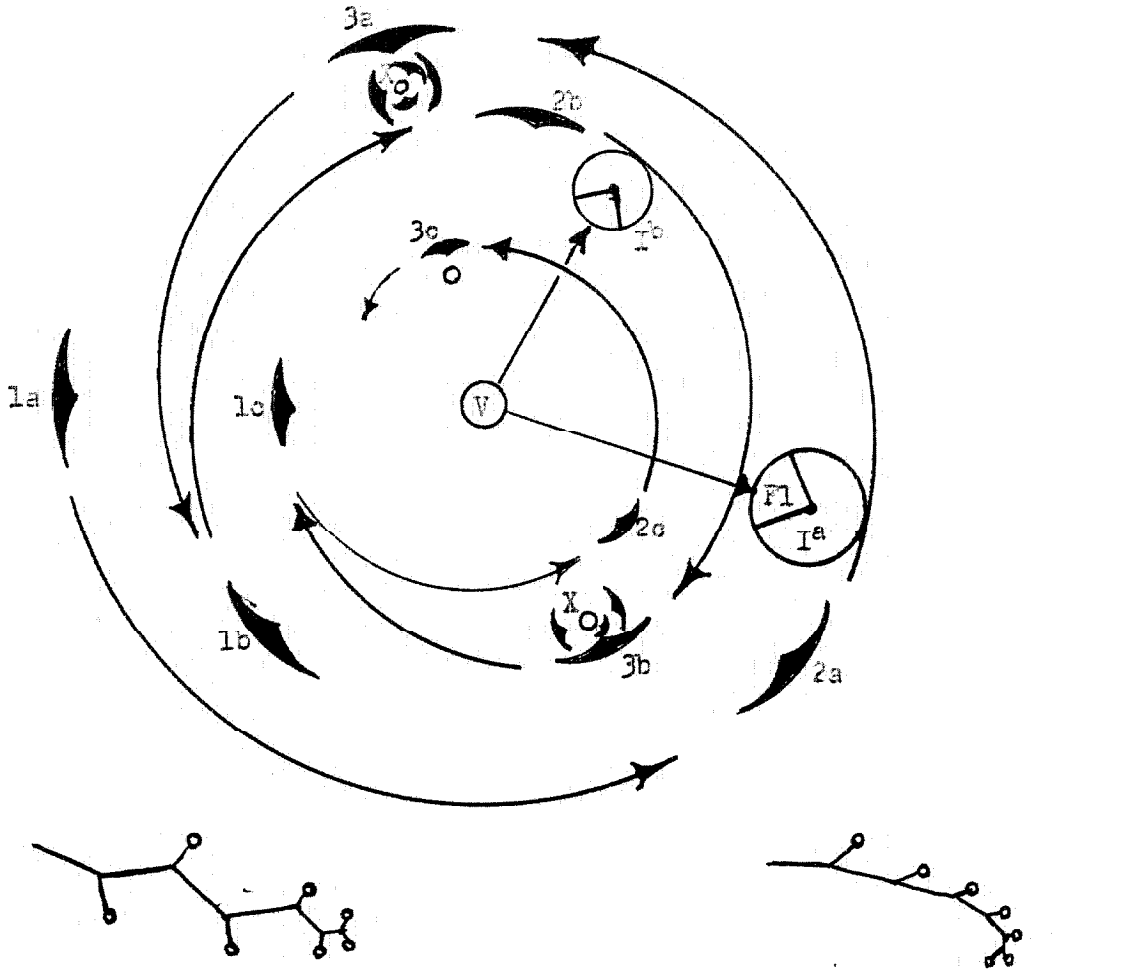


Figure 5. Growth diagram of mature vegetative and reproductive axes of Lycopersicon pimpinellifolium. V, vegetative axis; I, inflorescence (axial view); numbers, order of leaf formation; a, b, c, successive growth cycles; X, vegetative axis of axillary bud; Fl, floral sector of inflorescence. Arrows indicate direction of phyllotactic spiral.

the point of least resistance where the next lateral organ will be formed. That it does not do so for the first leaf may be interpreted as further evidence of dichotomy and simultaneous origin of both the inflorescence and the axial primordia. In this matter, also, the first flower primordium on the inflorescence is determined by the two previous leaves. As the first flower primordium commences growth, however, it comes in contact with the axial meristem and in conjunction with the first leaf, it thereby defines the position of the second leaf and shifts the leaf spiral.

In summary, the initiation of a flower in L. pimpinellifolium would seem to be governed by a complex of "spatial effects," hereditarily determined and residing in the apical meristem. Thus a flower is not usually formed unless a certain amount of vegetative growth has taken place (attainment of the ripeness-to-flower condition). The number of leaves produced may then determine the position of a bifurcation of the apical meristem. The relative positions of the preceding leaves determine which of the two forks will be capable of producing flowers and also the position of the first flower.

The physiological explanations of this growth pattern remain to be elucidated. Auxin relationships have been implicated in L. esculentum by de Waard and Roodenburg (86) and by Zimmerman and Hitchcock (87). In L. pimpinellifolium, also, the writer has found that application of triiodobenzoic acid to plants in early seedling stages, when no more than four leaves are present, results in the initiation of flowers at an earlier physiological age than untreated controls. The flowers initiated by this treatment, however, have not developed further. Accompanying vegetative malformations have made interpretation of results exceedingly

difficult.

Flower Development.

Under normal developmental conditions the ontogeny of the individual flower of red currant tomato does not appear to differ from that of the common tomato which has been described by Cooper (88) and Hayward (78). It develops first of all as a clear, dome protuberance in the plane of bifurcation. The first sepal primordium appears on the side nearest the vegetative sector of the inflorescence and the others follow in succession, clockwise if on the right and counterclockwise if on the left of the inflorescence axis (both Cooper and Hayward give "clockwise" for L. esculentum). Further development of the sepals, including dorsal acceleration of growth and connivance of the sepal tips over the floral meristem, appears to be the same. So also does the acropetal differentiation of petals (corolla), stamens, and pistil. The only difference, which is minor, is one of number. L. pimpinellifolium is invariably pentamerous and has a bicarpellate ovary. L. esculentum is usually described as being hexamerous with several carpels and frequently shows fasciation of the first flower of each inflorescence.

Because of its indeterminate nature the growing inflorescence usually bears a large number of flowers in various stages of development. It appears that floral differentiation takes place at a fairly uniform rate, at least until the anthesis of the first flower. The latter phenomenon occurs when the growth cycle to which the inflorescence belongs approaches maturity, i.e. when the three leaves below have fully expanded and the internodes have completed the major part

of their elongation. This is generally true, for the plants studied in this investigation, when this growth cycle is the fourth complete one below the apical meristem. As a typical example a terminal vegetative meristem exhibited two leaf primordia. The inflorescence of the first growth cycle below had at this time developed four flowers—a floral dome (youngest), a dome with the first sepal primordium, one with sepal primordia distinct but of unequal length, and one with evident tip connivance. The next lower inflorescence possessed ten flowers, the third fourteen, and the fourth twenty with the first formed in a condition of full bloom. A much older inflorescence showed that the first three flowers (in order of initiation) had abscissed; the next 6 were in various stages of developing fruits; one abscission; one in the initial stages of anthesis; one abscissed bud; one bud less than 1 mm in length (underdeveloped); two buds (ca 3 and 2.5 mm in length, respectively); and five buds less than 1.5 mm; total, 20 flowers. The axis primordium was present as a browned and shriveled mass. Checks of a number of other old inflorescences also revealed 19-22 flowers before collapse of the axis meristem. It appears, then, that under these growing conditions reproductive organs are originated and go through the various stages of development in approximately the same length of time as do vegetative structures, and that the inflorescence as a whole has a duration of activity corresponding approximately to the time necessary to reach vegetative maturity. Maturity measured in morphological terms is attained after four growth cycles. Flowers are formed at a rate of approximately five per cycle.

Anthesis and Pollination.

The ultimate changes in the development of the flower which can be determined by casual observation are the same whether the flower produces a fruit or falls from the plant. These changes are those concerned chiefly with the corolla.

Just prior to anthesis the corolla commences rapid growth at the base, the bud swells, and the corolla tips are pushed and elongate upward forcing the calyx tips apart. The corolla lobes themselves finally separate forcefully (stage of beginning anthesis). Continued growth of the corolla is of such a nature that the corolla tips become so strongly reflexed that they eventually cross each other below the level of the receptacle (stage of reflexed corolla). The latter condition has been variously termed full flower, full bloom, and full anthesis for L. esculentum. (The term "anthesis" has been variously used in the literature to refer to the condition just described, to the initial opening or splitting of the bud, and to the whole sequence of events from initial opening to the fully reflexed condition. In the present work "anthesis" will apply to the entire process of opening.)

The reflexed corolla continues to grow and expands in a plane perpendicular to the floral axis (stage of revolute corolla). Eventually the corolla tips reconnive (stage of late corolla) and the whole corolla rapidly withers and dries out. It then either dehisces with the attached stamens or remains attached to the receptacle and the whole flower abscisses (flower drop) at a preformed zone of the pedicel. At this same zone, also, a marked bending of the floral axis occurs during anthesis and the subsequent stages of corolla development (senescence), resulting in a pendulous habit of the flower. This series of changes

and the stages of "beginning anthesis," "reflexed corolla," "revolute corolla," and "late corolla" are shown in Figure 6.

Roberts and Struckmeyer (89) have determined the time of pollination in relation to a similar delineation of stages in L. esculentum. It normally occurs between the time of the half-opened flower and the stage corresponding here to "revolute." Their stage 8 (full flower), which corresponds to the stage of reflexed corolla, is stated to be best for pollination. Judkins (90), however, has said that the stigma is receptive to pollination two days prior to "anthesis" (not clearly defined) and that the anthers dehisce when the petals first open. Microscopic examination of flowers of L. pimpinellifolium has revealed that shortly after anthesis of the bud commences free pollen grains can be found adhering to the stigma and style. However, firm attachment of the pollen in large numbers through growth of the pollen tubes is not evident until just prior or at the reflexed stage.

It may be said, then, that the development of the flower of Lycopersicon pimpinellifolium, from initiation to maturity, is essentially the same as that of L. esculentum.

Figure 6. Stages of flower development in Lycopersicon pimpinellifolium. Upper row, stages leading up to pollination; left, a group of developing buds; right, anthesis of the flower. The first flower of the right hand group is "beginning anthesis" and the last is "reflexed corolla." Lower row, left to right, flower slightly after reflexed corolla, two stages of "revolute corolla", two stages of "late corolla," and finally "flower drop" (top) and dehisced corolla or first stage of obvious fruit development (bottom). X 3/4.

Development of Fruits in situ.

Flowers have been collected at various stages of development for use in the in vitro experiments. It is desirable, therefore, to characterize the development of equivalent flowers on the plant and to isolate insofar as possible the factors affecting normal fruit growth. The following tagged-flower experiments have been carried out.

The first two tagging experiments were conducted on plants which were growing outside of the Dolk Greenhouse. On July 23, 1949, a number of flowers were tagged in both the revolute and late stages but flower drop at the time was so heavy that the experiment was repeated on July 30 with a larger number of revolute flowers. After the establishment of new plants in the Earhart Laboratory under controlled greenhouse conditions a much more extensive experiment involving flowers at several stages was started on May 19, 1950. The last experiment was commenced on May 5, 1951, after an air-filtering system had been installed for the Earhart Laboratory.

Floral changes and time of fertilization. -- In the 1950 experiment, flowers were tagged at the stages of beginning anthesis, revolute corolla and late corolla (see Figure 6) and were observed daily for changes in stage of development. After dehiscence of the corolla the diameters of the developing ovaries were measured weekly until the onset of the changes in pigmentation which mark the attainment of maturity. A number of the flowers of each group abscissed and a few failed to produce normal fruits. In determining the time sequence of stages of floral development (Table 2), only those flowers which did produce normal seeded fruits have been considered.

TABLE 2. Time sequence of the stages of flower development. Groups of twenty flowers initially tagged at stages of beginning anthesis, fully revolute corolla, and late corolla. Results reported for only those flowers of each group which eventually produced mature fruits. Figures represent number of days to pass successively from one stage to the next. Standard errors of the means (S.E.) given for total days to dehiscence.

Tagged at	No. Fls.	Mean number of days: Anth.-Reflexed	Refl.-Revol.	Revol.-Late	Late-Dehisc.	Tagging-Dehisc.	S.E. ±	S.E. as % Mean
Beginning Anthesis	8	1.9	1.9	1.4	1.4*	6.4*	.42	6.6
Revolute	9	-	-	1.1	1.0	2.1	.11	5.2
Late Corolla	7	-	-	-	1.6	1.6	.37	23.1
Overall Means	8	1.9					.23	12.1
	8		1.9				.32	16.8
	17			1.2			.14	11.7
	24				1.3		.13	10.0

*Values given are minimal values, since dehiscence of one half of the corollas of this group had not occurred by the 7th day. The next observation was made on the 14th day.

The data in Table 2 show that approximately two days are required for the opening bud to reach the fully reflexed stage and two more to attain the stage of revolute corolla. Slightly over two days later the corolla will dehisce. Therefore, in a period of slightly over six days all parts of the developing flower attain physiological maturity, the corolla and stamens pass through the stages of senescence, and fruit development is initiated in the ovary. Nitsch (67) has found the same time intervals to prevail in L. esculentum.

When, however, does fertilization occur? It has previously been stated (p. 37) that pollinations are not abundant until just prior to the stage of reflexed corolla. Cooper and Brink (77), who probably employed hand pollination, observed that some fertilizations were accomplished within 24 hours in selfed diploid plants of L. pimpinellifolium but found a slightly longer period was required in selfed tetraploids or in diploid crosses involving pollen from L. peruvianum. At the time of tagging of the flowers mentioned above and reported in Table 2, the corollas, stamens, and styles were prematurely removed from additional groups of twenty flowers each, which were at the stages of revolute and late corolla. Seeded fruits were obtained from three of the flowers treated at the revolute stage and from four treated at the late stage. Since removal of the style should prevent further fertilizations, this demonstrated that fertilization had been accomplished in at least 1/3 of the flowers which, on the basis of the number of fruits obtained from untreated controls (Table 2), should have developed into seeded fruits. The time of fertilization is consequently placed prior to the revolute stage but after the reflexed, probably by one day.

Several points which pertain to the in vitro investigations are to

be found in the above experiment. Flowers which are collected after the stage of reflexed corolla can be considered as pollinated and at least some of those at the revolute and late stages are undoubtedly fertilized. From the standpoint of uniformity of subsequent development in situ, flowers collected at the revolute stage represent the most uniform material for culture. This is shown in Table 2 by the much smaller standard error (S.E.) of the mean days to dehiscence for flowers tagged at this stage.

Growth of the fertilized ovary. — For comparing the development of all fruits in the various tagging experiments, the diameters of the ovaries, measured with a millimeter rule, have been tabulated in Table 3 and plotted in Figure 7. All measurements have been referred to the stage of revolute corolla. For flowers tagged before this stage, time reference was started on the day of its attainment; those tagged in the late stage were considered to be one day past the revolute stage, as determined in Table 2. Values for the initial and one-day diameters (marked with *) were separately determined by measurement of equivalent revolute and late ovaries under a dissecting microscope with an eye-piece micrometer. Only one dimension has been used since Houghtaling (45) has shown that the growth of red currant tomato fruit is essentially spherical throughout its development.

An examination of the data of Table 3 and of Figure 7 reveals that, despite considerable variability, the character of the growth is quite uniform throughout the entire period of development, whether determined with flowers in different initial stages or at different times, seasons, or years. The growth curve shows a slight sigmoid shape similar to that of the common tomato (23, 90). For practical purposes, however, growth

TABLE 3. Normal fruit development of Lycopersicon pimpinellifolium. Results of experiments of 1949, 1950, and 1951. Flowers tagged at stages of beginning anthesis, revolute corolla, and late corolla. Values reported are mean diameters determined at various times after the revolute stage. Figures in parentheses represent the number of fruits from which the mean was calculated.

Date of Experiment:	July 23 1949		July 30 1949	May 19 1950			May 5 1951	Overall means
Stage at tagging	Revolute	Late	Revolute	Beginning anthesis	Revolute	Late	Revolute	
# Tagged	4	8	20	20	20	20	20	
# Developed (%)	3(75%)	4(50%)	12(60%)	8(42%)	9(47%)	7(39%)	18(90%)	
Days after revolute corolla			Mean diameters (mm)					
0								1.03 (34)*
1								1.12 (200)*
2			1.4(12)					1.4 (12)
3	1.0(3)			1.5(1)				1.1 (4)
4		2.2(3)		1.7(2)				2.0 (5)
5			2.9(12)					2.9 (12)
6								
7	2.5(3)				2.8(9)		4.0(9)	3.3 (21)
8		4.3(3)		3.2(1)		3.2(7)		3.4 (11)
9	3.4(3)			3.8(1)				3.5 (4)
10		5.1(3)		3.4(4)				4.1 (7)
11				5.0(2)				5.0 (2)
12	4.9(3)							4.9 (3)
13		6.2(4)	7.0(3)					6.5 (7)
14			6.3(9)		7.1(8)		6.5(9)	6.5 (26)
15				5.8(1)		6.0(7)		6.0 (8)
16				6.8(1)				6.8 (1)
17				6.9(4)				6.9 (4)
18				7.8(2)				7.8 (2)
21	7.3(2)				8.4(9)			8.1 (11)

TABLE 3 (continued)

Date	July 23 1949		July 30 1949	May 19 1950			May 5 1951	
Stage at tagging	Revolute	Late	Revolute	Beginning anthesis	Revolute	Late	Revolute	
Days after revolute corolla	Mean diameters (mm)							Overall means
22		8.6(3)		8.0(1)		7.9(7)		8.1 (11)
23				9.0(1)				9.0 (1)
24				8.0(4)				8.0 (4)
25				9.0(2)				9.0 (2)
28					9.5(9)			9.5 (9)
29						8.8(7)		8.8 (7)
30			9.7(8)					9.7 (8)
37	9.6(3)							9.6 (3)
38		10.0(3)						10.0 (3)
43				10.5(1)				10.5 (1)
44				11.0(1)				11.0 (1)
45				9.5(4)				9.5 (4)
46				10.1(2)				10.1 (2)
49					10.8(9)			10.8 (9)
50						10.0(7)		10.0 (7)
76			10.6(6)					10.6 (6)
Pigmentation	days after revolute corolla							
green	37(1)			43,44,45(3)	49(1)	50(1)		
gr.-yel.				45,46(2)	49(2)	50(1)		
yellow					49(1)			
or.-yel.				45,46(2)	49(1)			
orange	37(2)	38(3)		45(1)	49(1)	50(3)		
or.-red					49(3)			
red			76(6)			50(2)		

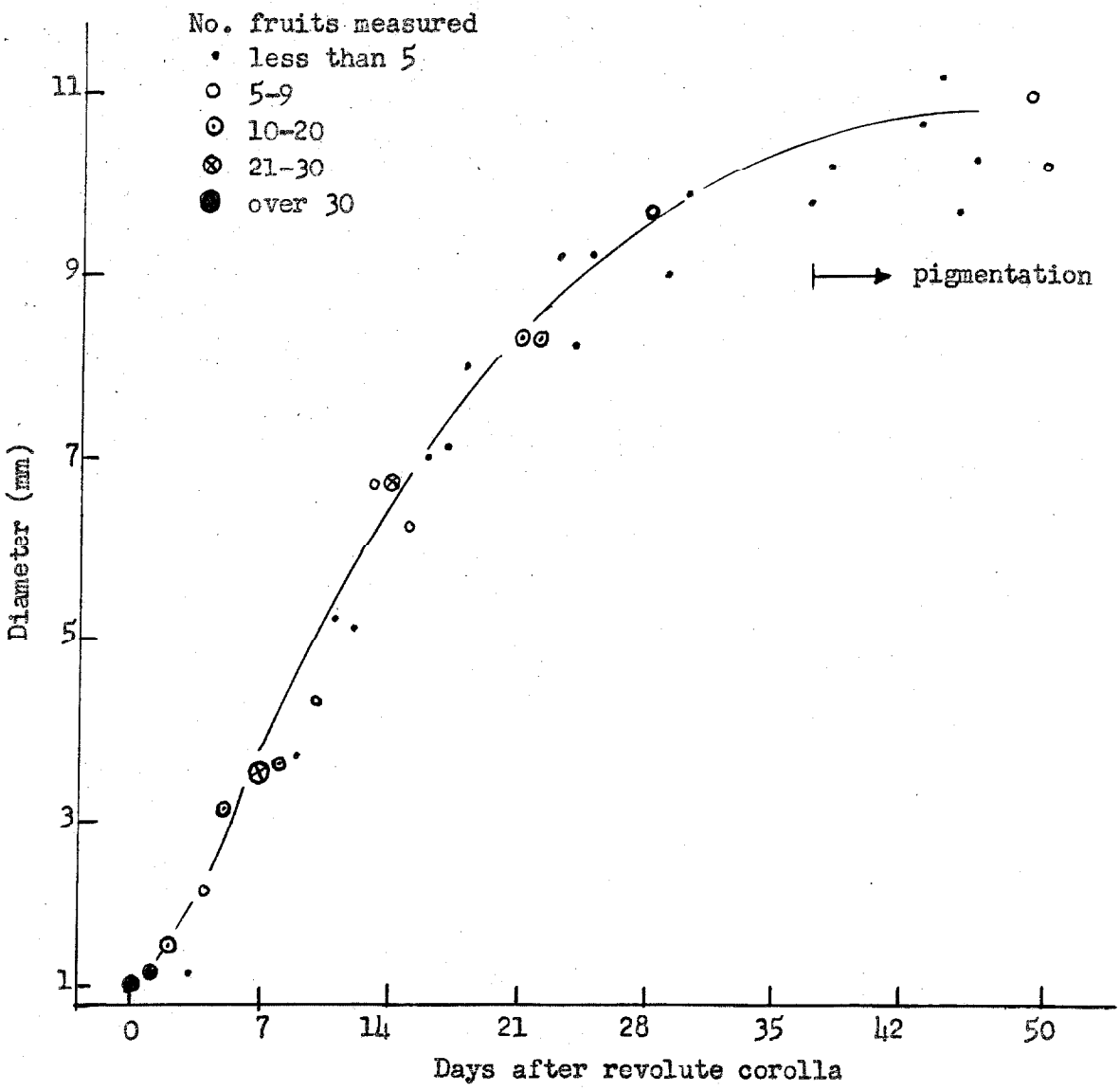


Figure 7. Growth of fruits of Lycopersicon pimpinellifolium. (Data of Table 3). Points represent overall means of fruit diameters determined on the plant at various times after the stage of fully revolute corolla.

in diameter is essentially linear for the first 14 days after attainment of the stage of revolute corolla. Houghtaling (45) has analyzed the growth of several tomatoes from the standpoint of cell enlargement and cell divisions. She has found that during the period of most rapid increase in size all varieties of L. esculentum examined grow by cell enlargement but that L. pimpinellifolium evidently grows by cell divisions as well, since the relative increase in fruit size is greater than that of the individual cells of the pericarp. While the overall character of fruit growth of the two species is the same, the processes which constitute this growth are not.

Mention has been made that the corollas, stamens, and styles were prematurely removed from some of the flowers tagged in 1950 and that some of these treated flowers also produced fruits. The data for the development of these fruits are given in Table 4. If these data are compared with those for fruits from untreated flowers in Table 3, it can be seen that the fruits from the mutilated flowers are in every way comparable to the untreated. No explanation can be given for the variability in the ripening of fruits. Similar variation in final pigmentation has been noted in the regular tomato (90, 91, 92); fruits attached to the plant, although of comparable age and treatments, do not ripen uniformly.

Relationship of fruit development to seed development. -- Dissection of a number of small-sized ripe fruits, which were occasionally observed on the plants, invariably revealed that these contain but few seeds. The tagging experiment in 1951 was consequently designed in a manner that the number of developing seeds could be checked at the end of the linear period of growth. Fourteen-day old fruits were picked

TABLE 4. Development of fruits from mutilated flowers. Corolla, stamens, and styles removed prematurely. Experiment of May 19, 1950.

Condition of flower at time of mutilation	No. Treated	No. Developed	% Developed	Diameters (mm) No. Days after Treatment 7	14	21	28	49	Final Color
Revolute	20	3	15%	4.0	7.5	9.5	11.2	11.8	orange-red
				3.0	6.8	9.0	10.2	11.5	orange
				3.5	6.5	9.0	10.0	11.5	yellow-green
Means				3.5	6.9	9.2	10.5	11.6	
Late	20	4	20%	3.0	5.5	7.5	8.0	9.2	orange
				3.0	6.5	8.5	9.8	11.0	orange-yellow
				2.0	3.8	6.5	8.0	9.2	yellow-green
				2.0	3.5	5.0	6.0	6.8	greenish-white
Means				2.5	4.8	6.9	8.0	9.1	

and measured under a binocular dissecting microscope with a calibrated eyepiece micrometer. Each ovary was also dissected and the number of developing ovules counted. The results, shown in Table 5, have been analyzed according to the methods of Snedecor (93) and the following regression equation has been obtained:

$$E = 5.33X - 8.19$$

in which E is the number of developing ovules (seeds) and X is the ovary diameter in mm on the 14th day after the stage of revolute corolla. By calculation, a 14-day old seedless fruit should have a diameter of 1.54 mm; the one observed was 1.30 mm. The correlation coefficient for these data, 0.97, is highly significant at the 1% level. It would seem that the number of successful fertilizations is one of the most important factors determining the size of the fruit as it develops on the plant.

Natural parthenocarpy. — In each of the tagged flower experiments three types of flower development were observed. The majority of flowers either developed into mature fruits or abscissed. A few, however, showed dehiscence of the corolla but subsequently failed to develop into large fruits. Approximately one half of the abnormal ovaries failed completely to develop further. The remainder, on the other hand, grew slowly, at least for a time. Two of these ovaries obtained a maximum diameter of 2.0 mm, one by the 10th day and the other by the 8th day after the stage of revolute corolla. Both were dead by the 22nd day.

In only one instance has one of these small fruits survived the entire period of normal fruit development. This fruit developed from a flower tagged at beginning anthesis. Two days after tagging, the

TABLE 5. Influence of seed development on fruit development *in situ*. Analysis of 9 fruits developing on the plant 14 days after tagging of flowers in fully revolute condition. Experiment of May 5, 1951.

	X diameter mm	Y no. of seeds developing	XY
	8.00	31	
	6.45	28	
	7.80	36	
	7.50	38	
	8.00	32	
	8.00	32	
	6.50	26	
	3.23	6	
	1.30	0	
Sums	56.78	229	1694.75
n=9			
Means	6.31	25.44	

Squares
x^2 46.88 y^2 1418.22 xy 250.01

Regression equation: $E = 5.33X - 8.19$
Correlation coefficient: $r = 0.97$.
Significance of r: highly significant; for significance at 1% level, 7 df, $t = 2.67$, found 10.60.

flower had almost reached the reflexed stage and two days later it was fully revolute. Up to this point development seemed perfectly normal. Dehiscence of the corolla, however, did not occur until the 10th day after revolute corolla and at this time the ovary had a diameter of 1.0 mm. On the 17th day after the stage of revolute corolla the diameter of the ovary was 1.8 mm. If growth from the 10th to the 17th day was linear, on the 14th day the diameter would have been about 1.45 mm, very close to the theoretical seedless diameter of 1.54 mm. During the remainder of the experiment growth of this ovary continued, evidently at a uniform rate of about 0.2 mm/wk. On the 45th day, when the experiment was stopped, the diameter was 2.5 mm, the color was still green, and dissection revealed no developed ovules. This was without doubt a true case of natural parthenocarpy in L. pimpinellifolium. Clendenning (94) has applied the term "laggard fruits" to similar slow-growing parthenocarpic fruits of commercial greenhouse tomatoes, which develop both naturally and from some auxin-treated flowers.

Influence of Environmental Factors on Reproduction.

Light and temperature. -- During the summer months of 1949 the plants which were being grown outside of the greenhouse under the normal climatic conditions of Pasadena were obviously not responding well in either vegetative or reproductive development. When the facilities of the Earhart Laboratory (Phytotron) became available, an experiment was conducted to find the optimal growing conditions for the red currant tomato, such as had been done for the common tomato by Went (38, 95, 96).

Plants were germinated and grown as described previously. On January 8, 1950, all plants had produced at least one mature inflo-

rescence and the first flowers had opened. They were then trimmed to one stem and one mature inflorescence, randomized into lots of six, and placed under the conditions listed in Table 6. Stem lengths were measured at the beginning of the experiment and again 10 days later when any shock effect from change of environment should be over. For the first few weeks the plants were kept trimmed to one stem. However, it was observed that removal of the axillary shoot which normally develops in the axil of the third leaf of each growth cycle (see p. 29) apparently releases the inhibition of the buds in the axils of the first and second leaves. More error was therefore introduced by this practice than was prevented, even more so since removal of all buds led to rapid formation of numerous adventitious buds in each axil. The pruning was consequently discontinued. When the experiment was terminated on March 1 and 3, 1950, all lateral shoots were removed and discarded, the main axes were measured, and leaves of the main axes were pooled from each group. Roots of each group were washed free of all gravel and also pooled. Dry weights of roots and leaves were determined after drying in a 70° forced-air oven. In addition, the number of inflorescences, flowers, fruits, and buds were counted for each plant. The results are given in Tables 6 and 7 and the data are plotted in Figures 8 and 9.

Perhaps the most striking fact which the data of Table 6 reveal is the generally better growth under the natural photoperiod with fluctuating temperatures. When a thermoperiodic differential of 6° is employed, the condition of 23° day—17° night is optimal for dry weight production in both leaves and roots regardless of photoperiod; however, stem elongation under these conditions in minimal. Under an eight hour photoperiod, stem length and number of inflorescences increase with an

TABLE 6. Vegetative growth of Lycopersicon pimpinellifolium under controlled conditions. At the start of the experiment the plants used were 65 days old and all showed one to two inflorescences below the terminal bud under conditions of 20° day - 14° night - normal daylength. After trimming to one mature inflorescence per plant, groups of 6 plants were transferred to the conditions indicated. Experiment started with the night temperatures on January 8, 1950.

Experimental Conditions			Stem Length (cm) Total of 6 plants			Tip growth rate mm/day/plant		Final dry wts. Total of 6 plants grams		No. new inflorescences	
Day T°C	Nite T°C	Light hrs.	Init.	Increases 10 days	Increases 54 days	first 10 days	next 44 days	Leaves	Roots	per group	per plant
26	20	N	53.5	127.5	1131.0[a]	21.25	38.01	18.72	7.46	63	10.5
	26	8	52.0	140.5	812.0[a]	23.42	25.44	10.65	2.23	59	9.8
	20	8[b]	51.0	113.5	721.0[a]	18.90	23.02	13.49	4.35	55	9.2
23	17	N	48.0	91.5	979.5	15.25	33.63	22.67	8.04	59	9.8
	23	8[c]	63.5	129.75	788.3	21.63	24.60	14.94	4.27	60	10.0
	17	8	62.5	76.5	625.5	12.75	20.80	19.15	7.89	49	8.3
20	14	N[c]	66.0	57.0	1024.5	9.50	36.65	16.29	5.25	54	9.0
	20	8[b]	64.0	84.5	704.0	14.10	23.45	15.21	5.22	56	9.3
	14	8	50.0	44.0	563.5	7.33	19.68	17.62	5.90	41	6.8

N - Normal daylength
a. Plants harvested on 52nd day; 54-day increments calculated from growth rate.
b. Figures for 6 plants calculated from results obtained on 5 plants.
c. Figures for 6 plants calculated from results obtained on 4 plants.

TABLE 7. Flower and fruit development of Lycopersicon pimpinellifolium under controlled conditions. Number of reproductive parts still developing after 54 days of treatment. Plants and conditions as in Table 6.

Experimental Conditions Temperature Day	Night	Light hrs.	Inflorescences per 6 plants	Individual parts Opened Buds*	Flowers	Fruits	Total
°C							
26	20	N	63	0	1	4	5
	26	8	59	0	0	0	0
	20	8	55	0	1	0	1
23	17	N	59	22	17	55	94
	23	8	60	0	0	0	0
	17	8	49	0	1	12	13
20	14	N	54	0	1	0	1
	20	8	56	2	0	2	4
	14	8	41	2	1	2	5

*Only healthy pre-anthesis buds larger than 1 mm in length have been considered. Many yellowed buds in various stages of development were present in all treatments.

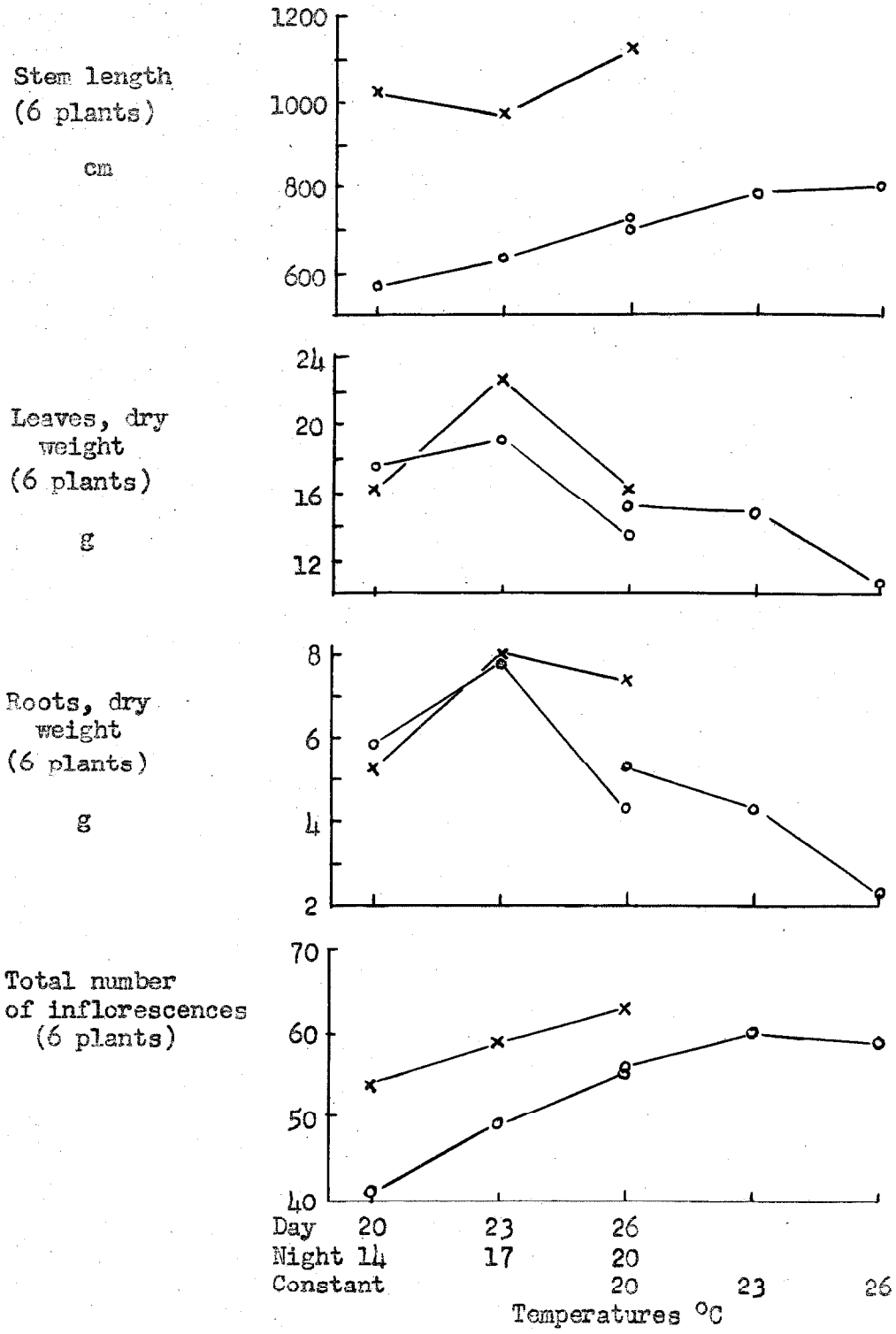


Figure 8. Growth of Lycopersicon pimpinellifolium at different temperatures. Data of Table 6. Circles, 8 hour photoperiod; crosses, natural daylength (average 10.75 hours).

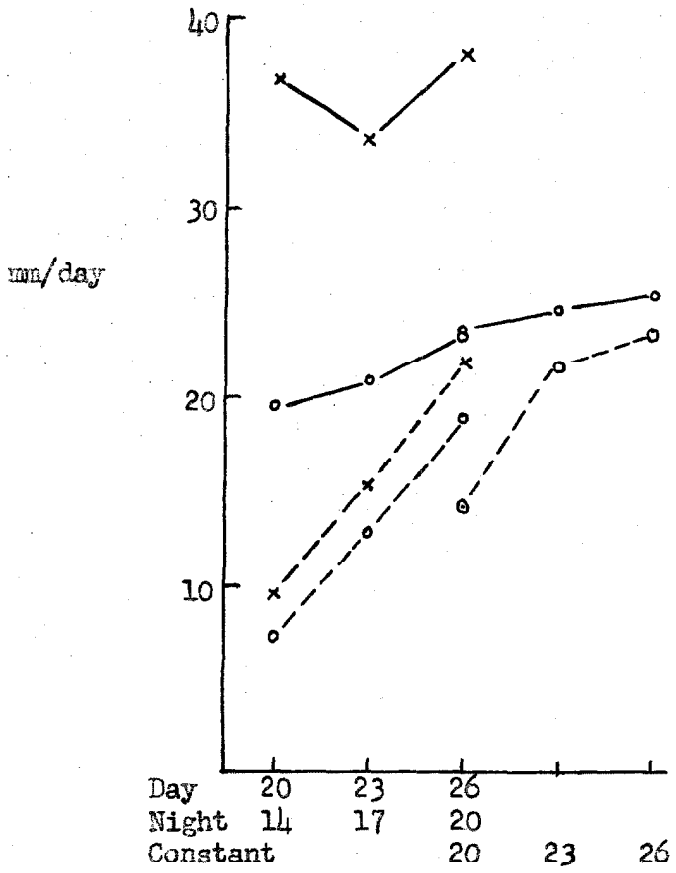


Figure 9. Relationship of growth rate of stem tip to temperature and age of plant. Circles, 8-hour photoperiod; crosses, natural daylength. Broken lines, growth rate during first 10 days; solid lines, growth rate during next 44 days. Data of Table 6.

increase in night temperature but are independent of day temperature. The ratio of total elongation to number of inflorescences (growth cycles) remains almost constant. As calculated from the data of Table 6, these ratios, according to night temperatures during 8-hour photoperiod groups, are as follows:

Night Temperature	Stem Length / Growth Cycle
	mm
14°	13.7
17	12.8
20	12.6, 13.0
23	13.1
26	13.8

Under natural photoperiod these ratios for the first three night temperatures are 19.0, 16.6, and 18.0 mm, respectively.

It seems then that in Lycopersicon pimpinellifolium the overall top growth rate (that is the number of growth cycles formed) is temperature dependent. The dependency is chiefly on the night temperature, and under an 8-hour photoperiod an increase in the night temperature within the range of 14°-26° results in an increase in top growth. A flat optimum is evident between 23-26°. Increasing the photoperiod from 8 hours to the natural daylength (average 10.75 hours during the course of the experiment) results in uniform increases in top growth rate at all temperatures studied. The rate of reproductive development (formation of inflorescences) is identical with the top growth rate. Vegetative development, however, is differentially affected by changes in either temperature or photoperiod. At low night temperatures, stem

growth is preferentially increased by increase in photoperiod; the dry weights of both leaves and roots are little affected at this temperature. Above 17°, however, the nature of the relative increases effected by lengthening the photoperiod is such as to suggest that stem growth is in manner of a competitive process with leaf and root growth.

The rate of stem elongation at different ages and its marked dependence on the temperature of the nyctoperiod under an eight-hour photoperiod are very similar to the findings of Went (38, 96) for Lycopersicon esculentum. In L. pimpinellifolium, however, there is no thermoperiodic stimulation of the stem growth rate, except in younger plants. On the other hand L. esculentum shows practically no response to photoperiod, whereas this is one of the most prominent features of this experiment. Another difference in the two species is found in the influence of temperature on the rate of differentiation of new structures from the apical meristem. Went (96) observed only a slight effect of night temperature on the number of stem nodes between inflorescences or on total number of nodes. In this experiment the number of leaves per growth cycle did not vary -- three leaves per inflorescence --, but the total number of growth cycles was increased with increase in night temperature. This type of response (change in rate of meristematic activity) has been found for a number of other plants, however, even though it does not apply to the common tomato (97).

The data of Table 7 would indicate that for flower and fruit production there is an optimal night temperature at 17° C and a photoperiodic response as well. However, it should be remembered that each inflorescence produces about 20 flowers during its development. The group with the smallest total number of inflorescences should consequently

TABLE 8. Flower and fruit development under different environmental conditions. Summarized from tagged-flower experiments of 1949, 1950, 1951.

Date of Expt.	July 23 1949	July 30 1949	May 19 1950	May 5 1951
Environment	Outdoors	Outdoors	Phytotron	Phytotron (filtered air)
		Numbers		
Flowers tagged	12	20	60	20
Identity lost	1	0	4	0
Considered	11	20	56	20
Seeded Fruits	7	11	24	17
"Seedless" Fruits	2	3	2	1
Flower Abscissions	2	6	30	2
		Percentages		
Abscissions	18%	30%	43%	10%
"Seedless"	18	15	4	5
Seeded Fruits	65	55	54	85

have had a minimum of 800 flowers. Only 5 had developed. Very heavy flower drop and necrosis of flower buds was observed. Apparently some factor, not yet under control, was influencing reproductive development after initiation of the inflorescence.

Smog. -- At the time that the above experiment was being conducted, some smog damage was occurring in the Phytotron to a number of plants which are known to be smog-sensitive. Many of the symptoms of such damage were quite evident on the red currant tomato. In the spring of 1951 an air-filtering system was installed for the Phytotron. The improvement in growth and development of the plants of red currant tomato was spectacular. Within a week differences in vegetative growth were noted. All new leaves were larger, greener and showed no evidence of damage. The mild chlorosis of stem tips, which had been frequently noted before, could no longer be found and in subsequent growth every leaf which developed on a stem remained alive and healthy for long periods of time. Fruit set which had seldom been greater than six to eight fruits per inflorescence increased to as many as fifteen.

A comparison of reproductive development under different environmental conditions has been made in Table 8. Data of the various tagged flower experiments have been reassembled and the types of flower development classified. There is little doubt that the overall improvement of both vegetative and total reproductive development is due to the elimination of smog. Since the 1951 experiment was used to demonstrate the effect of seeds on fruit size, however, the detrimental effects of smog must be on some process prior to fertilization. Three explanations immediately come to mind. First, smog may be lethal to pollen; second, it may effect the growth rate of the pollen tube; or third, it may,

through its recognized oxidizing character, perhaps cause destruction of some essential growth factor(s). No evidence exists as to whether or not any one of these is the correct interpretation; however, the last seems most likely in view of the extensive killing of buds in all stages of development. It apparently does not affect fruit growth, except through its prevention of fruit initiation.

2. Standardization of Culture Techniques and Elimination of Variables

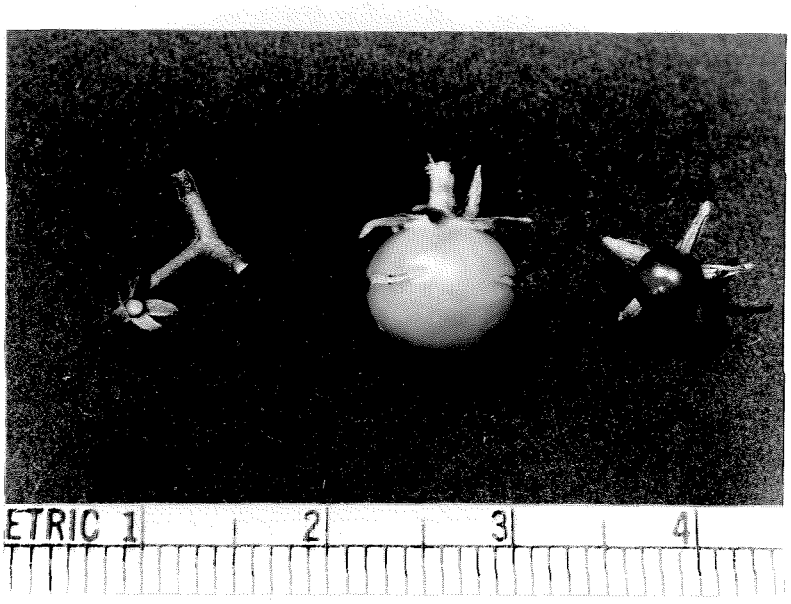
In exploratory experiments to obtain fruit development *in vitro* complex media were employed. Many of the various growth factor complexes, such as yeast extract, malt extract, casein hydrolysate, vitamins, and plant hormones, which had been used in preliminary investigations of other tissues and organs, were included in these media in a number of different combinations. Initial success with flowers of *L. pimpinellifolium* on one of these consequently provided a stepping stone for more critical examination of the growth factor requirements. Before this could be conveniently undertaken, however, it was necessary to establish standard conditions for cultivating these fruits. Technical questions concerning the development of fruits *in vitro*, such as, how best to measure growth, detrimental factors arising from practices employed, selection of uniform material, etc., all required investigation in an effort to eliminate the variation in growth responses initially obtained. These points will be taken up in this section after first describing the original conditions.

Original Medium and Conditions.

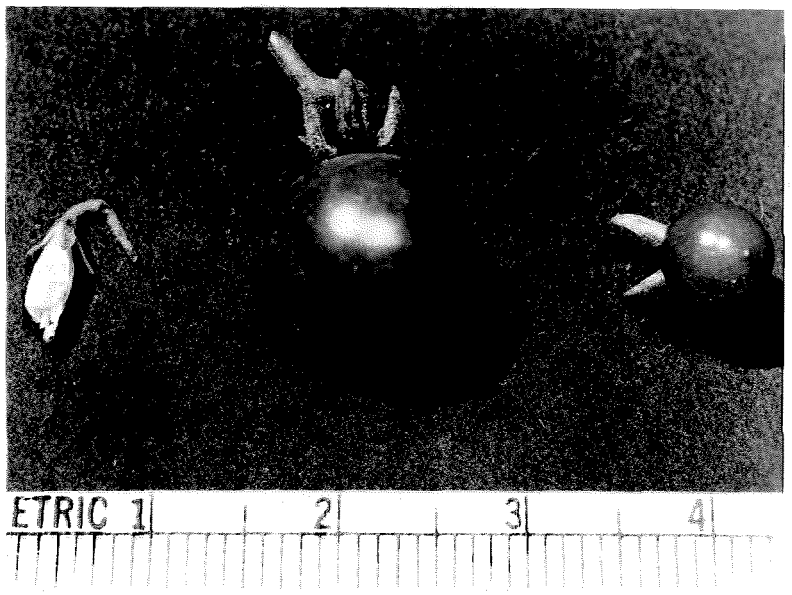
First success in culturing the flowers of red currant tomato was achieved in the following manner. A number of inflorescences were collected from the plants and each was dipped basally into petrolatum immediately after excision. They were then brought to the laboratory in a covered dish lined with moist filter paper. Sterilization was accomplished by immersing each inflorescence for five to ten minutes in

1% NaOCl to which a small amount of detergent (approximately 50 mg Dreft/ 50 ml solution) had been added. Flowers were excised individually above the abscission zone of the pedicel and were immediately planted in test tubes containing sterile nutrient media. Flowers at the stages of beginning anthesis and revolute corolla and developing ovaries of flowers from which the corolla had just dehisced were used in this initial planting. The media consisted of solutions of either Bonner's or Gautheret's inorganic salts, trace elements, 4% sucrose, amino acids (casein hydrolysate) 100 mg/l, a mixture of B-vitamins (used in Neurospora research), and indoleacetic acid (IAA) 10 µg/l. One portion of each of the two types of media was solidified with 0.75% agar and the rest was used in liquid form with glass wool as a supporting substrate. The pH was initially adjusted to 5.5. All cultures were kept in a dark cabinet, except when removed for examination. The room temperature was regulated at approximately 25° C.

Development of the excised flowers was obvious in all cultures after several days. Most of the flowers initially in the stage of beginning anthesis had opened up completely, those at the stage of revolute corolla showed evidence of corolla dehiscence, and one from which the corolla had already dehisced at the time of planting showed significant enlargement of the ovary after the fifth day. The initial diameter of this ovary was approximately 1 mm. Ten days after planting it was 3 mm, and by the 56th day it was beginning to turn yellow on one side and the diameter was about 7 mm. On the 84th day it was uniformly orange in color and its appearance was normal, except for the absence of seeds. Both of the cultured fruits shown in Figures 10 and 11 developed on liquid media, the larger (described above) developed on a medium



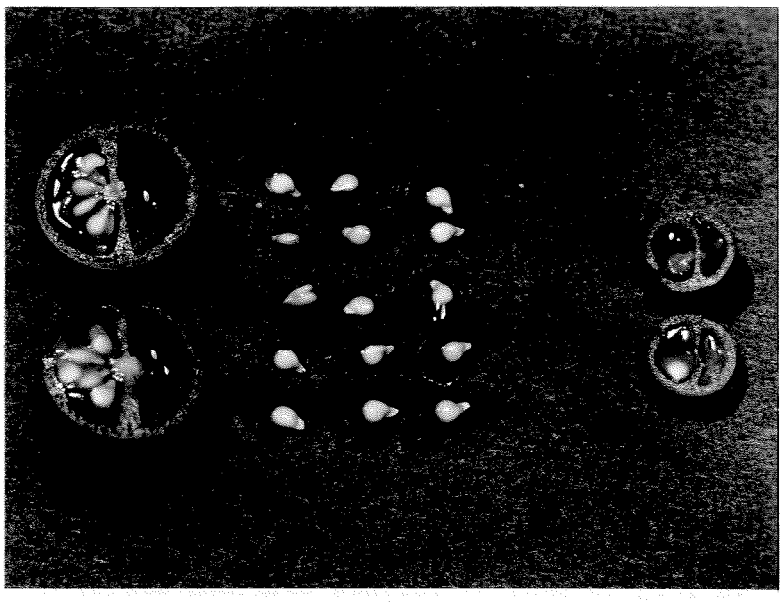
A



B

Figure 10. Fruits of L. pimpinellifolium. A. Fruits grown in culture. B. Fruits developed on the plant. Initial size of the ovary and stage of flower development (late corolla) originally planted shown on left in A and B, respectively.

A



B

Figure 11. Same fruits as in Figure 10 A, B, cut open to show contents. A. Fruits grown in vitro. B. Fruits developed on the plant.

containing Gautheret's salts and the smaller, on Bonner's salts. In the latter case the initial stage of the flower was revolute. A third fruit of approximately the same size and red coloration developed on Gautheret's liquid medium from a revolute flower, and two smaller fruits, one from a revolute flower and the other from an already developing ovary, grew to maturity on Bonner's agar medium. Pigmentation of all of the smaller fruits was well advanced by the 56th day of culture.

Criteria for Measurement of Growth.

As soon as it became evident that growth of the excised flower was possible, a program for the systematic study of the cultural conditions was initiated. Two criteria for the convenient measurement of developing ovaries were at hand. Since the diameter of the fruits increases equally in all directions throughout their entire period of growth, a single measurement of diameter should be representative of the fruit volume at any given time. An alternative method was that of weight. Because of the small initial size of the ovary, however, it was necessary to resort to an eyepiece micrometer for linear measurements and to use an analytical balance for weight determinations. Even with the analytical balance it was necessary to employ groups of ovaries for accuracy. In separate determinations of three groups of ten ovaries, excised from flowers at the revolute and late stages, weights of 4.5, 4.5, and 4.6 mg/group were obtained. However, since not all ovaries grew in culture, it seemed necessary to measure them individually. Thus the first stages of ovary growth, starting from an initial weight of 0.5 mg/ovary, would involve measurements which approach the limit of sensitivity of the balance. The weight method did not seem promising.

During the first phase of experimentation, however, both diameter and weight determinations were made in order to compare the two methods. Results obtained are given in Table 9. The statistics revealed an almost perfect linear correlation between weight and the cube of the diameter. The methods were therefore equally representative but, for convenience, the diameter was chosen for further work.

The results of the experiments included in Table 9 disclosed a number of facts which suggested changes in the design of further experiments. First of all, it can be seen that growth increments amounting to a 250-fold increase in volume or 90-fold increase in weight were possible. Not all flowers developed, however. In fact a total of 125 ovaries were included in these cultures, but even on the medium containing all components many were dead by the third day. By the seventh day, ovaries which survived could be separated into two categories, one group which showed considerable increase in diameter and another in which growth was quite slow. Finally, there was a rather wide range of fruit sizes obtained in the longer periods of culture. Elimination of these variable responses seemed imperative.

Relationship of Ovary Diameter at Time of Planting to Subsequent Development.

Since so few flowers actually grew in culture and since it was found that the initial diameters of ovaries varied, it seemed entirely possible that the fruits which developed might represent only the larger of these ovaries. In continuing the investigations it was found possible to obtain good growth from the ovaries of flowers from which the corolla was prematurely removed at the time of planting. For

TABLE 9. Relation of growth in weight to growth in diameter of fruits cultured on various media. Experiments of May 12, June 18, and June 22, 1949. Standard errors are given for group means of smaller ovaries.

Medium: Sucrose	Casein hydrolysate	B-Vitamins	IAA 10 g/l	Days in culture	Number of Fruits	Diameter (micrometric units) mm	Diam.^{3} X mm^3	Weight Y mg	XY
				0	39	0.80 ± .02	0.5	0.5	
4%	X	X	X	3	8	1.20 ± .10	1.7	1.05	
				7	12	1.08 ± .04	1.2	0.8	
				7	1	1.65	4.5	2.4	
				7	1	1.88	6.9	3.75	
				42	1	3.83	57.1	21.7	
				42	1	3.83	57.1	22.8	
8%	X	X	X	42	1	2.93	22.5	9.9	
4%	X	X		42	1	3.15	31.3	13.5	
				42	1	2.93	22.5	11.2	
				42	1	5.10	132.7	46.8	
4%		X	X	42	1	3.15	31.3	13.0	
				42	1	2.25	11.4	5.3	
				42	1	2.40	13.1	5.4	
				42	1	3.30	35.9	14.5	
				42	1	3.45	41.1	15.6	
4%	X		X	42	1	2.10	9.3	4.3	
				42	1	2.48	15.0	7.2	
				42	1	3.90	59.3	17.5	
Sums					75		598.5	252.35	12,469.55

Sum of squares x^2 29,551.54
y^2 3,833.58
xy 10,455.80

Correlation coefficient: r = 0.982.
Significance of r: highly significant; at 1% level, for 73 df, t = 2.645; found, 95.24.

reasons to be discussed later, it was also found desirable to change from liquid to agar media. By planting such mutilated flowers on agar in Petri dishes, the cultures could be easily maneuvered under a dissecting microscope and the growth of individual fruits followed by micrometric measurements without affecting sterility.

In Table 10 are given the statistics of data obtained in the above manner for individual ovaries on two different media. There is no correlation between the initial diameters and the size attained in fourteen days by the cultivated ovaries, nor is there any statistical difference between the initial diameters of those which develop into fruits and those which fail to develop. A comparison of the fruits grown in vitro with equivalent fruits which develop on the plants has also been made in Table 10. The range of 14-day diameters of the fruits cultured on the glutamic acid medium was from 1.58-3.38 mm and on the tryptophan medium from 1.50-3.00 mm. All but the smallest of these, then, are larger than the 14-day old natural parthenocarpic fruits (1.54 mm). It is probably quite significant that the variability of the cultivated fruits is no greater than that of the natural seeded fruits, among which variation in size has been correlated with the number of effective fertilizations.

Inasmuch as no significant correlation can be shown between the initial diameter and the size of the cultured fruit which developed, it is felt that the variation in initial diameter shown in Table 11 is not important. Since the standard error is expected to decrease with increase in the size of the sample, the revolute stage represents a much more uniform material with respect to the initial diameter of

TABLE 10. Analysis of relationship of size of cultured fruits to diameter at time of planting and to equivalent fruits on the plant. (See Table 5).

Experiment No.	VIII-9	VIII-10	Normal Fruits on the Plant
Medium: mineral salts and 4% sucrose plus	Glutamic acid 10 mg/l	Tryptophan 10 mg/l	
Ovaries which failed to develop			
Number	10	10	
Mean initial diameter	1.09 ± .04	1.07 ± .06	
Ovaries which developed			
Number	6	6	9
Diameters			
Initial (X)	1.12 ± .03	1.03 ± .02	-----
14-day (Y)	2.45 ± .29	1.99 ± .18	6.31 ± .81
Correlation coefficient (r_{xy})	0.30	0.46	-----
Level of significance of r_{xy}	n.s.*	50%	-----
Standard error of 14-day diameter as % of mean	11.8%	8.8%	12.8%

*n.s. = no significance at 50% level

TABLE 11. Natural variation in ovary diameters of flowers collected for culture at various times. Figures in brackets represent the number of replicates.

Floral Stage at Time of Collection	Date	Mean Diameter mm
Late	June 22, 1949	0.80 ± .15 (39)
Late	July 14, 1949	1.10 ± .02 (200)
Revolute-Late	July 16, 1949	1.07 ± .01 (119)
Late	August 6, 1949	1.00 ± .01 (34)
Revolute	January 16, 1951	1.04 ± .01 (16)

the ovary. This stage has also been previously shown to be a much more accurate indicator of further development of the flower on the plant than is the late stage. Revolute flowers have consequently been employed in most of the later experiments. The initial diameter of the ovaries of these flowers, for all practical purposes, can be said to be roughly 1.0 mm.

Sterilization of Flowers and Fruits.

The five-minute sterilization of flowers which was effective in the original experiment later proved to be too short for the elimination of contaminations, particularly in flowers collected from the outdoor plants. In an experiment commenced on June 24, 1949, longer immersion times were employed in order to eliminate this difficulty. Periods of ten, fifteen, and twenty-five minutes were all effective against contamination but twenty-five minutes was apparently sufficiently long to adversely affect development in culture (Table 12). In subsequent experiments a ten-minute sterilization was employed but even this duration seemed to be somewhat detrimental. Out of one group of 130 flowers, fruits developed from only three and one contamination occurred. Of 127 planted on July 11, 1949, 92 were definitely necrotic by the seventh day of culture and most of those remaining showed some brown necrosis of the stylar scar area. Use of 0.5% NaOCl-Dreft did not eliminate this stylar necrosis and was less effective against contaminations.

Zephiran Chloride* was therefore tried. In early trials using

*Zephiran Choride is a product of Winthrop-Stearns, Inc., New York. The manufacturer's pamphlet describes the compound as a brand of benzal-

TABLE 12. Effect of duration of sterilization with 1% NaOCl-Dreft on development of ovaries in culture. Pooled results of flowers planted on several growth-supporting media after each of the following times of sterilization. Equal numbers of the variously treated flowers were planted on each medium. Experiment of June 24, 1949.

Minutes of Sterilization:	10	15	25
Number of flowers planted	81	41	30
Number of fruits developed	20	10	5
% Development	24.7%	26.8%	16.7%

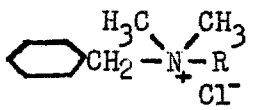
this preparation in dilutions of 1:20,000, 1:10,000, and 1:5000 for ten minutes no stylar necroses were observed; however, only the 1:5000 dilution was effective against contaminants. In addition, since this agent is a cationic detergent in itself, its use was much preferred to the hypochlorite-detergent mixture. In later experiments the length of sterilization periods was gradually increased from 10 minutes to 25 minutes with no measurable effect on fruit development. With flowers from plants grown under filtered-air conditions use of Zephiran Chloride in a concentration of 1:5000 for 25 minutes, followed by a sterile rinse, has given 95-100% sterility with regard to contaminations and from 80-100% of the ovaries have developed in culture.

Smog-temperature History of Flowers.

In the same manner as with the development of fruits on the plants the influence of smog has been detected in growth responses of fruits in vitro through the use of flowers from plants developing in an air-filtered atmosphere. In most of the early experiments it had been possible to distinguish three types of development — ovaries which failed to grow almost completely, ovaries which developed for a short time (7-14 days) but only very slowly thereafter, and those which continued to increase for periods of 6-8 weeks. If, then, we designate these developmental types as 0, I, and II, respectively, it becomes

---

konium chloride, having the following structure:



R represents a mixture of the alkyls C_8H_{17}- to $C_{18}H_{37}$-, as in the fatty acids of coconut oil.

possible to make some comparisons between cultures at various times and from materials of different history. By way of a more precise definition, the Type I developments represented a significant increase in diameter; however, after 14 days of culture these fruits seldom exceed 1.5 mm. In addition, the following distinctions have been noted.

At the time of planting, ovaries of flowers in the revolute and late stages have a rounded pentagonal outline and a dull-textured surface which is discernible under the microscope. As the ovaries commence to grow, those which have been classified as Type II not only do so at a more rapid rate but also rapidly lose the pentagonal shape and develop a lustrous appearance. The Type I ovaries undergo all of these changes at a much slower rate. Developing fruits (ovaries) have been assigned the Type I classification, in general, only when at least two of these criteria -- slow rate of growth, small diameter, pentagonal shape, or dull-textured surface -- are apparent at the end of the first two weeks of culture.

In Table 13 are assembled the growth response data for fruits grown on the minimal medium (mineral salts and sucrose) at various times during the course of these investigations. There is no doubt about the effect of filtering the air, it resulted in almost complete elimination of the growth failures (Type 0) in culture. Previous high temperature exposure (hot summer weather), however, seems to have been less important, if at all, since the flowers collected under natural environmental conditions of Pasadena did not differ materially from those from the controlled, non-air-filtered conditions of the Phytotron. Variations in the experimental technique also seem unimportant, either

TABLE 13. Effects of previous smog-temperature history and variation of experimental techniques on cultural development of fruits of red currant tomato. Sterilization: 25 min. with 1:5000 Zephiran Chloride. Minimal medium: (mineral salts, sucrose). For explanation of growth types see text. Abbreviations: (l), late flowers; (r), revolute flowers; (n) natural environment of Pasadena; (c), controlled environment, 20° C day temp., natural photoperiod, 14° C night temp.

Date	Floral History: Environment	Floral History: Filtered Air	Floral Stage at Collection	Corollas Removed	Number of Flowers Planted	Growth Types: 0	I	II
						Number of ovaries		
6 August 1949	n	-	l	/	37	24	1	12
2 February 1950	c	-	l	/	83	77	5	1
5 April 1950	c	-	l	/	30	22	1	7
7 April 1950	c	-	r	/	50	20	4	26
17 January 1951	c	-	r	-	80-100*	22-42	16	42
3 May 1951	c	/	r	-	20	0	1	19**
11 May 1951	c	/	r	-	10	0	1	9
16 May 1951	c	/	r	/	10	0	0	10
4 April 1952	c	/	r	-	38	3	14	21

* At this time from 40-50 flowers were being planted in large Petri dishes. After dehiscence of the corolla in culture, only those flowers which were evidencing growth and which were not contaminated were transferred to new media. The figures represent two such initial dishes.

** Compare with corresponding tagged-flower experiment in Table 8.

with regard to stage of flowers planted or to mutilation by premature removal of the corolla.

Effects of Various Manipulations on Growth of Ovaries.

Use of liquid media was abandoned early because of detrimental effects on growth of the ovaries in culture. Submersion of the flowers during or after planting or accumulation of a capillary film of liquid in the calyx around the base of the ovary resulted in almost complete prevention of growth. Nitsch (67) has observed similar detrimental effects of liquid films on cultivated ovaries of L. esculentum. Clendenning (91, 94) has shown that all gas exchange in the common tomato takes place through a lenticular ring at the base of the fruit. Microscopic examination has shown that the epidermis of the fruits of L. pimpinellifolium, like that of L. esculentum (78), is without stomates, so that the explanation that prevention of gas exchange prevents growth undoubtedly holds for this species as well. This may also be the reason for repeated failure to obtain growth of ovaries excised from flowers, since such ovaries must of necessity be seated in the medium.

The above observations pertain to several points of technique. First, use of media solidified with agar reduces danger of entry of free water into the calyx cup; 1% agar has been found best. Second, flowers must be thoroughly drained after sterilization. On several occasions, however, it has been noted that small amounts of water do not prevent later growth, providing evaporation takes place within a few days. On one such occasion, increase in diameter of a partially flooded ovary was delayed until the second day, but from the second

to the third day the growth rate (0.3 mm/day) of this delayed ovary was greater than the initial rate (0.21 mm/day) of six other fruits on the same medium.

The following points regarding manipulation of the flowers have not been found to influence development in vitro in any assessable measure: elimination of the petrolatum dip at time of collection, collection of individual flowers rather than inflorescences, and sterilizations of the flowers with Zephiran Chloride before or after removal of the corolla, stamens, and style (the mutilation practice). The effect of mutilation itself, however, is shown in the following paragraphs.

Effect of Mutilation of Flowers at Different Stages on Growth in Culture.

The relationship of the stage of flower development on fruit development in vitro was determined in the following experiment. Flowers were collected at the stages of beginning anthesis, reflexed corolla, revolute corolla, and late corolla. The flowers were mutilated after sterilization and planted on either minimal medium (M) or minimal medium supplemented with B-vitamins and casein hydrolysate (MVC). The 7-day diameters of ovaries which developed are given in Table 14. Those which grew further by the 14th day are marked (*). Thus the data starred represent Type II ovaries, the others Type I.

Although the data of Table 14 show distinct differences in distribution of the growth responses due to stage of flower development and to supplemental growth factors, they do not reveal effects of mutilation. The parallel experiment using non-mutilated flowers planted at

TABLE 14. Growth of ovaries of excised flowers collected at various stages and mutilated at time of planting. M, minimal medium; MVC, minimal, B-vitamins, and casein hydrolysate. Diameters measured after seven days; fruits which developed further by 14th day designated (*). Experiment of April 18, 1950.

Floral stage	Beginning anthesis		Reflexed corolla		Revolute corolla		Late corolla	
Medium	M	MVC	M	MVC	M	MVC	M	MVC
Growth response:								
Type 0	12	11	13	10	9	8	12	5
Type I	1	2	0	0	1	3	0	2
Type II	0	0	0	3	3	2	1	6
Diameters (mm)	1.50	1.28 1.50	--	1.68* 1.70* 1.85*	1.50 1.75* 1.90* 1.93*	1.38 1.50 1.50 1.83* 1.90*	1.68*	1.43 1.50 1.80* 2.08* 2.15* 2.23* 2.30* 2.50*

the stage of beginning anthesis (Table 15) should be considered in conjunction with the data of Table 14 in this regard. It appears that the Type II response is restricted to flowers which had reached at least the reflexed stage when the flowers were mutilated at planting. Younger flowers, planted whole, from which the corolla was allowed to dehisce naturally in culture, developed almost entirely as Type II fruits. Since neither Type 0 or Type I ovaries were eliminated at any stage nor in the non-mutilated treatment, the effect of mutilation would appear to be limited to the Type II fruits. Supplements in the medium, while not obligatory for growth of the Type II ovary, seem to have a promotive effect which is particularly evident on the ovaries of the late-stage mutilated flowers and on those of flowers planted whole. In these cases the growth supplements not only bring about absolute increases in growth of the Type II ovary but also a redistribution of the various responses in favor of Type II.

Significance of the Growth Response Types.

The following points have been established from observations and from experiments with flowers attached to the plant: 1) pollination occurs just prior to the stage of reflexed corolla; 2) fertilization is accomplished, at least in some ovaries, by the time of attainment of the revolute stage; 3) mutilation results in a reduction in the number of seeded fruits obtained from pollinated flowers; and 4) "elimination of smog" brings about an increase in number of seeded fruits and a decrease in both flower drop and number of "seedless" fruits. Also, it has been shown that the diameter of the 14-day old seedless fruit is approximately 1.5 mm.

TABLE 15. Growth of ovaries from flowers planted at the stage of beginning anthesis without removal of the corolla, stamens, and style (non-mutilated). Diameters measured after 14 days in culture. Media same as in Table 14. Experiment of April 20, 1950.

Medium	Minimal (M)	Supplemented (MVC)
Number of flowers planted	18	20
Growth response:		
Type 0	6	3
Type I	3	0
Type II	9	17
Mean diameters (mm)		
Type I	1.55 mm	---
Type II	2.19 mm	2.66 mm

How well do the facts concerning the cultivated ovaries correspond to those of development *in situ*?

First of all, in view of their almost complete elimination through the use of flowers from plants grown under filtered-air conditions, those ovaries which fail to grow in culture, Type 0, can be dispensed with in further discussions as "smog-damaged." It seems probable, however, that they may be equivalent to those which absciss (flower drop) but experimental maltreatment cannot be overlooked. On the other hand, "elimination of smog" results in a sharp increase in the number of Type II developments, and mutilation of flowers at time of planting largely limits the Type II distribution to the revolute and late stages of flower development. This evidence would indicate that Type II ovaries are probably fertilized. The diameter of these fruits after fourteen days of culture would also support this premise, since it exceeds that of the 14-day old seedless fruit.

The criteria which are used to distinguish Type I from Type II growth responses are actually those which would indicate lack of fruit set, for which pollination is normally necessary (39). Since mutilation of flowers at the stage of beginning anthesis should prevent pollination, it is to be expected that only Type I responses are obtained *in vitro* from such flowers. If pollination and/or fertilization is a limiting factor in ability of the ovary to grow at the increased rate, the predominance of Type II responses obtained from flowers planted whole at beginning anthesis suggests that pollination and possibly fertilization can also be accomplished *in vitro*. However, the fact that no seeds have developed in the fruits grown from Type II ovaries, does not permit us to say definitely that these are fertilized. Also,

Type I responses are given by ovaries of flowers collected at stages where pollination is observed. In the experiments reported in the next section the flowers have usually been collected at stages later than pollination. All ovaries are therefore considered pollinated and will be discussed specifically according to type of response given, i.e. as pollinated ovaries, Type I, and pollinated ovaries, Type II.

Standardized Technique.

The technique which was eventually developed and which has yielded the most significant results in the study of the growth factors governing development of pollinated ovaries is as follows:

a) Collection of material -- Flowers, usually at the stage of revolute corolla, are collected by excision from the inflorescence close to the inflorescence axis.

b) Sterilization -- As soon as possible, the collected flowers are immersed in Zephiran Chloride solution (1:5000) in a beaker covered with one half of a Petri dish. The time interval necessary to bring the flowers from the greenhouse to the laboratory is therefore used as a part of the sterilization period. The solution is changed once or twice in the laboratory and the flowers are subsequently rinsed with distilled water. The changes can be made by pouring off the solution through the lip of the beaker with the Petri dish in place. Additions can be accomplished in a sterile manner by lifting the edge of the Petri dish in standard aseptic fashion.

c) Planting -- After draining the sterilized flowers between sterile paper towels or filter papers, a fresh cut is made above the pedicellar abscision zone of each flower and it is immediately planted on previously prepared media in Petri dishes. From twenty to forty flowers can be planted per dish with a reasonable degree of safety.

d) Transplanting and transfer selections -- The corollas will usually dehisce naturally by the following day. Removal of the dehisced corollas and transfer of the ovaries to new media has generally been delayed until the fourth day. After a four day incubation most contaminations are evident but, in the case of fungi, no spores have been formed. It is thus possible to select ovaries which are not contaminated for transfer to new dishes of the same or different media. After four days it is also possible to make a fairly accurate selection of the various types of growth.

Completely sterile cultures for detailed studies can thus be obtained.

3. Growth of the Ovary Under Culture Conditions

Although the results of many of the early experiments demonstrated qualitative differences in growth of pollinated ovaries on various media, quantitative responses were not easily detected because of low survival of the flowers planted in culture. The value of the early work, then, lay chiefly in the standardization of the technique and recognition of the growth response types. In the course of the investigations, however, a simple medium (minimal medium) was found which would support growth of the pollinated ovary. Fruit development on this medium under a given set of environmental conditions was arbitrarily adopted to serve as a standard control.

The minimal growth requirements were not definitely established until the quality of the flowers collected for culture was improved by growing the parent plants under filtered-air conditions. Therefore, in the following pages, growth of the pollinated ovaries on the minimal medium will first be characterized. The factors influencing development in the cultures have been investigated a) under different conditions of light and temperature, and b) by changing certain conditions of the medium, namely, pH and sucrose concentration. The growth of the two types of pollinated ovaries on other media has then been taken up to determine more precisely the growth factor requirements of these ovaries. However, since only a few Type I ovaries occur in the better experimental material, the experiments are largely limited to studies of the Type II response.

Establishment of the Minimal Medium for Growth.

No one of the three organic, non-carbohydrate constituents of the original medium (IAA, B-vitamins, or casein hydrolysate) was found to be obligatory for growth over an extended period of time (see Table 9), since fruits grew and ripened in the absence of each. Since sucrose and mineral salts were the only constituents common to the various media, it appeared that one or both of these were all that were required. To see if this were so, the following experiment was carried out. Flowers were collected at the stage of late corolla and were planted on 0.5% agar media to which the following components of the original medium had been added:

a) none,

b) mineral salts only,

c) 4% sucrose only,

d) mineral salts and 4% sucrose,

e) same as d) but with other supplements.

Since the flowers were mutilated before being planted, growth of the ovaries was followed from the start of the experiment. The diameters measured at various times after the day of planting, July 9, 1949, are given in Table 16. The dish containing ovaries growing on d) was accidentally broken near the beginning of the experiment; consequently, data obtained in an equivalent mineral salts-sucrose treatment from an experiment started on July 14 have been substituted for d) in the table. The increments in diameter obtained for these treatments have been plotted in Figure 12.

It is clear from these results that both mineral salts and sucrose must be present for sustained growth of the ovary of red currant tomato.

TABLE 16. Growth and development of pollinated ovaries of red currant tomato on various components of the original medium. Figures in parentheses are mean diameters of ovaries which were dead on 14th day. Experiments of July 9 and 14, 1949.

Addenda to 0.5% agar			Number of ovaries planted	Number growing after		Mean diameters mm			
						Day of culture			
Mineral salts	Sucrose 4%	Other*		2 Days	14 Days	0	2	4	14
-- none --			11	4	0	1.17	1.28	1.33	(1.33)
X			11	5	0	1.08	1.16	1.22	(1.38)
	X		11	7	1	1.14	1.56	1.69	(1.67) 2.00
X	X	X	10	7	7	1.23	1.68	2.01	2.75
X	X		22	--	6	1.10	--	--	2.39

*Other addenda consisted of thiamine 25.0 mg/l, pyridoxine 12.5 mg/l, nicotinic acid 50 mg/l, and glutamic acid 50 mg/l.

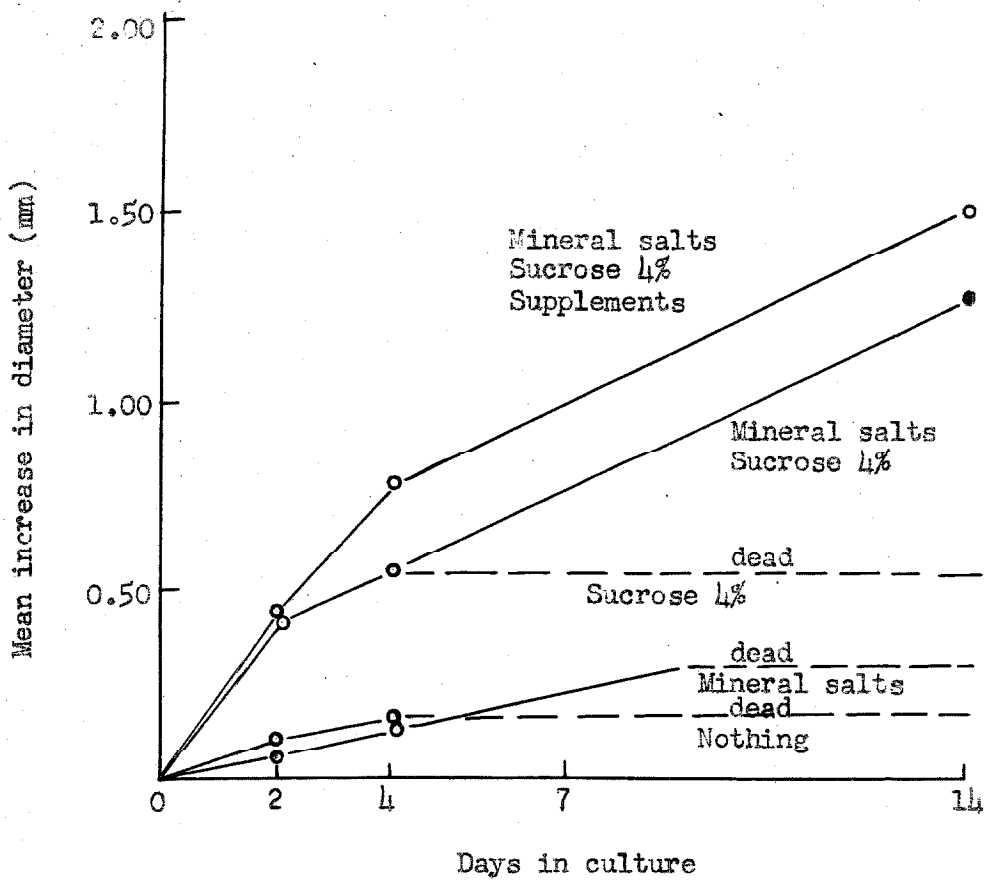


Figure 12. Growth of ovaries of excised flowers on various components of the original medium (Mineral salts, Sucrose, B-vitamins, Casein hydrolysate, and IAA). Supplements consisted of vitamins B_1, B_6, nicotinic acid, and glutamic acid. Data of Table 16.

The ovaries must contain a certain amount of endogenous substrate, however, since in the first four days the diameter increased approximately 10% on agar alone. On sucrose alone the diameter increased considerably during this same period (by more than 50%), but also after four days some other factor apparently became limiting, both for continued growth and for the survival of the ovary. Mineral salts, however, which alone were no better than plain agar, permitted growth to continue for periods longer than 14 days when they were combined with sucrose. The supplemented medium (e) apparently did not increase the initial growth rate but merely delayed its falling off.

Growth of Pollinated Ovaries on Minimal Medium under Standard Conditions.

Characterization of the growth curve. -- Following the demonstration that mineral salts and sucrose (minimal medium) would support continued growth of the pollinated ovary, this medium was included as one of the controls in every set of experimental treatments. The standard conditions for growing these control cultures consisted of placing them in a 25° C constant-temperature incubator in darkness. In most of the early experiments so few fruits grew that a characterization of the growth on this medium was possible only by observation of the individual fruit or by pooling the results from the various controls. This situation was strikingly improved, however, when flowers from plants grown under filtered-air conditions were planted in culture.

In the first control which involved use of the better experimental material, sixteen non-contaminated, Type II ovaries developed from a lot of twenty flowers initially planted. These flowers were collected at the stage of revolute corolla and planted whole (non-mutilated).

On the 34th day of culture only four were still white (non-pigmented); two were in stages of yellowing; one was yellow-orange; six, orange; and three, reddish-orange. The diameters of these fruits, determined at weekly intervals, are given in Table 17. The data are found to fit the curve

$$E_t = .000055t^3 - .0055t^2 + .217t + 1.04$$

in which E is the estimated diameter in millimeters at time t in days. In figure 13 the cumulative increments in diameter ($E_t - 1.04$) have been plotted along with corresponding values for the development of fruits attached to the plant. Comparison of fruit development *in vitro* and *in situ* thus shows that the Type II ovary grows at a rate which is intermediate between that of a large, seeded fruit on the plant and that of the parthenocarpic fruit. The growth curve of the cultivated fruits, thus obtained, also constitutes a standard curve of reference for growth of ovaries under other conditions of culture. It should be stated, however, that the results for this group of fruits are the best yet obtained for growth of the Type II pollinated ovary on this medium. Equivalent flowers, both mutilated and non-mutilated, which were collected and planted at later dates, show a significantly smaller diameter after 14 days of culture (Table 18).

Influence of Environmental Factors on Growth of Pollinated Ovaries on Minimal Medium.

Effect of light. — Early attempts to determine the effect of light on development in culture gave equivocal results because of low survival of the flowers planted. When flowers from plants grown under filtered-air conditions became available, the following experiment was

TABLE 17. Growth of Type II pollinated ovaries on minimal medium. Data for sixteen ovaries of flowers collected at stage of revolute corolla and planted whole. Initial diameters (*) determined in corresponding group of 16 flowers.

Days after planting	Mean diameter
	mm
0	1.04 ± .01*
7	2.33 ± .05
14	3.14 ± .11
21	3.66 ± .10
34	4.15 ± .11

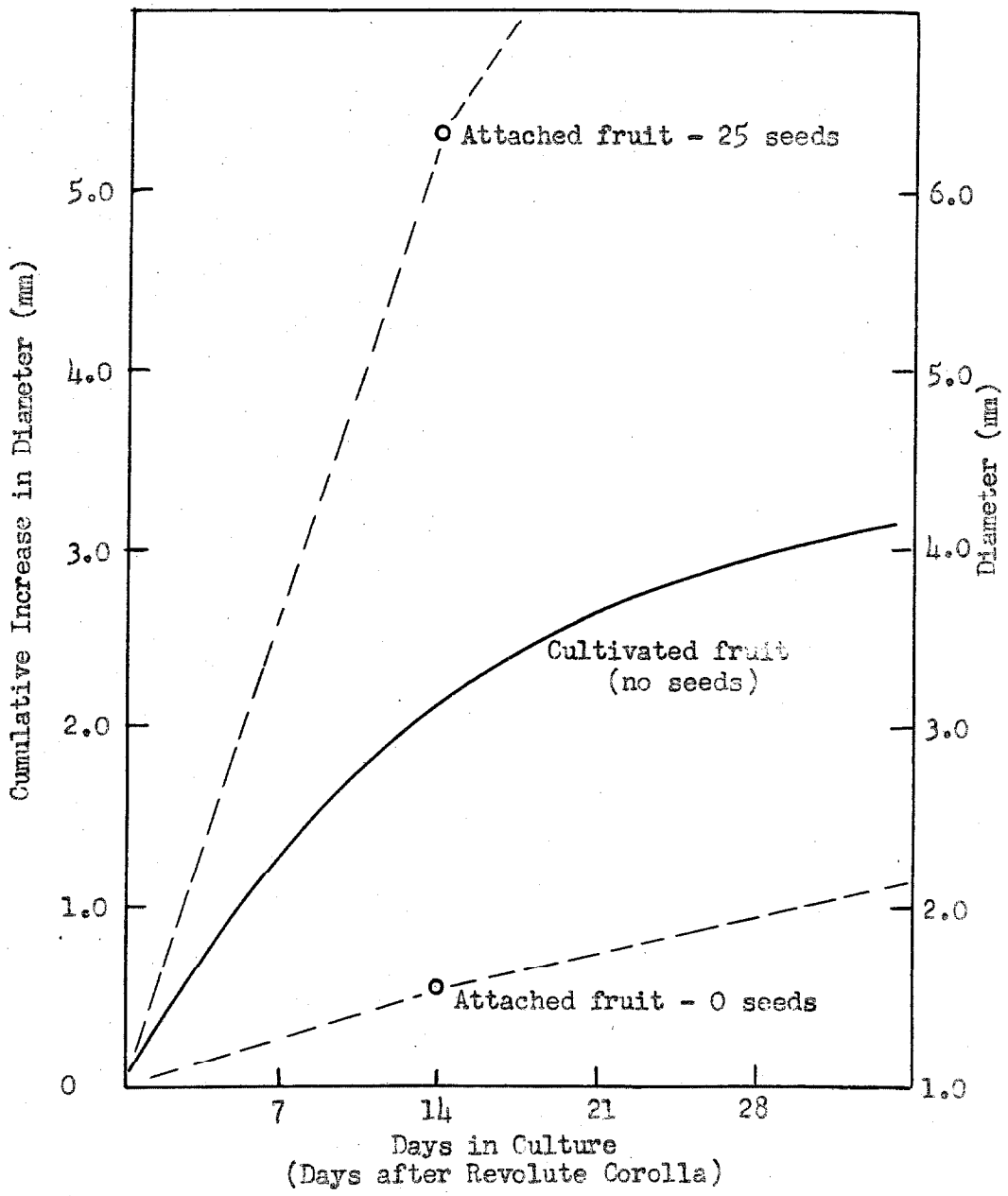


Figure 13. Growth of fruits cultivated in vitro and of fruits attached to the plant.

TABLE 18. Growth of Type II pollinated ovaries on minimal medium in relation to distribution of growth response types and to mutilation at time of planting. Flowers collected at stage of revolute corolla and planted whole or after removal of corolla, stamens, and styles (mutilation).

Date	Muti-lated	Number planted	Growth Response 0	I	II	% Type II	Diameter of Type II ovaries after 14 days of culture
May 3, 1951	no	18	0	2	16	88.8	3.22 ± .11
May 11, 1951	no	10	0	1	9	90.0	2.60 ± .18
May 16, 1951	yes	10	0	2	8	90.0	2.54 ± .09
April 10, 1952	no	38	3	14	21	55.3	2.43 ± .09

carried out. Non-mutilated flowers, collected at the stage of revolute corolla, were planted on minimal medium and incubated under standard conditions for five days. Ovaries exhibiting Type II response were then transferred at random to a new minimal medium, ten ovaries per plate. Four plates were then randomized and two were placed at room temperature (approximately 25° C) under continuous artifical illumination from a bank of fluorescent lights. The other two plates were placed back under the standard conditions. Diameters of the fruits, measured on the fourteenth day of culture are given in Table 19.

It is quite evident that these light conditions have no effect on growth of the fruit in diameter. All of the light-exposed ovaries, however, had developed chlorophyll, whereas the dark controls were completely non-pigmented. Dissection of the fruits showed no seed development, except in the largest of the light-grown ovaries (3.83 mm). In this fruit a number of cup-shaped ovules with necrotic centers were observed. The largest of these malformed ovules was 0.23 mm in length. The presence of the photosynthetic machinery in the pericarp, then, is ineffective either in increasing the fruit size by supplying additional substrate or growth factors or in supporting embryo development.

Effect of temperature. -- For the study of the growth of the cultivated fruits at different temperatures, use was made of the same temperature conditions of the Phytotron under which the growth of the plants had been previously studied. Aside from its relationship to the experimental method, such a study should also show whether temperature controls the growth of the attached fruit directly or indirectly through the plant.

TABLE 19. Effect of light on growth of Type II pollinated ovaries.

Treatment	Number of ovaries	Diameter after 14 days of culture
5 days dark - 9 days continuous light	20	2.70 ± .10
Dark controls	20	2.69 ± .08

In the first experiment in the investigation of temperature, one-day old growing ovaries of flowers planted at the stage of revolute corolla were transplanted at random to new dishes of minimal medium, 18 ovaries/dish, without regard to growth response. All dishes were wrapped in heavy paper and randomized before being placed under the various temperature conditions. After two additional weeks of culture the dishes were removed for measurement. Unfortunately Petri dishes proved to be unsuitable containers to use for such an investigation. The air in the Phytotron is under constant and rapid circulation. Thus, a considerable marginal contamination of the cultures by both fungi and bacteria resulted, despite additional protection provided by wrapping the dishes in paper. Although minimal medium does not support extensive growth of most microorganisms, their presence introduces an undesirable element. In a few instances, also, some fungi overgrew the ovaries. These ovaries have not been considered in the data.

In view of this contamination another experiment was carried out in which four-day old ovaries of flowers which had been collected from plants growing under the smog-free conditions were transplanted to media in flasks which were then plugged with cotton. In this case, however, the temperature exposure was reduced to ten days. The results of both of these experiments are given in Table 20.

The data of the 1951 experiment show that there are no differences in the growth of Type II pollinated ovaries at fluctuating temperatures. Differences significant at the 1% level are however found for those growing under constant temperature regimes. The optimum appears to be above 26° C. In the second experiment only constant temperatures

TABLE 20. Growth of excised pollinated ovaries under various temperature conditions in darkness. Number of ovaries given in parentheses.

Experiment of 1951

Daily temperatures, °C	8 hrs. 20° / 16 hrs. 14	23° / 17	26° / 20	20° / 20	23° / 23	26° / 26
Contamination	Marginal	Single spot	None	Marginal	General	Marginal
	Mean diameters (mm) after 15 days of culture, 14 days on conditions					
Growth response						
Type I	<1.50 (1)	<1.50 (1)	1.64 (4)	<1.50 (1)	— (0)	<1.50 (1)
Type II	2.50±.08(10)	2.21±.09(13)	2.25±.03(14)	2.02±.06(11)	2.20±.07(8)	2.57±.08(17)

Experiment of 1952

Temperatures °C (constant)	14°	17°	20°	23°	26°	30°
	Mean diameters (mm) after 14 days of culture, 10 days on conditions					
Growth response						
Type I	1.54 (3)	1.60 (1)	1.58 (1)	1.51 (6)	1.43 (5)	1.47 (4)
Type II	2.15±.20(7)	2.16±.07(19)	2.47±.11(9)	2.58±.28(4)	2.44±.14(15)	2.17±.13(16)

were used and the range was extended in an attempt to establish the optimum temperature. The data, plotted in Figure 14, show a flat optimum between 20° and 26°. The suggested optimum at 23° is not significant; however, the Type II ovaries grown at both the 20° and 23° temperatures are significantly different at the 5% level from those at 17°. Thus the temperature which had been arbitrarily selected for the standard conditions was at or near the optimum temperature.

The investigations on the growth of the plant showed that both root and leaf dry weight production, and perhaps growth and development of flowers and fruits also, were optimal at a night temperature at 17° C. They were apparently independent of the higher day temperature (p. 50ff). This seems to indicate that the effect of temperature on growth of the attached reproductive organs is exerted through control of vegetative processes. Optimal temperatures for translocation have been shown for other species (99), and since we have seen that sugar is one of the most important factors in the growth of fruits in vitro, it may be that translocation of sugar is one of the limiting factors in the development of attached fruits of L. pimpinellifolium. This remains to be investigated.

Growth of Pollinated Ovaries in Response to Changes in Conditions of Minimal Medium.

Influence of pH. — Minimal medium, mixed as usual and containing the trace element solution previously described, has been found to have a pH value close to 5.3. In the early experiments all media were adjusted to pH 5.4-5.6 by the addition of KOH before autoclaving. When

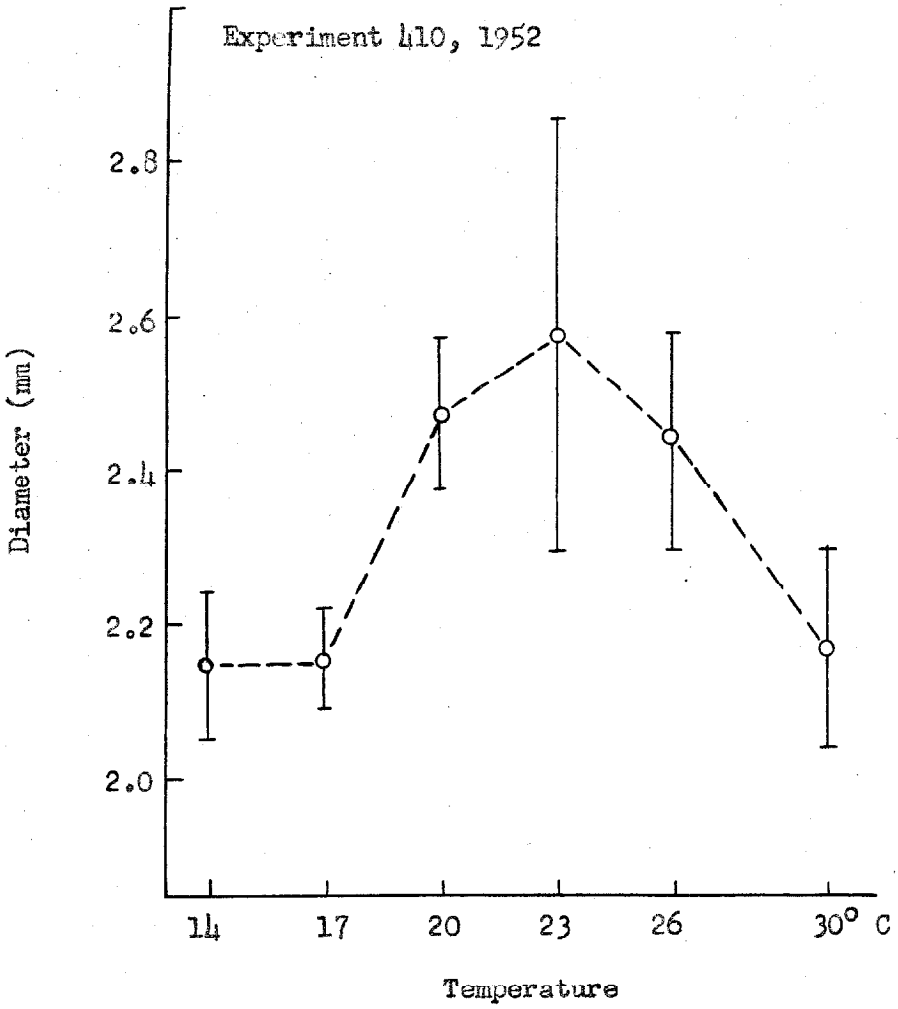


Figure 14. Effect of temperature on growth of Type II pollinated ovaries cultivated in vitro. Fourteen-day diameters of ovaries of excised flowers. Temperature exposure, 10 days.

Berthelot's trace element solution (55) was used, the pH before adjusting was found to be about 3.5. In an experiment designed to check the effect of pH on growth, minimal medium was prepared with Berthelot's trace elements. Sufficient M/3 K_2HPO_4 was added to bring the pH of aliquots of the unadjusted medium to 4.0, 4.5, 5.0, 5.5, and 6.0. In order to offset the effect of phosphate on availability of iron, extra ferric tartrate solution was added. Fifteen growing ovaries from 3-day old cultures of revolute flowers were transferred at random to each of these media. After 12 additional days of culture the fruits were measured (Table 21). The growth was identical over the whole pH range tested. This indicates that the earlier procedure of adjusting the pH initially was unnecessary. Gautheret (98) has shown that changes in pH of the medium between pH 4.0-9.0 have no effect on growth of carrot tissue in vitro. In subsequent experiments with fruits, careful control of the pH was abandoned. Only for those media which fell outside of the range 5.0-6.0 was the pH adjusted.

Sucrose concentration. — Since it has been shown that absence of sucrose in the medium resulted in death of the ovaries in from four to seven days, media were prepared containing inorganic constituents and also casein hydrolysate at a concentration of 500 mg/l. This latter component was added to provide a possible source of energy to sustain life of the ovaries which were to receive no sucrose. Sucrose was added in concentrations of 0, 2, 4, and 6%. Flowers were planted at the stage of revolute corolla and the diameters were measured after seven and fourteen days of growth (Table 22 and Figure 15). Ovaries of all flowers planted on the sucrose-free medium were dead by the seventh day. However, some growth occurred before death took place, since the

TABLE 21. Effect of pH on growth of Type II pollinated ovaries.

Initial pH	Number of Type II ovaries	Diameter after 15 days of culture mm
4.0	10	2.36 ±.10
4.5	12	2.28 ±.08
5.0	11	2.22 ±.09
5.5	12	2.27 ±.09
6.0	13	2.40 ±.09

TABLE 22. Effect of sucrose concentration on growth of pollinated ovaries in vitro. Flowers planted at stage of revolute corolla on mineral salts-agar media containing casein hydrolysate (500 mg/l) and various concentrations of sucrose. Figures in parentheses are number of Type II pollinated ovaries, except for those on sucrose-free medium. All of latter were dead by the 7th day. Initial diameter, approximately 1.0 mm.

Days in culture	7 days		14 days	
Sucrose concentration %	Diameter	Differences mm	Diameter	Differences mm
0	1.50 ± .04(20) dead		—	
2	2.08 ± .10(10)		2.85 ± .17(10)	
		.31 ± .12 s		.30 ± .19 ns
4	2.39 ± .07(10)		3.15 ± .09(10)	
		.20 ± .12 ns		.24 ± .13 ns
6	2.19 ± .09(16)		2.91 ± .12(8)	

s, significant at 5% level
ns, not significant

diameters had increased approximately 50%. Ovaries on sucrose-containing media more than doubled their diameter during the first week and grew almost as much during the second. No significant differences in diameters of ovaries on the different concentrations were obtained, except between the 2% and 4% treatments at 7 days. Ovaries on 4% sucrose increased about 30% more in diameter than those on 2%. Although the relative differences remain more or less constant on the different media, the significance is lost by the 14th day. It seems, therefore, that the effect of sucrose at these different concentrations must be on the initial rate of growth.

Two facts, then, are revealed in this experiment. First, and most obvious, sucrose at a concentration of 4% or greater is apparently best for growth of the ovaries. It is fortunate that this concentration had been arbitrarily selected for all of the earlier work for it thereby increases the value of the results.

The second fact requires consideration of a previous experiment. In the investigation in which the minimal requirements were established (p. 85), growth in the absence of sucrose was very slight (10% increase in diameter) and death of the ovaries occurred after four days. It was suggested that this small increase in diameter resulted from expenditure of some endogenous substrate. When sucrose alone was added, death also occurred after about four days, but the amount of growth was considerably greater, amounting to a 50% increase in diameter. In the experiment reported here (Table 22) we again see that on sucrose-free medium death occurs early (prior to the seventh day) but a 50% increase is obtained. Two possible explanations come to mind: 1) casein hydrolysate can substitute for sucrose in the minimal medium but is limiting for growth at this concentration (500 mg/l); or 2) in the first experi-

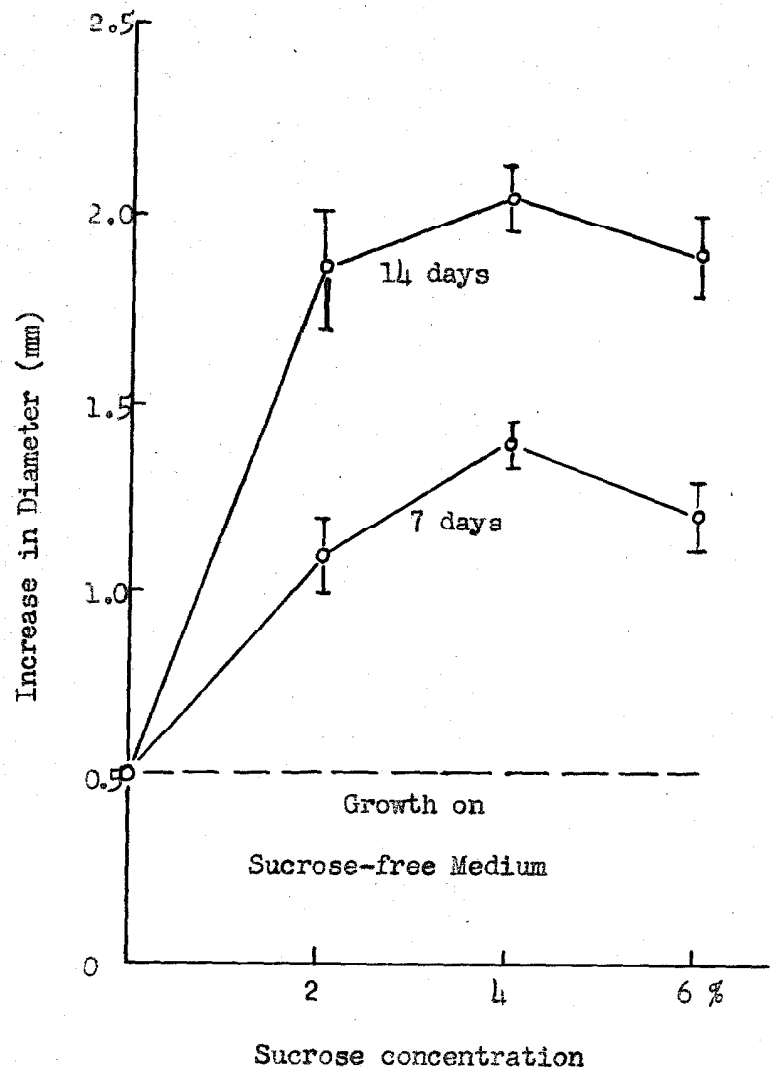


Figure 15. Effect of sucrose concentration on growth of Type II pollinated ovaries in vitro. Standard errors of 7- and 14-day diameters shown by vertical lines through each point. Initial diameter 1.0 mm. Data of Table 22.

ment the type of ovary was different. In other words, according to the second explanation, the first experiment may have involved predominantly Type I ovaries whereas in this experiment the ovaries were almost entirely of Type II (four Type I responses were obtained out of 20 flowers planted in the 6% sucrose treatment). Therefore, the following experiment was set up to clarify the situation.

Flowers collected at the stage of revolute corolla were planted whole on plain agar medium (no mineral salts or sucrose) and were allowed to develop for two days to use up endogenous substrate. The corollas dehisced naturally and were removed on the second day. The diameters of a random sample of 26 ovaries were measured and found to be 1.07 ±.03 mm. Assuming an initial diameter of 1.0 mm, this represents a growth rate of only 0.035 mm/day. The corresponding rate for the Type II ovary, calculated from the standard curve (Figure 13), is 0.22 mm/day.

After this preliminary starvation, the ovaries were transferred at random to either minimal or supplemented media and were allowed to grow for an additional fourteen days. The results obtained are given in Table 23 along with the data from a parallel group of non-starved ovaries grown on minimal medium for fourteen days only.

The results are striking. The Type II growth response is completely abolished among the prestarved ovaries. Two days of starvation, however, does not prevent the ovaries from continuing to grow. When the treated flowers were transferred to a medium containing sucrose and mineral salts, they did not attain the growth rate of non-prestarved ovaries grown on this medium but continued to grow at the same rate as during the starvation period (0.035 mm/day). Thus one can calculate

TABLE 23. Effect of two days of prestarvation on subsequent growth of pollinated ovaries. Diameters after 14 days of growth on minimal and supplemented media. Figures in parentheses are number of replicates.

Medium	Mean Diameter after 14 days of culture mm
Ovaries prestarved, 2 days	
Minimal	1.45 ± .05 (9)
Minimal plus B-vitamins	1.51 ± .15 (12)
Minimal plus casein hydrolysate	1.54 ± .10 (8)
Minimal plus B-vitamins plus casein hydrolysate	1.52 ± .07 (24)
Minimal plus B-vitamins plus casein hydrolysate plus ascorbic acid (20 mg/l)	1.47 ± .04 (17)
Ovaries not prestarved (control)	
Minimal (35)	
Type I ovaries	1.48 ± .03 (14)
Type II ovaries	2.43 ± .09 (21)

that on the 16th day they should have reached a diameter of 1.56 mm and in fact none of the groups differed significantly from this figure.

In considering the results of all three experiments in which sugar-free media or prestarvation treatments have been used, one can conclude that the Type I ovary contains a certain amount of some endogenous materials which will support growth for four days, but after this time an external supply of substrate, which can be supplied by minimal medium, is required. It appears that the difference between the Type II and Type I ovaries is that the Type II contains some additional factor(s) which is concerned with the greater initial growth rate of the ovary in the presence of organic substrates, when the minimal requirements for prolonged growth are not met. Also, this factor must be involved in the characteristic, continued increase in diameter given by the Type II ovary on minimal medium. This growth can be fitted to an equation which is third order with respect to time. The Type I growth is linear. This factor(s) is either inactivated or ineffectively expended by the starvation treatment and cannot be replaced by B-vitamins, a mixture of amino acids (casein hydrolysate), or ascorbic acid, used individually or in combination. It seems highly probable that this factor is contributed or established by either pollination or fertilization, and supports the evidence presented in the previous section that the Type II ovary has been pollinated and probably fertilized. In this case the growth response given by the Type I ovary is common to all ovaries--fertilized, pollinated and non-pollinated.

Growth of the Pollinated Ovary on Other Media.

Utilization of other carbohydrates. -- Since sucrose has been shown to be one of the most important constituents of the minimal medium required for growth of pollinated ovaries, it is of interest to know whether other carbohydrates can substitute for this requirement.

Media containing each of the following were prepared: 2% glucose, 2% fructose, 2% galactose, and 2 and 4% sucrose. These media were planted with flowers collected at the stage of revolute corolla. After four days of incubation, non-contaminated, growing ovaries were transplanted to new plates of the same media. The diameters were measured after 14 days of culture.

The results (Table 24) indicate even more clearly than those given in the preceding discussion (p. 106) that we are dealing with two different types of growth response and that the mechanisms for these responses differ in two respects. Growth of the Type I ovary is not affected by changes in sucrose concentration but can be supported by several carbohydrate sources, including galactose. The mean 14-day diameter of Type I ovaries on all of these media falls just short of the 1.5 mm diameter used to characterize this type of response. Type II ovaries, on the other hand, show a dependence on sucrose concentration and cannot utilize galactose. The diameter of ovaries on 4% sucrose is greater than those on 2% by $2.5 \pm .10$ mm. This difference is significant at the 5% level. On galactose, Type II ovaries apparently revert to Type I. Either 2% fructose or 2% glucose, however, can substitute with equal effectiveness for 4% sucrose in supporting the growth of the Type II ovary. The growth resulting on these media amounts to an increase in diameter of 130-140% in fourteen days. Since the molarities of these

TABLE 24. Growth of pollinated ovaries on various carbohydrate substrates. All media contain mineral salts. Diameters measured after 14 days of culture. Figures in parentheses are the number of ovaries of each growth type.

Substrate	Number of ovaries	Diameter (mm) Type II	Type I
Sucrose, 4%	35	2.43 ±.08 (21)	1.48 ±.03 (14)
Sucrose, 2%	33	2.18 ±.06 (24)	1.45 ±.04 (9)
Glucose, 2%	38	2.32 ±.05 (34)	1.47 ±.07 (4)
Fructose, 2%	33	2.34 ±.06 (32)	1.38 (1)
Galactose, 2%	33	—	1.40 ±.02 (33)

three sugars are almost equal at the concentrations mentioned and since 2% sucrose, with half of this molar concentration, results in an increase of only 118% after 14 days, the evidence again points to an effect on the initial growth rate. Galactose is not effective in establishing rapid growth; therefore, the sugar requirement of the Type II ovary appears to be specific towards sucrose or its constituent monosaccharides.

Effects of mixtures of B-vitamins and amino acids. — Several of the early experiments had suggested that B-vitamins and casein hydrolysate supplements in minimal medium could bring about an increased growth in diameter over that which could be obtained on minimal medium alone. After it was possible to grow plants under the filtered-air conditions of the Phytotron, this point was immediately reinvestigated.

Flowers, collected at the stage of revolute corolla from these plants, were grown on the following media:

a) Minimal (control),

b) Minimal plus B-vitamins, and

c) Minimal plus B-vitamins and casein hydrolysate (500 mg/l).

The cultures were incubated under the standard conditions and the diameters of the ovaries were measured at weekly intervals after planting. The results, given in the first part of Table 25, show that addition of casein hydrolysate and B-vitamins indeed promoted the growth of these fruits as compared with minimal medium. The diameter of fruits grown on medium (c) surpassed that of fruits on minimal by approximately 0.15 mm throughout the entire period of growth; on the 14th day of culture this amounted to about 10% of the total increase obtained on

TABLE 25. Effect of B-vitamins and casein hydrolysate on growth of pollinated ovaries. Figures in parentheses are the number of Type II ovaries.

Experiment 503, 1951

Days of culture:	7	14	21	34
Supplements	Mean diameter (mm)			
a) None —(minimal control)	2.33 ± .05 (16)	3.14 ± .11 (16)	3.66 ± .11 (16)	4.15 ± .11 (16)
b) B-vitamins	2.26 ± .08 (19)	2.94 ± .09 (19)	3.56 ± .09 (17)	contaminated
c) B-vitamins casein hydrolysate (500 mg/l)	2.48 ± .06 (17)	3.37 ± .09 (17)	3.81 ± .10 (17)	4.30 ± .11 (17)

Experiment 512, 1951

Days of culture:	7		14	
Supplements	Mean diameter (mm)			
a) None —(minimal control)	2.05 ± .13	(9)	2.60 ± .18	(9)
b) B-vitamins	1.97 ± .10	(17)	2.61 ± .23	(9)
c) B-vitamins, casein hydrolysate (500 mg/l)	2.14 ± .09	(16)	2.88 ± .09	(10)
Casein hydrolysate				
d) 100 mg/l	2.24 ± .05	(18)	3.09 ± .13	(10)
e) 300 mg/l	2.16 ± .08	(19)	2.75 ± .17	(10)
f) 500 mg/l	2.39 ± .07	(10)	3.15 ± .09	(10)
g) 1000 mg/l	2.32 ± .05	(20)	3.15 ± .13	(10)

minimal medium alone. This difference is significant at the 5% level for only the first week of growth. Since the difference in diameters remains constant throughout the entire period of culture, growth in volume must be decreasing relatively more rapidly on the medium containing the vitamins and amino acids. In other words, as the diameters increase, the relative differences in volume become less. Thus the additional constituents of medium (c) must be affecting the initial rate of growth. The diameters of fruits which developed on the B-vitamin medium did not differ significantly from those on minimal medium at any time of the growth period. But it is not possible to say from this experiment whether the promotion given by the more complex mixture (c) was due entirely to casein hydrolysate or whether the latter was effective only in combination with the B-vitamins.

The experiment was therefore repeated, using casein hydrolysate and B-vitamins, both alone and in combination. Also, the number of treatments were extended to include several concentrations of casein hydrolysate. The results are given in the second part of Table 25.

The second experiment demonstrates again the lack of effect of B-vitamins by themselves but shows very slight promotion due to the combination of vitamins and amino acids (medium c); only the constancy of the differences in diameters seem significant. On the 7th and 14th days of culture, the relative increases in diameter of ovaries on (c) were only 9% and 6%, respectively, of the total increases obtained on minimal alone by each of these days. The data also show this effect to be due entirely to the casein hydrolysate. It even seems that addition of B-vitamins decreases the benefits derived from the amino acid mixture by itself. Equal concentrations (500 mg/l) are about two to four times more effective in increasing the actual amount of growth

(volume) when used alone than when used in combination with the mixture of B-vitamins. The promotion (increase in diameter) effected in ovaries grown on the higher concentrations of casein hydrolysate (500 and 1000 mg/l) amounts to 35% of the total increment in diameter attained on minimal medium alone after 14 days of culture. These promotions are significant at the 1% level. Lower concentrations of casein hydrolysate are less promotive. Treatments (a) and (c) of the first experiment and (a) and (f) of the second (in either case, minimal medium and one containing casein hydrolysate at a concentration of 500 mg/l) have been plotted in Figure 16. A comparison of identical or similar treatments of the two experiments shows that on neither the minimal medium nor casein hydrolysate medium of the second experiment did the fruits attain the amount of growth reached on the corresponding media of the first experiment; however, the relative increases effected by the addition of the casein hydrolysate are greater in the second case.

The nature of the casein hydrolysate promotions of growth. -- It has already been suggested in the above experiments that casein hydrolysate acts on the growth of these fruits by increasing the initial growth rate. However, if we review a number of experiments in which either the initial growth rate has been affected or casein hydrolysate has been employed in the media, it seems probable that this supplement is playing two roles in the growth of the Type II ovary.

a) When excised flowers were planted on a medium containing sucrose alone (without mineral salts), the amount of growth of the ovaries which occurred during the first two days was not different from that made by

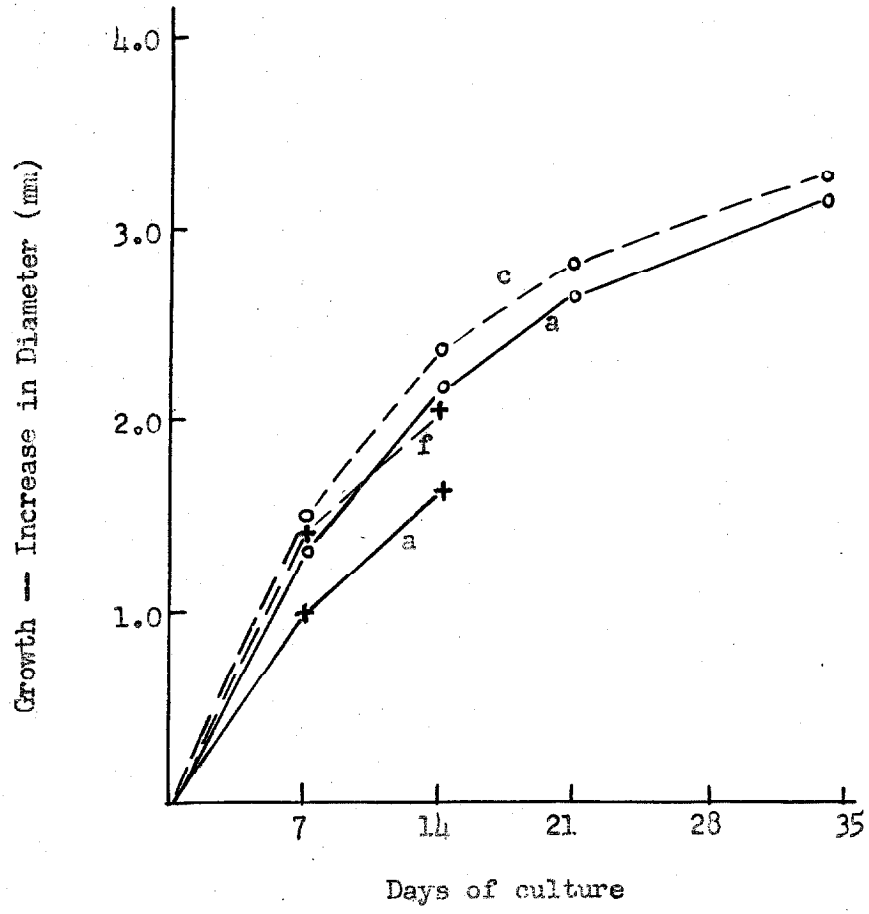


Figure 16. Promotion of growth of Type II pollinated ovaries by addition of casein hydrolysate (500 mg/l) to minimal medium. Results of two experiments, designated with circles and crosses. Broken lines, supplemented medium; solid lines, minimal medium. Letters refer to data of Table 25. Initial diameter, 1.0 mm.

ovaries on media containing sucrose and mineral salts; but growth stopped abruptly after about four days. The diameter at this time represented an increase of about 50%. (Table 16).

b) Approximately this same amount of growth (50% increase in diameter) was obtained by ovaries grown on a sucrose-free medium containing casein hydrolysate and mineral salts. This increase all occurred prior to the seventh day and all ovaries died unless sucrose also was present. (Table 22).

c) In the absence of both sucrose and casein hydrolysate, ovaries increased in diameter only 7% in two days and continued to increase at the same rate (that of the Type I ovary) even when afterwards supplied with both of the organic constituents. (Table 23).

It has also been shown that sucrose, glucose or fructose are specifically required for the growth of the Type II ovary. We must conclude, then, that casein hydrolysate can substitute for the sucrose requirement of the Type II ovary under conditions in which the minimal requirements for continued growth are not met, i.e. sucrose and mineral salts. Continued growth in both cases must be limited by other materials contained in the ovaries. For ovaries on sucrose alone the limiting factors would appear to be some essential mineral requirement. For those on casein hydrolysate, however, it is the amount of substrate available for growth. Since the sucrose requirement is specific, it also seems probable that establishment of this characteristic growth of the Type II ovary requires some essential organic substance or the

energy obtained from a particular mechanism. From what we know of general metabolic reactions, it seems possible that phosphate and Krebs cycle acids are involved in these two cases.

The above factors, however, are not sufficient in themselves for growth of the Type II ovary. Prestarvation of the ovaries results in complete obliteration of this type of growth, but the ovaries can continue to grow as Type I. Pollination or fertilization is the apparent difference in the two types. Hence, both sucrose and casein hydrolysate must exert their action in establishment of the Type II growth rate through some factor originating from one of these physiological processes. This, then, is the pollination-fertilization factor. The differences in the relative amounts of growth obtained in different experiments using identical media and lengths of culture suggest that the amount of this factor varies and that it is actually the prime determiner of Type II growth. The addition of casein hydrolysate to minimal medium in some way supplements this factor in determining the initial rate.

The probable manner in which the supplementary action of casein hydrolysate is obtained is perhaps best revealed in the experiments with mutilated flowers given in Section 2 (Tables 14 and 15). When flowers were mutilated at the stage of beginning anthesis (unpollinated flowers), only Type I ovaries were obtained. This is to be expected, since removal of the style would prevent pollination and fertilization. Similar flowers planted without mutilation produced predominantly Type II ovaries on both minimal and casein hydrolysate-supplemented media. On the latter, however, both the number of Type II fruits and also their diameters were increased over those which grew on minimal. This suggests

that casein hydrolysate is capable of bringing about a greater amount of pollination and fertilization, thereby increasing the amount of the pollination-fertilization factor. Its effect may be only indirect, however, mediated through either better pollen growth or through a promotive action on fertilization.

Effect of auxins. -- In the early experiments indoleacetic acid (IAA) was included in media in concentrations ranging up to 100 µg/l. No measurable effect on growth was observed. Some ovaries always grew, even in complete absence of auxin. A number of auxins, however, are known to be very effective in the production of parthenocarpic fruits, and since it is well known that certain of the auxins are definitely polar in their movement, it might easily be that the auxin originally added was not able to reach a site where its action could be exerted.

To check this possibility, an experiment was run in which auxin was supplied to the apices of the ovaries. Flowers, collected at the stage of revolute corolla, were planted on minimal medium and were allowed to develop for six days. After this preliminary culture period, the dehisced corollas were removed and the styles were broken off at their point of attachment to the ovary. Sterile agar blocks, containing indoleacetic acid (IAA) or naphthaleneacetic acid (NAA) in concentrations of 0, 0.1, 0.3, 1.0, 3.0, 10.0, and 30.0 µg/l, were then seated firmly against the stylar surface of the ovaries and the cultures were replaced in the incubator for an additional period of eight days. The 14-day diameters are given in Table 26.

Both IAA or NAA affect the growth of the Type II ovary. The optimal concentration for growth promotion by both auxins appears to be

TABLE 26. Effect of auxins on growth of Type II pollinated ovaries *in vitro*. Naphthaleneacetic acid (NAA) and indoleacetic acid (IAA) applied in agar blocks to the stylar region of six-day old fruits cultivated *in vitro*. Number of replicates shown in parentheses. Initial diameter approximately 1.0 mm.

Auxin concentration	Control (no agar blocks)	IAA	NAA
µg/l	Mean 14-day diameters (mm)		
0	2.72 ± .10 (9)	2.66 ± .09 (10)	
0.1		2.60 ± .10 (8)	contaminated
0.3		2.52 ± .14 (8)	2.48 ± .12 (9)
1.0		2.73 ± .14 (10)	contaminated
3.0		2.91 ± .16 (9)	2.93 ± .20 (9)
10.0		2.56 ± .15 (9)	2.49 ± .12 (10)
30.0		2.41 ± .12 (9)	2.60 ± .05 (10)

Analysis of variance
(IAA and controls)

df	Sum of squares	Variance	
71	10.48		
7	3.93	.56	F = 5.49
54	6.55	.102	significant at 1% level

3.0 μg/l; higher concentrations are inhibitory. The amount of growth (increase in diameter) brought about by the optimal concentrations is 20-40% greater than that obtained by the lower concentrations or controls. These differences are significant at the 5% level; those at other concentrations are not. Growth of the pollinated ovary in vitro, while apparently not completely limited by an external source of auxin, can be increased to a slight extent by the application of physiological concentrations of IAA or NAA. Similar auxin-induced increases in growth of poorly pollinated and self-pollinated fruits, developing on intact plants, have been demonstrated by Howlett (33) in commercial greenhouse tomato varieties.

Effects of other supplements. — Investigation of the effects of a number of other factors and growth metabolites failed to reveal any growth promotions in the early experiments. Trans-cinnamic acid, an antiauxin, failed to prevent growth of fertilized ovaries. Fruits cultivated on yeast extract, malt extract, juice of green and of ripe tomatoes, and non-autoclaved coconut milk were not markedly different in size from the controls. Extracts of lyophilized leaves of red currant tomato, made with ether or aqueous alcohol, proved toxic to the growth of fertilized ovaries; the residue from such extractions was without effect (at seven days 16 ovaries on minimal medium were 2.33 ± .05 mm in diameter; 18 on minimal plus leaf residue, 2.24 ± .05 mm).

Pigmentation. — At the time of the dehiscence of the corolla from flowers of Lycopersicon pimpinellifolium developing on the plant, the ovary is more or less non-pigmented. During the normal course of

development, however, it rapidly becomes green through the development of chloroplasts in the pericarp. At maturity the green color is intensified through the loss of a whitish opaqueness of the surface layers of cells. Finally, the color of the fruits changes through stages of greenish yellow, yellow-orange, and red-orange to bright red. The orange and red pigments occur in rod-shaped chromoplasts, which are particularly concentrated in the inner cell layers of the pericarp and in the gelatinous placental tissue which surrounds the seeds.

Fruits, planted in vitro and grown in dark for five days, remain colorless but will develop chlorophyll if transferred to continuous artificial illumination after 5 days. However, if the dark period is prolonged for two weeks, the ability of the ovary to develop chlorophyll is lost. Older cultures never become green even when left exposed to light for weeks. Daily illumination of young cultures for short periods is also ineffective in causing visible greening.

The earliest development of the pigments of maturity which has thus far been observed in fruits grown in vitro is 34 days, approximately the same length of time in which the earliest pigmentation has been observed on the plant (36 days). Periods of total darkness up to two weeks have not prevented the development of orange and yellow pigments in some fruits older than 21 days at the time of exclusion of light. In addition some fruits have been observed to remain alive for prolonged periods without becoming pigmented even when exposed to light. The longest period recorded is 141 days. There appears to be no correlation between fruit size and ability to form pigments, nor between nutrients and either degree or quality of pigmentation. In some of the early cultures yellow fruits only developed. These were on minimal medium supplemented with

biotin 5 µg/l and IAA 10 µg/l. Similar fruits were later observed on minimal only and minimal plus other supplements. The yellow color in these cases has been found to be restricted to the epidermal cell walls. Fruits with other pigments have also been found to have the yellow epidermis but the color is masked by the more intense red and orange pigments.

The development of pigments in fruits either on the plant or in culture does not mean the end of growth in size. Increase in diameter may continue for much longer periods, but the rate is always slow. Both seeded fruits attached to the plant and fruits developed from Type II pollinated ovaries in culture have shown rupture of the epidermis after extended periods of time. The attached fruits, though, soon become overripe and begin to rot. In culture, the splitting continues completely through the pericarp and may take place in several directions. Subsequently the inner cell layers of the pericarp tissues continue to grow, and in so doing produce a curling back of the strips of tissue. Thus the fruit is literally turned inside out. It would seem then that one of the limiting factors in ultimate fruit size is the extent of growth of the epidermis. Hayward (78) states that the number of cells in the epidermis of the tomato fruit is the same as in the ovary, indicating that the epidermis grows only by cell enlargement.

Root and callus formation. — In contrast to the excised flowers of Lycopersicon esculentum, which were used by Nitsch (20, 67) and which develop roots when cultured in the dark on a medium containing thiamin, those of L. pimpinellifolium do not form roots on minimal media supplemented with thiamin alone or with most of the other supple-

ments employed in these investigations. In fact roots have been observed on only four media -- minimal supplemented with thiamin (0.1 mg/l), and adenine, guanine, cytosine and uracil (1 mg/l each); minimal with adenylic acid (0.1 mg/l); minimal plus non-autoclaved coconut milk (10%); and minimal plus trans-cinnamic acid (1 mg/l). In none of these media did rooting occur on all flowers or even on all with growing ovaries. The presence of roots has not been observed to have any appreciable effect on the growth and development of the fruits, although a slight delay in development of pigments at maturity is suggested.

Callus formation, which was observed by Nitsch (20) on media containing either synthetic auxins or autoclaved coconut milk, has been obtained on the pedicels of the red currant tomato only on the coconut milk medium and on a medium containing trans-cinnamic acid. In the first instance small calluses are produced on all flowers which grow. This type of development on the trans-cinnamic medium is less extensive and much less uniform. No correlation seems to exist between rooting and callus formation.

E. Discussion.

In the preliminary discussions of this work, it was pointed out that the value of *in vitro* studies depends upon demonstration that the results obtained by this means compare favorably with the situation *in situ*. These studies have concerned growth. Hence, the best criteria for comparison are probably the growth rates. In the investigations on the growth of fruits attached to the plant it was found that two types of growth response can be obtained from the ovaries of presumably pollinated flowers. The apparent difference between these two types is whether or not they contain seeds. In all of the seedless fruits observed, the diameter on the fourteenth day after the stage of revolute corolla has been found to be of the order of 1.5 mm. This represents an increase in the initial diameter of 50%. In one instance, growth of one of these fruits continued for longer periods of time at a rate of approximately 0.2 mm/wk. (p. 50). Within the limits of accuracy with which these measurements could be made, both values represent a growth rate of 0.03 mm/day, which is linear throughout the period of growth. The sigmoid nature of the growth curve of the seeded fruits (Figure 7), however, indicates that increase in diameter of the seeded fruit is exponential. Since the size of the fruits which contain seeds can be correlated with the number of seeds developing on the 14th day (Table 5), the number of seeds must be a function of the growth rate and the mean growth rate of the fruits during the period of most rapid increase in size would be of the order of 0.40 mm/day.

None of the fruits grown in culture show development of seeds. However, it has been possible in several ways to distinguish two types of growth response in these fruits, too. Those ovaries which have been

designated Type I grow at a linear rate of 0.035 mm/day. Type II ovaries, on the other hand, increase exponentially, with initial rates ranging up to 0.22 mm/day. This growth rate gradually decreases. The variability in the sizes of these fruits grown in vitro is the same as that in seeded fruits grown on the plants (Table 10).

It is quite apparent from the growth rates, from the types of growth, and from the variability that the two types of cultivated ovaries represent the development in vitro of the two types which can be distinguished among fruits growing on the plant. This interpretation is further substantiated by the experiments with mutilated flowers and by the correlation existing between the stage of flower development and the types of growth response obtained. Mutilation of flowers after the time of pollination does not prevent the development of seeded fruits on the plant nor of Type II ovaries in culture. Mutilation before pollination, however, eliminates Type II ovaries in culture, but equivalent flowers, planted without mutilation, develop as Type II fruits and also the number of such fruits is increased over those of groups which were mutilated. It therefore seems evident that the Type II cultivated fruit is equivalent to the attached seeded fruit and that it is probably fertilized, whereas the Type I development represents that of either a non-pollinated or at least non-fertilized ovary.

What, then, have the in vitro studies told us about the growth requirements of these ovaries? In the first place, it seems that both types have obligate requirements for both mineral salts and a source of carbohydrate, if growth is to be continued for any length of time. In the absence of either of these requirements, some growth will take place for a short time, presumably at the expense of endogenous materials,

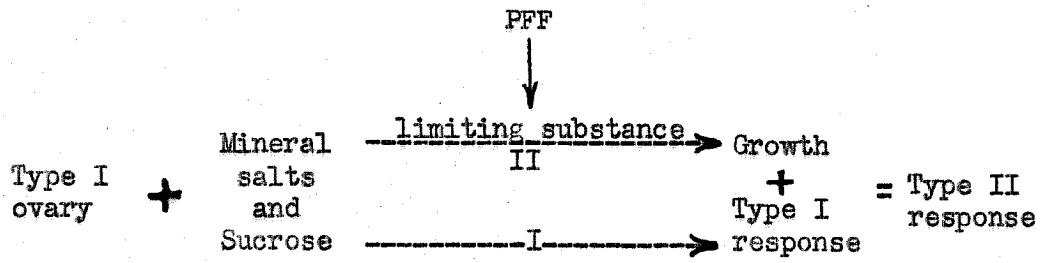
but death will occur after about four days. In the case of the Type I ovary, the carbohydrate requirement for continued increase in diameter can be satisfied by several sugars, including galactose. The amount of growth is not increased by raising the sucrose concentration above 2%. In the Type II ovary, on the other hand, the diameter can be significantly increased during the initial stages of growth by increasing the molar concentrations of sucrose up to M/10 and by the addition of casein hydrolysate to the medium. Another effect of casein hydrolysate will be discussed later.

Thus it seems that the growth in size of the Type II ovary is actually a composite growth resulting from two different processes, one superimposed on the other, and that the observed increase in diameter (or volume) which characterizes the Type II response can be separated into two parts -- one part due to a basal growth process (I), which is common to non-pollinated or non-fertilized ovaries and to those pollinated or fertilized; and another part which results from the establishment of an additional process (II) through pollination or fertilization. The fruits grown in culture do not attain the size of those developed on the plant; therefore, it seems that we must add still a third component in the growth of the normal attached fruit, namely, one attributable to a seed factor.

Since the basic difference between the Type I and Type II ovary seems to be that the latter possesses the pollination-fertilization factor (PFF), one might at first conclude that this factor and process II are the same. This does not appear to be the case, however, for two reasons: a) the pollination-fertilization factor seems to function in a quantitative fashion which is reflected in the magnitude of the

initial growth rate; and b) it also seems to function by means of an intermediary substance. The evidence which concerns (a) is that individual ovaries of any lot and groups of ovaries collected at different times have different capacities for responding under identical treatments. On the plant we know that a similar variation is associated with the number of seeds which develop. The facts which pertain to (b) are that in the absence of either sucrose or casein hydrolysate in media which only partially satisfy the minimal requirements of growth of the Type II ovary (mineral salts and sucrose) no Type II growth response can be obtained. If either of these organic materials are present, however, the Type II growth rate is established and can be maintained until the supply of endogenous materials in the ovary is expended. Thus, sucrose or casein hydrolysate can bring about this conversion of PFF to an active form, whereas galactose cannot. Fructose and glucose can substitute for sucrose, both for this conversion of PFF and for continued growth afterwards.

All of this evidence can be incorporated into the following scheme for growth of the cultivated ovary:



The various factors which have been shown to influence the growth of the cultivated fruits may now be classified into four types:

a) Qualitative factors -- such as, stage of flower development, mutilation practices, starvation treatment, and

smog -- all of which affect the nature of the ovary by determining presence or absence of the pollination-fertilization factor. Smog is generally detrimental to both types of ovaries.

b) Substrate factors -- Mineral salts, carbohydrates, endogenous materials in the ovary, etc. All materials which may be used for growth in either process.

c) Conversion factors -- Specific substances needed for changing PFF to the limiting substance: casein hydrolysate, sucrose, glucose, or fructose.

d) Promotive factors -- Temperature, carbohydrate concentration, auxins, casein hydrolysate.

In connection with the qualitative factors, it has already been mentioned that Type II growth responses are largely restricted to stages of flower development after pollination and fertilization when flowers were mutilated at planting. The number of such responses is greatly increased when flowers are not mutilated, particularly the number from flowers planted at beginning anthesis. Elimination of smog results in almost complete elimination of growth failures and redistribution of growth responses in favor of Type II. Starvation of the ovaries, however, apparently results in complete destruction of PFF, since no Type II responses can be obtained on subsequent transfer to media favorable for development, but starvation does not affect continued growth as Type I ovaries. All of these factors, then, exert their influence through determining in a qualitative fashion whether or not the limiting substance can be formed under otherwise favorable conditions.

In general, substances used as substrate for growth also function as conversion factors for the formation of the limiting substance. In a few instances, however, it has been possible to separate these two functions. Galactose, for instance can be used for growth of the Type II ovary (containing PFF) but only by Process I; thus only Type I responses are obtained. Casein hydrolysate, on the other hand, can bring about the formation of the limiting substance when growth substrate is limited to that contained in the endogenous materials of the ovary, but it apparently cannot function as a growth substrate itself, at least not to any great extent, since the ovaries die after four days just as they do when sucrose is supplied in the absence of mineral salts. In both of these cases, the typical Type II growth takes place during the first two days.

When the minimal conditions are satisfied for obtaining Type II response, the degree of response (amount of increase in diameter) can be increased by several means: increasing the carbohydrate concentration up to M/10, addition of casein hydrolysate in concentrations of 500 mg/l or greater, application of either IAA or NAA at 3 μg/l to the apex of the ovary, or raising or lowering temperature to the optimum range of 20-26° C. Since all of these changes are reflected in changes in the rate of growth of the Type II ovary, they probably are all acting through the limiting substance. The concentration of carbohydrate and casein hydrolysate seem definitely to do so, since growth of ovaries which exhibit Type I response is not increased by changes in carbohydrate concentration and is not supported by casein hydrolysate alone. One possible explanation for the action of these two factors is that they promote pollen tube growth in the ovary and perhaps make conditions

more favorable for fertilization, thereby increasing the amount of PFF which in turn provides for more of the limiting substance through which the rate is increased. In vitro studies on pollen germination and growth have shown that certain growth factors are necessary, including both sugars and amino acids for some pollen (24), and it is a well known fact that pollen tubes will burst in hypotonic solutions. The increased size of the Type II fruits obtained from non-mutilated flowers planted at the stage of beginning anthesis and cultured on a medium containing casein hydrolysate indeed suggests that this may be the correct interpretation.

The temperature relations in growth of fruits in vitro and for those on the plant are divergent. In vitro an optimum of 20-26° C is apparent while on the plant this seems to be about 17° C. Since temperature can affect so many different processes independently it seems probable that the processes affected in the cultivated fruit may be those leading up to the formation of the limiting substance, perhaps pollen growth or the conversion reaction. In the case of the attached fruits some other process, perhaps dealing with formation of substrate in the leaves or its transport to the fruit, is limiting.

The manner in which auxin promotes the growth of the Type II ovary is not yet clear. An external supply is certainly not necessary for the bulk of the growth made. Auxin may perhaps actually be the limiting substance and PFF may be a mechanism producing it. Pollination and fertilization have been shown to establish an auxin-producing mechanism in tobacco (27, 28, 29). At least some of the auxin thus produced moves down the pedicel of the flower. Some of the observations on root and callus formation in the present investigation would suggest

that this is also the case for red currant tomato. Both roots and calluses are known to require auxin for their initiation and to be inhibited above a certain concentration. Since these phenomena occurred on a medium containing an anti-auxin, trans-cinnamic acid, this suggests that the concentration of endogenous auxin was too high initially to permit these phenomena to occur and that the inhibitory action was counteracted by anti-auxin. On the other hand, these phenomena also took place on media containing unautoclaved coconut milk, which has been shown to be a good source of ether extractable auxin (100). The evidence, then, is somewhat contradictory and in any respect is actually not relevant to the auxin relations in fruit growth, except perhaps as a side process. The very small number of Type I fruits encountered throughout these investigations on the growth of the pollinated ovaries has not permitted a direct study of their auxin requirements separately under optimal growing conditions, i.e. on minimal medium. Some of the work of Nitsch, however, seems applicable to the situation.

Nitsch (20, 67) reported that pollinated ovaries of Lycopersicon esculentum (corresponding to those of flowers at the stages of revolute and late corolla used in these investigations) would increase in diameter about 133% in three weeks when grown on a mineral salts-sucrose medium and that unpollinated ovaries (from flowers at beginning anthesis) increased no more than 40-50% during the same length of time. In several other experiments with flowers presumably pollinated (planted at the stage which corresponds to late corolla for L. pimpinellifolium), Nitsch found that the ovaries also increased no more than 50% during a three week period of culture on the same mineral salts-sucrose medium and media supplemented with tryptophan (20, appendix III). On unautoclaved coconut

milk the increase amounted to 114% of the initial diameter. These results seem to indicate that Nitsch also was working with the Type I and Type II growth responses in pollinated ovaries, and that the Type I can be converted into Type II with some factor contained in cocnut milk. We know that coconut milk contains auxin. Furthermore, Nitsch found that non-pollinated ovaries could be made to develop like the better-growing pollinated ovaries by addition of the auxins, 2,4-D and naphthoxyacetic acid, to the medium. It would therefore seem, as suggested by Nitsch, that pollination in some manner results in auxin formation or liberation. The growth substrate requirements of the two types of ovaries of L. esculentum appear to be the same as those of the equivalent ovaries of L. pimpinellifolium. In addition, the maximum size of the pollinated ovaries of L. esculentum cultivated in vitro is only about half of that of the attached fruit. They likewise contain no seeds, which suggests that a seed factor is also involved in development of the attached fruit of the common tomato. It therefore seems probable that the mechanisms for fruit development in both species are identical and that the results obtained with either species are interchangeable.

The growth requirements of gherkin ovaries, also investigated by Nitsch (20, 67), are slightly different from those of tomato. Nonpollinated ovaries do not grow on the basal (mineral salts-sucrose) medium even when supplied with auxin. Pollinated ovaries, however, not only grow well without auxin but will also produce seeds if excision of the flowers is delayed until three or more days after pollination. Both callus production and increase in diameter are roughly correlated with the duration of this delay up to four days. After this time both of

these responses and also number of seeds become maximum. This evidence seems to emphasize quite clearly that the limiting substance for growth is derived from fertilization and that an auxin-producing mechanism is established but is not itself wholly limiting. This mechanism might be either this fertilization factor (FFF in the tentative scheme) or a seed factor. The case of gherkin also brings out another point which concerns this scheme. If process I exists, the factors which govern its operation in gherkin are more complex than in either of the tomatoes. In addition, the embryo growth factors are either very simple and can be satisfied by a common growth substrate or else they may be synthesized by mechanisms contained in the ovary itself.

Through these investigations of fruit development in vitro, certain basic mechanisms seem evident in the three species studied. Specific differences, however, also occur and much more thorough investigation of each is necessary before the identity of the mechanisms proposed can be confirmed. In addition, it seems necessary to ask how, and if, these influencing factors revealed for the cultivated fruits operate on the plant. Certain factors have already been demonstrated to operate in both cases with the fruits of red currant tomato. These are the detrimental effects of smog but divergent effects of temperature. It is of interest to know now where the natural growth substrate originates, to determine whether this is the factor influenced by temperature or whether some particular growth process is involved. Problems in the formation of the "limiting substance", identification of the auxin steps, isolation of the casein hydrolysate factors remain to be investigated.

PART TWO

STUDIES IN VERNALIZATION

A. Introduction.

The concept of vernalization has been defined earlier (pp. 3-4) and it has been stated that we distinguish two types of short-lived plants in which flowering is dependent on exposure to a period of low temperature. These types are the winter annuals and the true biennials. The principal difference between them is that the former, e.g. winter cereals, will eventually come into flower without previous cold-treatment, whereas the biennials have an obligate requirement for low temperatures. It is still dubious, however, as to which of the two groups certain plants should be ascribed and it may be that we must eventually establish still other groups of "cold-requiring" plants. Unequivocal representatives of the winter annual plants are the winter cereals; of the biennial plants, many varieties of sugar beets, turnips, and carrots and the biennial varieties of Hyoscyamus niger, which differ by one gene from annual forms of the same species (101). Another difference between the two plant types seems to be in the stage of development at which they become responsive to low temperatures. Winter annuals can be vernalized in the earliest stages of development, after the embryo has developed for more than five days in the fruiting ear of the parent plant, that is, long before the embryo is mature. Treatment of mature grain is effective, providing only that a slight amount of germination has taken place. Similar attempts to treat germinating seeds of biennials has produced little, if any, effect in this respect, but the

number of unequivocal biennial plants studied is yet too small to say that this difference in time of responsiveness is a distinguishing characteristic.

Physiological studies in vernalization which have been carried out in the last ten to twelve years have shown that the cold requirement can be understood as the result of the interaction of several temperature dependent processes, some promotive to flowering and some antagonistic. They apparently differ in such a way that at low temperatures the promotive processes can proceed at a more rapid rate than the antagonistic. It has also been shown that the vernalization of the winter cereals is effective only in the presence of oxygen and that the excised embryos can be as effectively treated as whole grain but that they require an external source of suitable energy substrate, which can best be supplied by sucrose. The interrelations between these last two requirements, however, have not been definitely established, and it was with this aim in mind that the present investigation was undertaken.

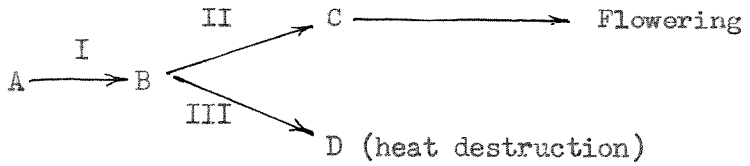
1. The Partial Processes of Vernalization.

The separation of vernalization into several partial processes has been achieved primarily through the study of the intimate temperature relations of these processes, particularly through the high-temperature reversal of the cold action (devernalization). This was first discovered by Gregory and Purvis (103) in attempts to dry out vernalized grain at various temperatures. Periods of high temperature, given immediately after the cold treatment, either abolished or greatly diminished the vernalizing effectiveness achieved at low temperatures. The phenomenon has since been more critically studied by Purvis and Gregory (102, 104), using winter rye, by Stout (105), with sugar beet, and by Lang and Melchers (106), with biennial Hyoscyamus.

A quantitative effect is achieved during the cold period--as the time of low temperature exposure is extended, the time to flowering is decreased until a minimal time to flowering is attained. Further extension of the cold period is without effect. In Petkus winter rye, increasing the length of treatment (1° C) from four days to twelve weeks results in a gradual decrease in time to flowering (anthesis) from more than 150 days to 50 days (107); longer periods are without additional effect and, in fact, the second six weeks period gives but a fraction of the promotive effect achieved during the first six weeks period. In biennial Hyoscyamus the flowering time decreases as the cold treatment is extended to 105 days, depending on the temperature (108). If the time of treatment is sufficiently long, almost any temperatures which are effective for vernalization seem to give the optimum level of effect (107, 108). These facts indicate that a vernalized condition gradually builds up in the cold-treatment plants.

The effect of devernalization is also a quantitive one, increasing with temperature and time (104, 106). However, the relative effectiveness of heat treatment decreases as the duration of the cold treatment is increased. Eventually, a point is reached beyond which no devernalization is possible (104, 110). This relationship between the length of cold treatment and the effectiveness of devernalization suggests that vernalization proceeds in two steps, one resulting in an unstable condition and the other in an irreversible condition. High temperatures are capable of destroying the first, but not the second, of these conditions. This conclusion is supported by the observation, made first in Hyoscyamus (106) and later also in rye (104), that short periods at an intermediate temperature rapidly abolish the possibility of devernalization. Thus in Hyoscyamus four to five days at room temperature almost completely reduce the possibility of reversing the effect of cold by five days at 32° C (106). Four days at 15° C prevents subsequent devernalization in winter rye (104). In both cases the lower temperature is apparently high enough to permit rapid establishment of the irreversible condition, but not high enough for the destruction of the reversible condition.

All of the preceding evidence can be fitted into the working hypothesis proposed by Lang and Melchers (5) and Lang (6).



We are therefore dealing with three processes (I, II, and III). Two of them (I and II) are involved in the formation of some condition necessary for flowering; the third is antagonistic by counteracting the activity of I. None of these processes require low temperature as such,

but I and II either have lower temperature coefficients or perhaps also lower temperature optima than III. The first two, then become effective only at low temperature.

As to the nature of the three processes, previous studies have supplied only indirect and rather general types of evidence. Stout (105) found in sugar beet that 17 days at 19.3° C are as effective as 36 days at 13.2° in reducing the effectiveness of vernalization. This corresponds to a Q_{10} of 3.3. In winter cereals revernalization of devernalized grain is possible. Purvis and Gregory (104) found, working with moisture-limited rye seeds and with threshold temperatures for devernalization, that two weeks of additional cold at 1.8° were necessary to overcome the effect of four days at 17° C. From these data they calculate a Q_{10} of 2.3 on the assumption that the second process is an actual reversal of the first. These temperature coefficients are characteristic of chemical processes. The implication, then, is that the processes underlying vernalization are biochemical (enzymatic). The "conditions" in which they result are perhaps definite chemical substances. Lang (6) stresses the point, however, that A, B, and C are not necessarily directly related in a straight-line series of chemical reactions. Process III (B→D) may consist in a sidetracking of the intermediary product B or in its outright destruction. Purvis and Gregory (104) consider it a reversal of I, since the winter cereals can be revernalized.

The nature of the substances which are produced in the course of the vernalization processes is still entirely obscure. Although the effect of the cold action is localized in the meristematic tissues where initiation of flowers occurs, either in the epicotyls of the cereal embryos or the growing points of the biennials (111, 112), graft experiments between cold-treated and non-cold-treated individuals of biennial

and photoperiodically sensitive plants (8) have shown that the ultimate outcome of the cold treatment is apparently the formation of a transmissible flowering substance. This substance is not identical with florigen, but seems to be necessary for the generation of the latter (Melchers, in 6). Melchers has called this substance "vernalin" (8). But the nature of vernalin, as well as that of florigen, is as yet quite unknown, and we also do not know how vernalin formation is related to the processes of vernalization proper. However, we do have some information of the chemical factors which enter into the process of vernalization.

2. Biochemical Requirements of Vernalization.

Our information about the biochemical requirements of vernalization is based almost entirely on the work of Gregory and Purvis with Petkus winter rye (102, 103, 104, 107, 109, 113, 114). This work has revealed two facts: first, vernalization is effective only if the tissues, in which the actual vernalization processes take place (the embryos), are provided with a suitable substrate. The function of this substrate is to permit the embryo to negotiate the first specific step of vernalization; second, vernalization is effective only if cold-treated seeds are supplied with oxygen.

The substrate requirement has been demonstrated through the use of excised embryos. As previously mentioned, the excised embryo can be vernalized as effectively as whole grains (114) but they show two striking differences in their response. Whereas in whole grains as few as 4 days of cold treatment produce a measurable acceleration of flowering, this is achieved in the excised embryo only after two to four weeks of low temperature. The second difference is that the embryos have to be provided with a suitable sugar (113) in order that rapid flowering after cold treatment will occur. That these two features are furthermore causally related is revealed from the fact that the sugar supply is essential only during that part of the cold period which is ineffective in promoting flowering (107). Once the cold treatment has extended beyond this lag period, the embryos can be treated in the absence of sugar. It appears from these facts that the embryo is supplied with some "ready-made" material from the endosperm of the whole grain which is necessary for vernalization. The embryo itself must contain the mechanism necessary for the production of this

substance, but its production requires time.

The question then is this, does this substance serve as a precursor (substrate) of the actual vernalization processes, or is it directly involved in vernalization at all? If the former is true, one might expect two things: first, the lag period should be abolished by growing the embryos for a few days on a sugar-containing medium at higher temperatures; and second, it should be abolished by leaving the embryos attached to their endosperms for the same periods of time and under the same temperature conditions. Experiments of Purvis (114) show that neither of these postulates is met: neither growing the embryos up to four days on sugar at 20° nor leaving them attached to the endosperms of imbibed grain one or two days at 20° had any marked effect on the lag period. But the lag period was completely abolished by leaving them attached to the endosperm for one week at 1° C. Therefore, the substance in question must persist in the embryos only at low temperatures. It is apparently identical with the first, or unstable "condition" reached in vernalization, (B) in the above scheme of Lang and Melchers--its non-stability at higher temperatures being based on the antagonistic process of vernalization (III). Sugar, then, must be required for the production of B. Once B is present, the subsequent steps of vernalization can take place without further sugar supply.

The second requirement of vernalization was demonstrated by the use of nitrogen atmospheres. Both continuous cold treatment under N-atmosphere and the interruption of vernalizing periods by periods of N-atmosphere at 20° completely suppressed the effect of low temperature (102). Alternate periods of N-atmosphere and air, both at low temperature, were effective towards vernalization only in proportion to the time spent under air. These findings show that the second promotive

process of vernalization (II) goes on only in the presence of O_2 while the antagonistic process (III) becomes fully effective even in the absence of oxygen. Thus, process II — the further conversion of B — involves some obligatory oxidative steps: process III — the loss of B — does not.

But the oxygen requirement of the first promotive partial process (I) remains unsettled. The N-atmosphere experiments of Gregory and Purvis (102) were done with whole grains, in which the substance representing B is supplied by the endosperm. They do not, therefore, permit one to decide whether process I is also strictly aerobic or not.

The present experiments were undertaken to settle this important point. Before entering into their description, however, one further point must be discussed. This is the interrelation of growth and vernalization. Cold treatment of grains of winter cereals is effective only if the grains have started germination. This is not surprising, since most biochemical processes cannot be carried out unless the cells are in an active state. But there is also a rather close correlation between the amount of growth made by the embryos being kept in the cold and the progress of vernalization. Devernalization of vernalized winter rye at ordinary temperatures, 20°, is apparently possible only if growth is in some way inhibited. This can be accomplished with anaerobic conditions, which also prevent process II from occurring (102), or under aerobic conditions when the moisture supply to whole grains of winter rye is limiting for growth (104). Thus the possibility of some causal interaction between growth and vernalization cannot be overlooked. This question will be taken up later.

B. Experimental

1. The Experimental Design.

Since the oxygen requirement of the first partial process of vernalization cannot be settled using whole grains, the experiments were done with excised embryos. A cold period of six weeks was chosen, since this period is sufficient to produce a high degree of vernalization. The period was broken into two periods of three weeks, and the embryos were maintained either under the same condition of sugar and oxygen supply throughout both sub-periods, or were transferred to different conditions after three weeks.

The work of Purvis (107) shows that three weeks are sufficient for the first process of vernalization to become effective, but not sufficient for any considerable degree of vernalization to be attained.

Several other points, also established by Purvis and Gregory, are pertinent to the design of this investigation and were consequently taken into account.

a) The optimal sugar concentration is 2% sucrose; this concentration was used in all sugar-containing media.

b) The response to sugar-supply becomes more clear-cut and the individual variability is reduced if the embryos are depleted of stored energy sources by growing them, prior to the cold treatment, on a sugar-free medium at room temperature. Therefore, one series of the treatments was run using freshly excised embryos, and another with embryos which had been prestarved for four days.

c) Treatments are comparable only when the day of planting

or day of removal from the cold is the same for all treatments. Excisions were therefore scheduled so that all treatments could be removed from the cold on the same day.

d) Fully vernalized winter cereals behave as long-day plants, but incompletely vernalized and non-vernalized plants may be promoted by short-day conditions. To avoid this complicating factor, the plants were maintained on photoperiods of at least 18 hours after transplanting to greenhouse conditions. In addition von Denffer (116) has shown that flowering in winter oats and barley is delayed under regimes of high mineral nutrition, particularly high nitrogen. Therefore, the mineral nutrition was kept at a low level, no plant received more than one liter of Hoagland's nutrient solution up to the end of the experiment. Provision was also made for measuring the growth which had occurred during the cold period.

2. Material and Methods.

Plant Material.

The grains used in this investigation were inbred lines of Petkus summer rye H-6204 and Petkus normal-straw winter rye H-6204 which were obtained from F. von Lochow-Petkus G.m.b.H., Bergen (Kreis Celle), Germany.

Embryo Cultures.

Media. -- The basal medium employed by Purvis (113) was used with the following minor modifications: substitution of ferric tartrate (1.5 mg/l) for ferrous sulfate (0.51 mg/l), increase of the agar concentration from 0.7 to 1.0%, and addition of the trace elements described in Part I. Sugar medium was prepared by adding 20 g of sucrose to a liter of basal salt solution. The pH of all media was adjusted to neutrality with KOH and HCl. After autoclaving at 15 lbs. pressure for 15 minutes, media are cooled to approximately 45° C and dispensed in 30 ml aliquots into 9 cm Petri dishes.

Sterilization and excision. -- After selection for uniformity and sound appearance, the grains were divided into lots of 100 and each lot was sterilized separately immediately prior to excision. For this purpose, the grains were put into 30 ml beakers and first covered with Zephiran Chloride, diluted 1:5000, and swirled for 30 seconds to moisten the surface. The detergent solution was then replaced with 25 ml of a calcium hypochlorite solution ((supernatant from adding 2.5 g $Ca(OCl)_2$ to 100 ml water)). After 1 hour of sterilization the grains were washed

once with sterile distilled water and drained but were allowed to remain in the beaker. Both during and after sterilization the beaker was kept covered with half of a small Petri dish. For excisions, five to ten grains were removed at a time and blotted between sterile paper towels. Embryos were excised on sterile glass slides and freed from all visible traces of endosperm which might remain on the embryos. These operations were done with the aid of specially ground nichrome wire knives. After excision the embryos were transferred to the dishes of media. Sterility of all instruments and slides was obtained by dipping in alcohol, flaming, and cooling between sterile paper towels. With some experience, one complete lot of grains could be excised while the next was sterilizing, so that a continuous schedule of excisions was maintained. Twenty-five embryos were planted in each dish and three dishes were used for each treatment.

All dishes of embryos which were to receive identical treatment during the cold period were placed in separate large vacuum desiccators. Each desiccator had been previously surface sterilized by swabbing with 70% alcohol and equipped so that all gas exchange required would take place through filters of sterile cotton. This was accomplished by placing a plug of cotton inside the cupola-like top of desiccators with top valves and securing it in place by means of a one-holed rubber stopper. A piece of glass tubing, of sufficient length to extend through the perforated porcelain plate and just clear the bottom of the desiccator, was inserted into the stopper (see Figure 17). All evacuation and replacement of the atmosphere was thus required to take place from the bottom compartment. More even dispersal of the incoming gas could also be expected in its passage through the perforated plate. In order to

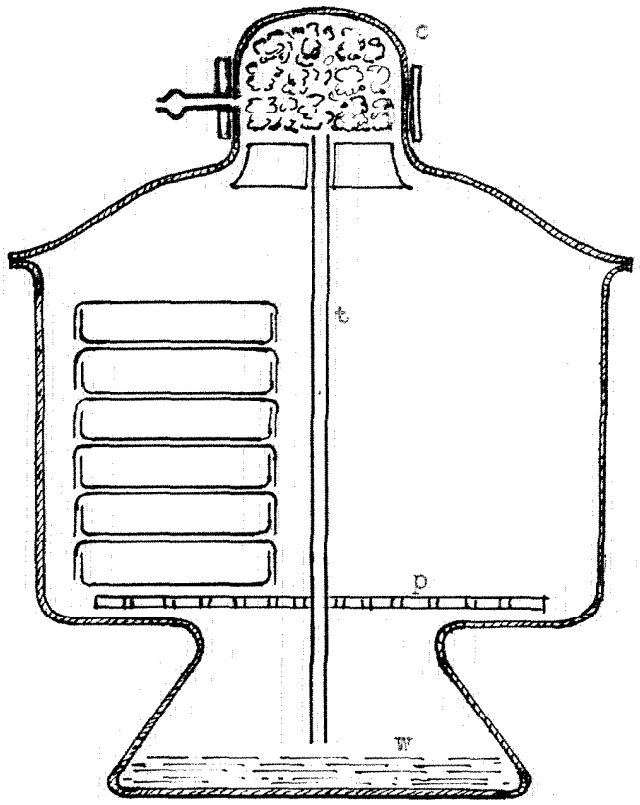


Figure 17. Desiccator equipped for vernalization of excised embryos under different atmospheres. c, sterile cotton in top of desiccator. t, glass tube extending from rubber stopper in top through perforated porcelain plate, p. w, layer of distilled water.

provide for a saturated atmosphere a layer of sterile water was placed in the bottom of each desiccator before final replacement of the lid.

Establishment of Nitrogen Atmospheres.

Each desiccator was separately evacuated to a pressure of approximately 12 mm of mercury. Moist nitrogen, obtained by bubbling the gas through a 15 cm column of distilled water, was allowed to slowly fill the desiccator until atmospheric pressure was reached. This evacuation and refilling was repeated twice. The third filling with nitrogen was allowed to proceed until a very slight positive pressure was built up inside the desiccator.

Temperature control.

No special attempt was made to control temperature conditions exactly but use was made of existing facilities, and temperature checks were made throughout the periods of embryo culture. All cultures which were to receive residual growth (prestarvation) treatment were placed for four days in a room kept at a constant temperature of 18° C. After subsequent transfer of the embryos to appropriate media, they were placed in desiccators, as described, along with an identical series of freshly excised embryos, and air or nitrogen atmospheres were established.

Low temperatures were given by placing the desiccators in a refrigerated work and storage room. During the first five weeks of cold the air temperature of the room varied between 3 and 4° C. At the start of the experiment one desiccator had been equipped with a thermometer, inserted through a rubber stopper closing a side arm. Temperatures

inside this desiccator also fluctuated between 3 and 4° but were frequently found to be about 0.2° lower than the room temperature. During the last week of the cold period the temperature was lowered to 1-2° C. At no time during the cold period did the temperatures exceed 5° C, the upper limit of most effective vernalization temperatures reported by Purvis (114).

Transfer to new media or changes of atmosphere after three weeks, which were required for all treatments to receive six weeks of total cold, were done at room temperature. These changes did not take more than 2 to 4 hours.

Post-cold treatments.--The procedure followed by Purvis (107) was to remove plants first to room conditions to avoid damage by sunlight and to permit an increase in size before planting. Roots of embryos from no-sugar regimes were also flooded with sucrose solution to improve the root system and to re-establish growth. Essentially the same methods were followed in this investigation.

All cultures were removed from both cold and anaerobic conditions on October 5, 1951, and were placed in an insulated room in which the temperature remained between 22-24° C for the entire period during which it was in use. In view of the most recent work of Purvis and Gregory (104), however, both this temperature exposure and the one given at the time of the three-weeks transfers were probably sufficient for some devernalization to occur in those groups in which the degree of vernalization was low and little growth was taking place.

In order to make a record of the growth during the low temperature period, embryos from each treatment were laid out on sterile glass

plates and shadowgraphed on Velour Black A-3 photographic paper. Embryos which had received no sugar or which had been under nitrogen during the second three weeks of the cold period were transferred to sugar media. Weak incandescent light from a 60 Watt bulb was given to all plants 12 hours daily, until the majority of those of each treatment had turned green and were of sufficient size for transplanting under greenhouse conditions.

Greenhouse conditions.

As soon as plants had reached an appropriate size under the conditions previously described, they were transplanted to a mixture consisting of equal parts of unwashed river sand and vermiculite in plastic quart containers. In general, only four plants were placed in each container and no more than fourteen pots were used per treatment. In those groups in which more than 56 plants had survived, five plants were used per pot. After transplanting, each group was watered with Hoagland's nutrient and was then placed in a lath house to become established. Supplementary light from batteries of incandescent bulbs was given from 1600 to 0300 daily. By October 25 all plants were well established and could be transferred to the heated glasshouse. Supplementary light was continued throughout the entire culture period. During the first six weeks after removal from the cold and subsequent to transplanting, watering was done with nutrient only as often as the pots showed evidence of drying. This occurred about every 10th day in the lath house and once a week in the greenhouse. Afterwards nutrient was withheld unless plants showed deficiencies; however tap water

was given by hose as often as required. No pots received more than one liter of nutrient during the course of the experiment.

Criteria for Measuring Effectiveness of Treatments.

Certain technical difficulties confront any study on the physiology of flowering, particularly those on the physiology of flower initiation (5, 6). Absence of visible flowers, for instance, does not necessarily mean ineffectiveness of a given treatment. Some of the early work of Gregory and Purvis showed this to be the case with both vernalized and non-vernalized winter rye and spring rye: under continuous short-day treatment no ear emergence took place although spikelet initials had been formed (109). In view of this, at the termination of this experiment, all "non-flowering" plants were dissected to determine the presence or absence of spikelets at the growing points and all doubtful cases were checked by microscopic examination.

Vernalization is, by definition, an acceleration of flowering resulting from exposure to periods of low temperature. Acceleration, however, cannot always be measured on a time basis. With most plants which respond to vernalizing treatments, increasing the duration of cold treatment within limits results in a gradual shortening of the vegetative phase which precedes the initiation of flowers. But environmental and nutritional factors which influence vegetative growth can greatly affect the duration of this vegetative phase, as measured by time. On the other hand, Gregory and Purvis have demonstrated that the number of leaves formed before the initiation of the ear can be a much more objective measure for differences in flower formation. Within limits, then, the leaf number is an accurate measure of vernalization.

There seems to be a maximum number of leaves which can be produced, however. After this number the plants eventually flower, although not at the same rate with respect to time.

To have as many good criteria as possible for recording the results of all groups the following procedure was adopted. In the first place, the time of ear emergence (appearance of visible flowering) was recorded. Furthermore, each plant was removed from the container at this time and the length of the main axis was measured to the base of the blade of the flag leaf (the last leaf formed before the inflorescence). Also, the number of leaves were counted on the main axis, since the terminal meristem of this axis was invariably the site of initiation of the first inflorescence, except when the main axis was killed or injured at an early age.

By the 49th day after removal of the plants from the cold, flowering (ear emergence) had occurred in all but one of the spring rye treatments and in several of the winter rye treatments, including those which had been supplied with sugar and air for the entire period of cold. All of these are treatments which are expected to flower early on the basis of the results of Gregory and Purvis. First visible flowering in both of the winter rye treatments which received no low temperature exposure had occurred by the 169th day. Since these groups cannot exhibit any effects of the low temperature treatment, the 169th day was defined as the limit of the period during which greatest effectiveness of cold treatment on flowering had been attained.

During a certain period of the experiment -- from the 75th to the 115th days after removal of the plants from the cold --, almost no flowering (ear emergence) occurred from the main axis. Many of the

plants, however, had unfolded the flag leaf, which normally precedes the appearance of the spike. Dissection showed that the ears had stopped growing at various stages of development prior to emergence and had eventually died. The reason for this phenomenon is not known; low light intensity, smog, leakage of gas fumes from the heating system in the basement of the greenhouse, and an aphis infestation, all may have been contributing factors. The killing was apparently limited to those plants in which flower formation had started; plants, which had remained strictly vegetative until the 115th day, subsequently flowered normally from the main axis. The affected plants, on the other hand, all flowered from tillers, either of the first, second, or third order. In the latter cases the parent shoots in each instance also showed partially developed, dead inflorescences. Regardless of the nature of flowering, that is, whether it was from the main axis or from a tiller, the date of flowering recorded for each plant was the date that ear emergence actually occurred.

No significant differences in the leaf numbers of plants flowering by the 169th day were found in groups in which little or no vernalization had been achieved. The leaf numbers in these groups were almost identical regardless of differences in flowering time. Since so few plants had flowered at the 169th day, the experiment was continued until at least 50% flowering had occurred in all groups and was terminated on the 203rd day after the end of the cold treatment. The actual extent of flowering which had occurred in these plants by the last day was determined by dissection as mentioned previously.

The results of the various treatments have been reported in the following ways. Since the tiller-flowering upset the measure of acceler-

ation by time, the rate of flowering (% flowering) during this period has been substituted. Many plants in groups which were effected by the inflorescence killing did not reach ear emergence until after the 169th day. However, the leaf number on the main axis remained low. The greatest vernalizing effectiveness is therefore determined by low leaf numbers during the first 169 days. Those groups which were but poorly vernalized or not at all vernalized were but little affected by tiller-flowering but could not be distinguished by leaf number. At the termination of the experiment the extent of flowering (%) coupled with the days to mean ear emergence consequently reflects the rate of flowering for these groups during the entire period. Those with low percentages of flowering and high number of days to ear emergence are the less vernalized.

3. Results.

The results obtained on the forty different treatments are presented in Table 27. The leaf numbers and extent of flowering (%) by the 169th day are also plotted in the diagrams of Figure 18.

In this figure, those treatments in which maximum, slight, and no vernalizing effectiveness are to be expected from the data of Gregory and Purvis have been marked with special symbols.

One fact becomes particularly obvious from the diagrams. Several of the treatments are sharply separated from all others and are clustered around the points for spring rye and for winter rye vernalized, which are the treatments in which rapid flowering is to be expected. These other treatments, then, have resulted in an acceleration which is equal, or almost equal, to the maximum vernalizing effect that can be obtained under the conditions of this experiment. The acceleration is evident both in the high percentage of flowering (above 60%) and the low leaf number (below 13-14 leaves).

In the case of the prestarved treatments either criterion gives an equally clear-cut picture; in the non-starved, the differences on both counts are less distinct. Many treatments are scattered between the expected vernalized and non-vernalized treatments. This emphasizes, though, the adequacy of the two measures, since a straight line correlation apparently exists. The inclusion of no-sugar treatments, which should largely prevent a perception of cold by the embryos, is expected to be reflected chiefly in embryos which were prestarved. Purvis (107) has shown that the non-starved embryos contain materials which can be used for both vernalization and growth at low temperatures but are used entirely for growth at high temperatures. Therefore, this material has

TABLE 27. Effects of sugar and oxygen (air) and time of application on the vernalization of excised embryos of Petkus winter and summer rye. Treatments: s, sugar; -, no sugar; a, air; N, nitrogen; ——, no cold treatment; separate periods of six weeks treatments separated by a slant (/), the first period is before the slant, the second after. Number of plants: T, total number surviving all treatments; 1, number flowering (Fl'g) by 169th day after removal from cold; 2, number flowering by 203rd day (termination of experiment). Leaves (Lvs.); number of leaves on main axis below inflorescence. Height (Ht.) of flag leaf, the last leaf formed on main axis. Mean days to flowering refers to those which had flowered (ears emerged from flag leaf) by the last day. (Continued on next page.)

TABLE 27 (cont.).

Treatment	PRESTARVED: No. pls. T	Fl'g 1	Fl'g 2	% Fl'g 1	Lvs. #	Ht. cm	% Fl'g 2	Mean days to flowering	Treatment	NON-STARVED: No. pls. T	Fl'g 1	Fl'g 2	% Fl'g 1	Lvs. #	Ht. cm	% Fl'g 2	Mean days to flowering
Petkus Spring Rye																	
—	33	26	33	78.8	8.6	26.0	100	108.7	—	39	34	39	87.2	8.3	31.4	100	92.3
sa/sa	48	30	48	62.5	8.2	28.5	100	111.0	sa/sa	34	21	34	61.8	8.3	29.5	100	117.0
sN/sN	33	23	33	69.7	8.1	28.0	100	105.8	sN/sN	42	38	42	90.5	8.3	22.5	100	100.1
Petkus Winter Rye																	
no cold																	
—	64	3	46	4.7	21.3	64.6	71.9	189.1±2.9	—	64	1	44	1.6	23	69.5	68.8	188.0
three weeks of cold																	
sa	64	17	57	26.6	23.7	66.6	89.1	180.3±6.2	sa	62	21	60	33.9	22.0	66.4	96.8	173.0
sN	53	4	40	7.5	17.3	68.5	75.8	190.3	sN	45	6	39	13.3	24.8	71.4	86.7	183.9
-a	49	2	38	4.1	26.5	75.3	77.6	190.5	-a	52	5	43	9.6	23.2	69.1	82.7	185.7
-N	3	0	2	0	—	—	66.7	203.0	-N	17	1	15	5.9	26	67.5	88.2	195.5
six weeks of cold																	
sa/sa	54	53	54	98.1	9.6	30.1	100	97.6	sa/sa	69	67	69	97.1	8.4	28.1	100	94.5
sa/-a	58	53	58	91.4	10.8	38.3	100	104.2	sa/-a	49	45	48	91.8	10.4	33.2	98.0	99.5
sa/sN	65	18	61	27.7	17.7	53.7	93.8	169.4	sa/sN	57	18	44	31.6	17.2	51.0	77.2	163.3
sa/-N	44	17	42	38.6	18.3	69.9	95.5	166.7	sa/-N	10	2	7	20.0	19.0	45.5	70.0	179.3
sN/sa	62	55	62	88.7	11.3	39.7	100	102.9	sN/sa	50	37	49	74.0	12.4	42.2	98.0	122.5
sN/-a	54	47	54	87.0	11.2	38.9	100	105.0	sN/-a	42	25	37	59.5	15.1	51.7	88.1	135.3
sN/sN	50	19	44	38.0	18.1	59.8	88.0	160.0	sN/sN	63	17	49	27.0	17.6	59.6	77.8	169.3
-a/sa	55	13	44	23.6	17.3	63.1	80.0	169.3±3.5	-a/sa	55	40	52	72.7	17.1	50.8	94.5	132.7
-a/-a	3	0	3	0	—	63.7	100	183.7	-a/-a	57	25	50	43.9	18.3	59.9	87.7	154.6
-a/sN	50	0	39	0	—	67.3	78.0	193.8	-a/sN	62	7	50	11.3	16.3	63.7	80.6	179.4
-N/sa	33	19	32	57.6	19.6	66.6	97.0	149.4	-N/sa	59	32	57	54.2	15.9	57.7	96.6	143.8
-N/-N	16	0	15	0	—	73.8	93.8	195.8	-N/-N	9	0	7	0	—	66	77.8	185.9

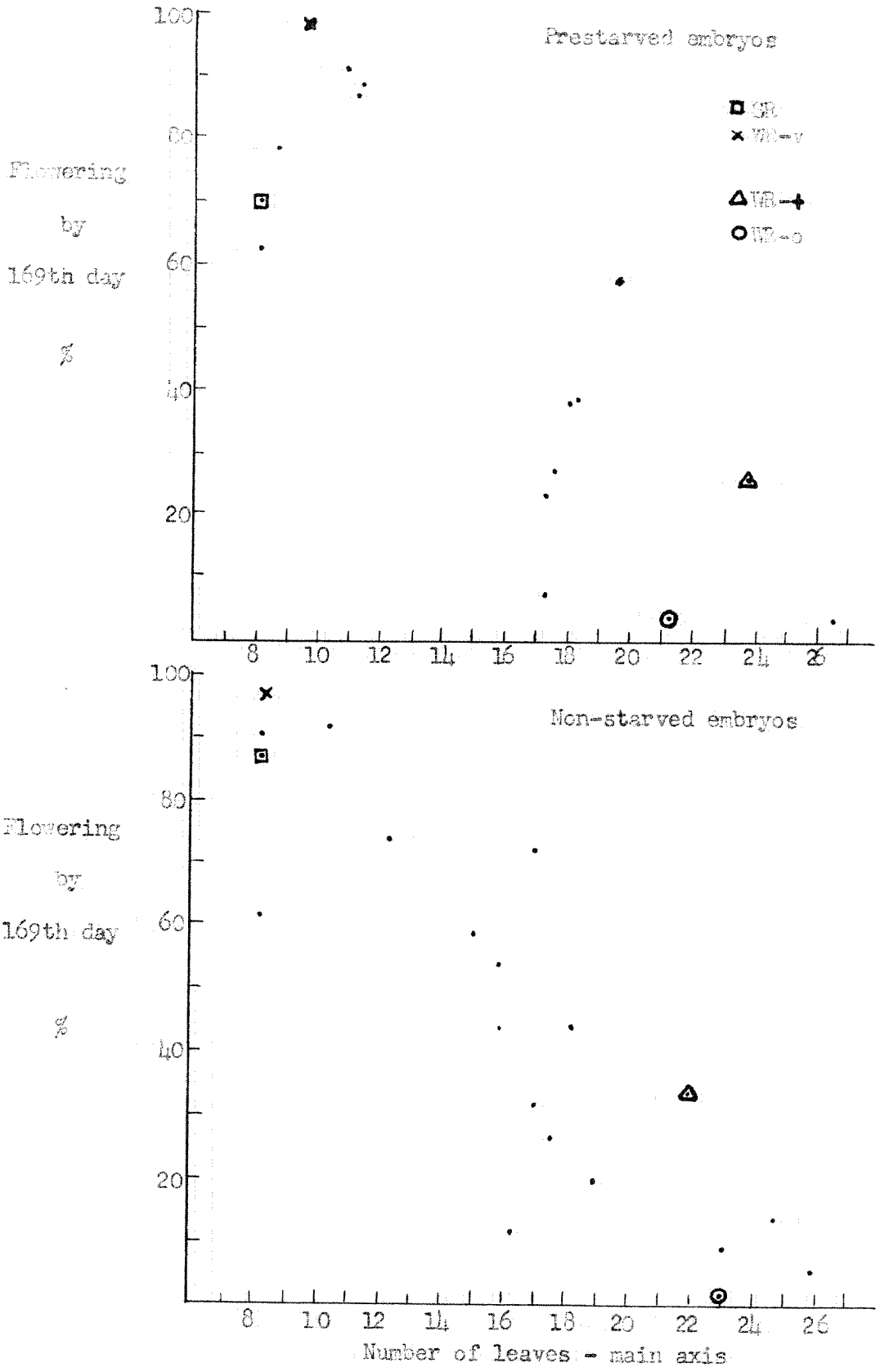


Figure 18. Relationship of leaf number to extent of flowering by 169th day in prestarved and non-starved embryos. SR - summer rye; WR - winter rye; v - vernalized (6 weeks of cold); + - slightly vernalized (3 weeks of cold; o - non-vernalized (no cold).

been largely eliminated in the prestarved embryos, whereas its presence in non-starved embryos is probably the cause of the greater effectiveness towards flower promotion and the intermediate groups seen in Figure 18. This will be taken into account in the discussions which follow.

One may ask how far are these effects specific? If either the lack of sugar during the first period, or the lack of air, is generally detrimental, then we might expect that flowering would be delayed but also that vegetative growth would be similarly slowed down. Such does not seem to be the case from the beginning. Even the prestarved treatments, which should perhaps be more affected by detrimental factors, show definitely that the vegetative growth must proceed uniformly with time. In other words, the longer the time before the plants were collected (low percentage of flowering), the greater the number of leaves. Any effect of treatment, then, must be upon the flowering response.

Effects of Experimental Treatments on Flowering of Spring Rye.

Spring rye is not expected to be affected by cold treatment; it flowers as rapidly without cold as when given the same treatment as winter rye (109). On the other hand, Gregory and Purvis have not utilized spring rye controls in excised embryo studies. Since they have shown that a slight "devernalization" (by one leaf) can be accomplished in spring rye through subjection to anaerobic conditions at high temperatures (102), the spring rye treatments used here stand in the way of controls for the measure of detrimental effects of treatments used. From the data in Table 27 for both starved and non-starved treatments, and for those subjected to anaerobic conditions, it is quite evident

that no detrimental effects were felt. The very low leaf numbers and low heights of the flag leaves are strikingly constant. The apparent differences in rate of flowering (%) by the 169th day cannot be considered significant because of the high percentage (50 to 80%) of tiller flowering which occurred in these groups. It is quite safe, then, to conclude that all treatment effects for winter rye groups must be upon the flowering response.

Conditions Necessary for Effective Vernalization.

If now we select out the winter rye groups in which flowering was equivalent to the spring rye groups, using high rate of flowering by the 169th day and low leaf numbers as our criteria for selection, the following treatments qualify as being almost maximally vernalized:

Prestarved	Non-starved
sa/sa	sa/sa
sN/sa	sN/sa
sa/-a	sa/-a
sN/-a	sN/-a

What then are the requirements for vernalization?

First, and perhaps foremost, all of these treatments involve six weeks of cold treatment. Moreover, non-starved (sa/sa) is in every way comparable to the spring rye treatments -- only 8.4 leaves to flowering, 97% flowering by the 169th day, 100% by the end of the experiment, fewer than 100 days to ear emergence, and less than 30 cm height at flowering. The others are nearly as effective. With regard to the first period of cold, it is easily seen that only sugar is necessary and that anaerobic conditions have no effect, except perhaps in the non-starved embryos

where flowering had been definitely promoted but somewhat less effectively than in the other groups listed. Any changes which occurred during the second period however, were apparently completely independent of the presence of sugar, but it is quite obvious that air must be present. No treatments qualified for vernalizing effectiveness in which anaerobic conditions obtained during the second period. These results, then, definitely permit us to say that effective vernalization of excised embryos of winter rye can be achieved during a sufficiently long cold period provided only that they receive 1) sugar during the first few weeks and 2) aerobic conditions during the remainder of the low temperature exposure. Also, the beneficial effects of sugar application can be achieved under anaerobic conditions. The obligatory requirements may therefore be symbolized as (s/a).

Armed with this information concerning the requirements for vernalization -- sugar during the first and air during the second period of low temperature --, it is now appropriate to inquire: how does the withholding of one or both of these factors affect the flowering response? May we expect no vernalization at all, or is some promotion still achieved? First, however, we should determine the limits of vernalization under the conditions of this experiment. Since cold is one of the prime factors in vernalization, how were the effects of experimental conditions perceived under limited periods of cold?

Effect of Various Treatments During Limited Periods of Cold.

Gregory and Purvis have variously reported values for the non-vernalized flowering response of winter rye as follows: days to first anthesis >150; days to mean anthesis >150; and leaf numbers to flowering >21, 23.0, 23.3, and 21.0. The "no cold" treatments listed in Table 27

are entirely comparable to the results obtained by Gregory and Purvis.

Inasmuch as these controls are comparable, we may actually make some predictions concerning the other treatments of the experiment. Purvis (107) has found that continuous application of sugar during the first two weeks of cold treatment of prestarved, excised embryos has no measurable effect on subsequent flowering and may or may not exert a flower-promoting influence from the second to the fourth week of cold. On the other hand, these same embryos grown in the absence of sugar for long periods of time (up to twelve weeks) eventually attain a degree of vernalization almost equal to that of embryos supplied with sugar. Two points are thus revealed which pertain to our understanding of the vernalization processes. The first is that we cannot expect these processes to proceed in a marked stepwise fashion. If conditions are favorable for the occurrence of vernalization, any amount of substance B, which is formed by process I, should immediately enter into a succeeding reaction. B may perhaps accumulate to a certain extent, if the rates of its formation and utilization differ, but given a sufficiently long period of time the stabilized condition can be attained even at low temperatures. This has been shown by the inability to devernalize cold-treated plants after prolonged periods of low temperatures (104). The second point to be made is this: the plant, or other organism, under even the most adverse conditions, may still derive energy for vital activities from endogenous sources, at least for a time. Hence, we must not necessarily expect a complete "all-or-none" effect of the critical conditions (sugar, oxygen). What do the results show?

It is apparent that only in those embryos which received both of the requisite conditions for vernalization simultaneously during a three-weeks period of cold was any measurable acceleration of flowering

achieved over that of the non-vernalized embryos. In both of the (sa) treatments this promotion is reflected chiefly in the relative increases in the rate of flowering which were maintained both during the period of most effective flowering and during the remainder of the growing period. This increased rate amounts to a 10 to 20% greater extent of flowering which had occurred by the two different days and a shortening of the time to ear emergence by approximately 10 days. In addition, it can be seen that the non-starved embryos were more effectively promoted to the extent of approximately one week than were those prestarved. Although these promotions are not statistically significant in themselves, the constancy of the difference between equivalent treatments would seem to be.

For purposes of making further comparisons, the three-weeks treatment (sa) of prestarved embryos may be considered as representative of the minimal degree of vernalization. The limits beyond which no promotions of flowering occur may be arbitrarily defined as follows: less than 20% flowering by the 169th day or less than 80% by the last day; more than 183 days to ear emergence; flag heights greater than 64 cm; and leaf numbers greater than 21.

Effect of Treatments During Two Three-week Periods of Cold.

In order to continue our discussion, all of the various treatment groups have been reassembled in Table 28 and values for relative effectiveness have been assigned. These values have been determined by comparing data for each group with the arbitrarily defined levels of no effectiveness (0) given above and with the corresponding data for the effectively vernalized lots (5). Non-starved groups have been further compared with the corresponding prestarved treatments and additional

TABLE 28. Relative effectiveness of various combinations of sugar and air on vernalization of Petkus winter rye embryos. Treatments as described in Table 27. Relative values: complete vernalization (5); no vernalization (0).

Conditions satisfied	Treatment	Prestarved embryos	Non-starved embryos
Both	sa/sa	5	5
	sa/-a	5	5
	sN/sa	5	5-
	sN/-a	5	5-
Both, but reversed	-a/sa	2	4
	-a/sN	0	1
Sugar only	sa/sN	2	2/
	sa/-N	2	1
	sN/sN	2	2
	-N/sa	3	3/
	sa	1	1/
	sN	0	0/
Air only	-a/-a	0	2/
	-a	0	0/
Neither	-N/-N	0	0/
	-N	0	0/

promotions have been designated (/). In addition the treatments have have been grouped in accordance with the manner in which they satisfy the requirements for complete vernalization.

Several facts are revealed in Table 28, the most obvious of which are to be expected. Particularly in the prestarved series, partial vernalization depends primarily upon the dual application of both sugar and aerobic conditions. However, in all six-weeks treatments which contained one such period flowering was accelerated to a greater extent than during the single period (sa) alone. This then implies that in such cases, even the non-effective period, with regard to the requirements of complete vernalization, also has a promotive effect on flowering. These treatments, however, all involve either a subsequent period of anaerobic conditions or a preliminary period of no-sugar treatment, both of which may be detrimental to growth. This will be discussed in the next section. Neither satisfaction of the aerobic requirement alone nor application of sugar under nitrogen during the last three weeks resulted in any degree of vernalization. In general, then, it seems that the degree of vernalization attained when only part of the conditions for vernalization are satisfied is proportional to the time spent simultaneously on sugar and under an atmosphere of oxygen. Two treatments, however, have special significance which will be discussed later. These are the treatments (sN/sN) and (-N/sa) in which a much greater effectiveness on flower promotion was obtained than one might anticipate.

With regard to non-starved embryos, the situation appears to be essentially the same except for a slight positive acceleration in nearly all treatments and a lesser dependence on sugar during the first period. One fact stands out in this respect. The lessened priority on sugar can

be explained by materials contained in the embryo which can be used for vernalization, as has been found by Purvis (107). We see here, though, that this material is incapable of acting in a single three weeks period of cold, air, and no sugar; treatment (-a) has an effectiveness of 0/ and treatment (-a/sN), 1. If the duration of the aerobic cold period is extended to six weeks, however, (-a/-a), the promotion of flowering is of the second order (2). It would seem, then, that this material, like sugar, cannot be used directly in vernalization but must also require a lag period.

Before discussing the significance of these findings it is necessary that we also examine the relationship of the various treatments to the growth of the embryo during the cold period.

Effect of Various Treatments on Embryo Growth During Cold Periods.

In general, it may be stated that both growth of the embryo during the vernalizing period and the progress of the reactions which lead to promotion of flowering proceed in a parallel fashion. In a few instances it has been possible to separate the two phenomena. Purvis (113) obtained differential responses through use of various carbohydrate substrates in the vernalization of excised embryos of Petkus winter rye. Ribose, particularly, was found to be much more effective for vernalization of excised embryos than for their growth, but it was not as effective as sucrose for the former process. Chouard and Poignant (117), on the other hand, were able to prevent vernalization almost completely without affecting growth through application of sodium arsenate at a concentration of 100 ppm. Sodium fluoride also caused preferential reduction of vernalization but did not prevent it entirely.

In the present study shadowgraphs of the embryos were made at the termination of the experimental period. Comparisons of the various treatments with regard to both growth and flower promotion showed that a rough correlation between the two phenomena was maintained. A few treatments, however, resulted in differential responses. These were the treatments, discussed in the preceding section, in which greater acceleration of flowering was achieved than was to be expected. The lengths of the epicotyls and the relative flower promotions among the various prestarved embryos have been compared in Table 29.

Separation of the two processes -- growth and vernalization -- is particularly obvious in treatment (-N/sa). Although the amount of growth which took place is significant at the 1% level from that of starved, non-cold-treated controls, it was certainly not great and is entirely out of proportion with the relative effectiveness in vernalization achieved in other treatments. Treatment (-a/sa) also largely prevented growth. It would seem that when the first period of cold treatment is sugarless, growth is almost completely abolished for the prestarved embryo. It is not resumed as might be expected on subsequent transfer of the embryos to sugar medium and aerobic conditions.

Anaerobic conditions during the cold period did not prevent growth. Treatment (sN/sN) demonstrates this. However, there is a considerable reduction in length which must be due to these conditions. The completely vernalized treatment (sN/-a) grew much more even in the absence of added substrate during the second period. Thus, as in vernalization, the effect of sugar application under nitrogen also has an after effect in growth. For prestarved embryos, then, it appears that growth during the period of low temperature is almost completely dependent upon immediate application of sugar, either under aerobic or anaerobic conditions.

TABLE 29. Relationship of growth made during the cold period to subsequent flowering of excised embryos of Petkus winter rye. Treatments and relative effectiveness on flowering as in Tables 27 and 28.

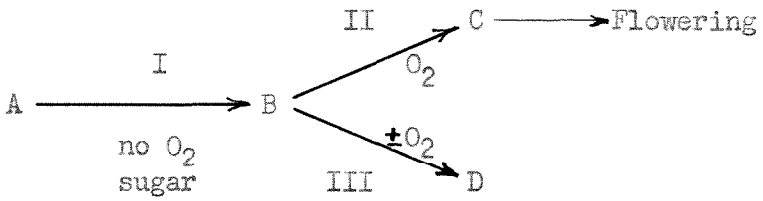
Prestarved treatment	Number of plants	Mean length of epicotyl mm	Relative effectiveness on flowering
----	83	8.11 ± .27	0
sa	72	15.49	1
-N/sa	51	9.49 ± .35	3
-a/sa	68	10.99	2
sN/sN	69	14.72	2
sa/-a	68	29.07	5
sN/-a	66	24.06	5

If the sugar supply is delayed until the second period, growth is not resumed at low temperatures. In this manner, then, the processes involved in acceleration of flowering can be preferentially affected by treatment during the second period. Furthermore, this suggests that growth and vernalization may be competitive processes under adverse conditions.

C. Discussion.

The results obtained in this investigation now permit us to say that the first partial process in the vernalization of excised embryos is mediated through more or less specific non-oxidative mechanisms which in some way require sugar. The second process, however, is oxidative and can take place without further application of sugar.

It is perhaps easiest to visualize these findings in the scheme of Lang and Melchers (6). On the basis of the present evidence this scheme now becomes



The overall process of vernalization, then, involves three processes having separate requirements for oxygen and temperature for their effective operation. In the case of the excised embryo an additional requirement for sugar is exhibited. Process I, for the prestarved embryo, can occur under either air or nitrogen and B will be formed, providing only that sugar is present. After this, Process II will take place, either during cold or at somewhat higher temperatures, if oxygen is present. An additional three weeks of cold is sufficient to elicit maximum promotion of flowering. Process III is independent of oxygen but requires much higher temperatures than does I.

By selective use of the sugar-no sugar and nitrogen-air treatments, however, it should now be possible to effectively study any one of the three processes separately. Thus supply of sugar under nitrogen permits process I to occur but blocks II. In addition, through the use of suf-

ficiently low temperatures, process III is also prevented from occurring. In this way we may anticipate a gradual accumulation of B during prolonged periods of low temperature. Through proper control of oxygen and temperature it may be selectively channelled into either II or III. It seems evident from the results of some of the treatments used in this investigation that this indeed occurs, and it can perhaps explain some of the unexpected promotions which occurred.

Three weeks of cold is the probable length of time necessary to reach a threshold level of B at the temperatures used. Treatment (sa) would indicate that the lag period had been passed, but barely so. The promotion of flowering caused by (sa), which indicates the extent of activity that has taken place through Process II, was very slight. On the other hand (sN) gave no acceleration as a single period, but was completely effective in eliciting the flowering response in (sN/-a). Hence, we might assume that transfer of the embryos to room temperature at the end of the cold permitted what little B was present in the embryos of (sN) to be dissipated by Process III. Longer extension of the sugar-nitrogen period, however, resulted in considerable acceleration of flowering -- (sN/sN), relative effectiveness 2. Therefore B may probably have accumulated to the extent that Process III was saturated and excess B was channelled into II. The acceleration of flowering by (sN/sN) may not necessarily have been achieved during the cold period.

Another possibility for explaining the response in (sN/sN) also comes to mind. It may be the II does not have an obligatory requirement for oxygen. Anaerobic respiration may also be able to supply the necessary energy for its function. As we have seen, such a system must undoubtedly function for growth which occurred in treatment (sN/sN).

Finally, the effect of growth during the cold must also be considered in vernalization. During a detrimental period (particularly no sugar) growth was largely inhibited and subsequent transfer to better conditions failed to elicit renewed growth under low temperatures. This might mean that some essential growth process had been temporarily inactivated. If, then, growth and vernalization should normally compete for some common substrate, perhaps B, at low temperatures, the prevention of growth would seem to selectively channel all of this substrate into vernalization. In this manner, then, the increased effectiveness of treatments such as (-N/sa) and (-a/sa) might be explained; all of the effectiveness would be restricted to the second period.

The possibilities of now studying in a more critical fashion each of the vernalization processes individually and the separation of growth and vernalization should make possible considerable advance in our knowledge of this process and the changes which occur. Determinations of temperature coefficients, for one thing, can perhaps now be more direct. Investigation of many more of the biochemical requirements of vernalization should also be undertaken, particularly of the role of sugar.

REFERENCES

1. SACHS, JULIUS von. 1906. History of Botany (1530-1860). Transl. H. E. F. Garnsey; revised by I. B. Balfour. Clarendon Press, Oxford. P. 568.

2. KLEBS, G. 1918. Über die Blütenbildung von Sempervivum. Flora 11:128-151.

3. GARNER, W. W., and H. A. ALLARD. 1920. Effect of relative length of day and night and other factors of the environment on growth and reproduction in plants. J. Agr. Research 18:553-606.

4. MURNEEK, A. E., and R. O. WHYTE, ed. 1948. Vernalization and Photoperiodism. Chronica Botanica Co., Waltham, Mass. P. 196.

5. LANG, A., and G. MELCHERS. 1948. Die Physiologie der Blütenbildung. Biol. Zentr. 67:105-174.

6. LANG, A. 1952. Physiology of flowering. Ann. Rev. Plant Physiol. 3:265-306.

7. CHAILAHYAN, M. H. 1936. New facts in support of the hormonal theory of plant development. Compt. Rend. (Doklady) Acad. Sci. URSS 13:79-83.

8. MELCHERS, G. 1939. Die Blühhormone. Ber. deut. botan. Ges. 57: 29-48.

9. WENT, F. W. 1944. Morphological observations on the tomato plant. Bul. Torrey Bot. Club 71:77-92.

10. POPHAM, R. A., and A. P. CHAN. 1952. Origin and development of the receptacle of Chrysanthemum morifolium. Amer. Jour. Bot. 39:329-339.

11. WENT, F. W. 1948. Thermoperiodicity. In A. E. Murneek and R. O. Whyte, ed. 145-157.

12. EGUCHI, T. 1937. Effects of the day-length upon the time of differentiation of flower bud and the subsequent development to flowering. Proc. Imp. Acad. (Tokyo) 13:332-333.

13. NITSCH, J. P., E. B. KURTZ, Jr., J. L. LIVERMAN, F. W. WENT. 1952. The development of sex expression in cucurbit fruits. Amer. Jour. Bot. 39:32-43.

14. LOEHWING, W. F. 1938. Physiological aspects of sex in angiosperms. Bot. Rev. 4:581-625.

15. HOWLETT, F. S. 1939. The modification of flower structure by

environment in varieties of Lycopersicum esculentum. J. Agr. Research 58:79-117.

16. RESENDE, F. 1949. Oscillation of auxin level during the year and flowering in Bryophyllum. Portugaliae Acta Biol. (A)2: 373.

17. LAIDACH, F. 1932. Pollenhormon und Wuchsstoff. Ber. deut. botan. Ges. 50:383-390.

18. WITTWER, S. H. 1943. Growth hormone production during sexual reproduction of higher plants. Mo. Agric. Exp. Sta. Res. Bul. 371. P. 58.

19. HATCHER, E. S. J. 1945. Studies in the vernalization of cereals. IX. Auxin production during development and ripening of the anther and carpel of spring and winter rye. Ann. Bot. NS 9: 235-266.

20. NITSCH, J. P. 1951. The role of plant hormones in fruit development. PhD Thesis. California Institute of Technology, Pasadena, Calif.

21. KUHN, E. 1941. Untersuchungen zur Frage einer hormonalen oder zellularen Geschlechtsdifferenzierung bei Blütenpflanzen (Pfropfungen bei zweihäusigen Arten). Planta 32:286-342.

22. JOHANSEN, D. A. 1950. Plant Embryology. Chronica Botanica Co., Waltham, Mass. P. 305.

23. MAHESHWARI, P. 1940. The role of growth hormones in the production of seedless fruits. Science and Culture 6:85-89.

24. YASUDA, S. 1939. Parthenocarpy induced by stimulation of pollination in some higher plants. Mem. Fac. Sci. and Agric., Taihoku Imp. Univ., Phytotechny No. 4, 27:1-51.

25. GUSTAFSON, F. G. 1942. Parthenocarpy: natural and artificial. Bot. Rev. 8:599-654.

26. WENT, F. W., and K. V. THIMANN. 1937. Phytohormones. Macmillan, New York. P. 294.

27. MUIR, R. M. 1942. Growth hormones as related to the setting and development of fruit in Nicotiana tabacum. Amer. Jour. Bot. 29:716-720.

28. ------. 1947. The relationship of growth hormones and fruit development. Proc. Nat. Acad. Sci. 33:303-312.

29. ------. 1951. The growth hormone mechanism in fruit development. In F. Skoog, ed. Plant Growth Substances. Univ. Wis. Press, Madison. p. 357-364.

30. WILDMAN, S. G., and R. M. MUIR. 1949. Observations on the mechanism of auxin formation in plant tissues. Plant Physiol. 24: 84-92.

31. GUSTAFSON, F. G. 1939. Auxin distribution in fruits and its significance in fruit development. Amer. Jour. Bot. 26:189-194.

32. NITSCH, J. P. 1950. Growth and morphogenesis of the strawberry as related to auxin. Amer. Jour. Bot. 37:211-215.

33. HOWLETT, F. S. 1941. Effect of indolebutyric acid upon tomato fruit set and development. Proc. Amer. Soc. Hort. Sci. 39: 217-227.

34. BERGER, J., and G. S. AVERY. 1944. Isolation of an auxin precurson and an auxin (indole acetic acid) from maize. Amer. Jour. Bot. 31:199-203.

35. STEHSEL, M. 1949. Thesis. University of California, Berkeley.

36. MURNEEK, A. E. 1951. Growth-regulating substances in relations to reproduction of some horticultural plants. In F. Skoog, ed. Plant Growth Substances. Univ. Wis. Press, Madison. p. 329-345.

37. REDEMANN, C. T., S. H. WITTWER, and H. M. SELL. 1951. The fruit-setting factor from the ethanol extracts of immature corn kernels. Arch. Biochem. 32:80-84.

38. WENT, F. W. 1944. Plant growth under controlled conditions. II. Thermoperiodicity in growth and fruiting of the tomato. Amer. Jour. Bot. 31:135-149.

39. LEOPOLD, A. C., and Frances I. SCOTT. 1952. Physiological factors in tomato fruit-set. Amer. Jour. Bot. 39:310-317.

40. NUTMAN, P. S. 1939. Studies in the vernalization of cereals. VI. The anatomical and cytological evidence for the formation of growth-promoting substances in the developing grain of rye. Ann. Bot., N.S. 3:731-757.

41. BRINK, R. A., and D. C. COOPER. 1947. The endosperm in seed development. Bot. Rev. 13:423-541.

42. TUKEY, H. B. 1936. Development of cherry and peach fruits as affected by destruction of the embryo. Bot. Gaz. 98:1-24.

43. ———. 1933. Growth of the peach embryo in relation to growth of fruit and season ripening. Amer. Soc. Hort. Sci. 30:209-218.

44. SINNOTT, E. W. 1945. The relationship of growth to size in cucurbit fruits. Amer. Jour. Bot. 32:439-446.

45. HOUGHTALING, Helen B. 1935. A developmental analysis of size and shape in tomato fruits. Bul. Torrey Bot. Club 62:234-252.

46. GUSTAFSON, F. G., and Elnore STOLDT. 1936. Some relations between leaf area and fruit size in tomatoes. Plant Physiol. 11:445-451.

47. GUSTAFSON, F. G., and Helen B. HOUGHTALING. 1939. Relation between fruit size and food supply in the tomato. Plant Physiol. 14:321-332.

48. van OVERBEEK, J. 1946. Control of flower formation and fruit size in the pineapple. Bot. Gaz. 108:64-73.

49. JANSEN, L. L., and J. BONNER. 1949. Development of fruits from excised flowers in sterile culture. (Abstract). Amer. Jour. Bot. 36:826.

50. NITSCH, J. 1949. Obtention de fruits charnus en culture in vitro. Compt. Rend. Acad. Sci., Paris 229:445-446.

51. -----. 1949. Culture of fruits in vitro. Science 110:499.

52. WHITE, P. R. 1951. Nutritional requirements of isolated plant tissues and organs. Ann. Rev. Plant Physiol. 2:231-244.

53. GAUTHERET, R. J. 1947. Plant tissue culture. Sixth Symposium on Development and Growth 21-23.

54. WHITE, P. R. 1943. A Handbook of Plant Tissue Culture. Jaques Cattell Press, Lancaster, Pa. P. 277.

55. GAUTHERET, R. J. 1942. Manuel Technique de Culture de Tissus Végétaux. Masson ed., Paris.

56. BONNER, J. 1948. The B vitamins as plant hormones. Vitamins and Hormones 6:225-275.

57. SCHOPFER, W. H. 1943. Plants and Vitamins. Transl. by N. L. Noecker. Chronica Botanica Co., Waltham, Mass. P. 293.

58. SKOOG, F., ed. 1951. Plant Growth Substances. Univ. Wis. Press, Madison. P. 476.

59. NĚMEC, B. 1935. Über Frucht- und Samenansatz bei Lilium candidum L. Z Věstniku Kral. Čes. Spol. Nauk. 2:1-16.

60. DAHL, A. O., R. SCHREINER, and Gertrude S. JOACHIM. 1949. Culture of Iris in vitro. Bul. Amer. Iris Soc. 114:99-101.

61. La RUE, C. D. 1942. The rooting of flowers in sterile culture.

Bul. Torrey Bot. Club 69:332-341.

62. van OVERBEEK, J., Marie E. CONKLIN, and A. F. BLAKESLEE. 1942. Cultivation in vitro of small Datura embryos. Amer. Jour. Bot. 29:472-477.

63. SMITH, P. G. 1944. Embryo culture of a tomato species hybrid. Proc. Amer. Soc. Hort. Sci. 44:413-416.

64. HAAGEN-SMIT, A. J., R. SIU, and Gertrude WILSON. 1945. A method for the culturing of excised, immature corn embryos in vitro. Science 101:234.

65. ZIEBUR, Nancy K. 1951. Factors influencing the growth of plant embryos. In F. Skoog, ed. Plant Growth Substances. Univ. Wis. Press, Madison. p. 253-261.

66. La RUE, C. D., and G. S. AVERY, Jr. 1938. The development of the embryo of Zizania aquatica in the seed and in artificial culture. Bul. Torrey Bot. Club 65:11-21.

67. NITSCH, J. P. 1951. Growth and development in vitro of excised ovaries. Amer. Jour. Bot. 38:566-577.

68. WENT, F. W. 1949. The Earhart Plant Research Laboratory. Chronica Botanica 12. Reprint No. 2.

69. BONNER, J. 1940. On the growth factor requirements of isolated roots. Amer. Jour. Bot. 27:692-701.

70. MURRAY, Mary Aileen. 1945. Carpellary and placental structure in the Solanaceae. Bot. Gaz. 107:243-260.

71. MULLER, C. H. 1940. A revision of the genus Lycopersicon. U. S. Dept. Agric. Misc. Pub. 382. P. 28.

72. POWERS, L. 1942. The nature of the series of environmental variances and the estimation of the genetic variances and the geometric means in crosses involving species of Lycopersicon. Genetics 27:561-575.

73. BAILEY, L. H. 1939. The Standard Cyclopedia of Horticulture. Macmillan, New York. 3 vols.

74. SMITH, P. G. 1944. Resistance of Lycopersicon spp. to spotted wilt. (Note.) Phytopath. 34:504-505.

75. LINDSTROM, E. W. 1932. A fertile tetraploid tomato cross-sterile with diploid species. J. Hered. 23:115-121.

76. GUSTAFSON, F. G. 1944. Growth hormone studies of some diploid and autotetraploid plants. J. Hered. 35:269.

77. COOPER, D. C., and R. A. BRINK. 1945. Seed collapse following matings between diploid and tetraploid races of Lycopersicon pimpinellifolium. Genetics 30:376-401.

78. HAYWARD, H. E. 1948. The Structure of Economic Plants. Macmillan, New York. P. 674.

79. BAILEY, L. H. 1949. Manual of Cultivated Plants. Macmillan, New York. P. 1116.

80. GOEBEL, K. 1887. Outlines of Classification and Special Morphology of Plants. Transl. by H. E. F. Garnsey; revised by I. B. Balfour. Clarendon Press, Oxford. P. 515.

81. CALDWELL, J. 1930. A note on the dichotomous branching of the main stem of the tomato (Lycopersicon esculentum). Ann. Bot. 44:495-498.

82. VENNING, F. D. 1949. Investigations on the morphology, anatomy, and secondary growth in the main axis of Marglobe tomato (Lycopersicon esculentum Mill.). Amer. Jour. Bot. 36:559-567.

83. VELENOVSKY, J. 1905. Vergelichende Morphologie der Pflanzen. 1 Teil. Verlagsbuchhandlung von Fr. Řivnáč, Prag. P. 1211.

84. SACHS, J. 1875. Textbook of Botany. Transl. by A. W. Bennett and W. T. T. Dyer. Clarendon Press, Oxford.

85. GOEBEL, K. 1900. Organography of Plants. Transl. by I. B. Balfour. Clarendon Press, Oxford. 2 Parts.

86. de WAARD, Jeanne, and J. W. M. ROODENBURG. 1948. Premature flower-bud initiation in tomato-seedlings caused by 2,3,5-triiodobenzoic acid. Proc. Kon. Akad. Wetensch., Amsterdam 51:248-251.

87. ZIMMERMAN, P. W., and A. E. HITCHCOCK. 1942. Flowering habit and correlation of organs modified by triiodobenzoic acid. Contrib. Boyce Thompson Inst. 12:491-496.

88. COOPER, D. C. 1927. Anatomy and development of tomato flower. Bot. Gaz. 83:399-411.

89. ROBERTS, R. H., and B. Esther STRUCKMEYER. 1944. The use of sprays to set greenhouse tomatoes. Proc. Amer. Soc. Hort. Sci. 44:417-427.

90. JUDKINS, W. P. 1939. Time involved in pollen tube extension through style and rate of fruit growth in tomato (Lycopersicum esculentum Mill.). Proc. Amer. Soc. Hort. Sci. 37: 891-894.

91. CLENDENNING, K. A. 1942. The respiratory and ripening behaviour of the tomato fruit on the plant. Canadian Jour. Research, C 20:197-203.

92. JANES, B. E. 1941. Some chemical differences between artificially produced parthenocarpic fruits and normal seeded fruits of tomato. Amer. Jour. Bot. 28:636-646.

93. SNEDECOR, G. W. 1937. Statistical Methods. Collegiate Press, Inc., Ames, Iowa. P. 341.

94. CLENDENNING, K. A. 1948. Growth studies of normal and parthenocarpic tomato fruits. Canadian Jour. Research, C 26:507-513.

95. WENT, F. W. 1944. Plant growth under controlled conditions. III. Correlation between various physiological processes in the tomato plant. Amer. Jour. Bot. 31:597-618.

96. WENT, F. W. 1945. Plant growth under controlled conditions. V. The relation between age, light, variety and thermoperiodicity of tomatoes. Amer. Jour. Bot. 32:469-479.

97. LEWIS, H., and F. W. WENT. 1945. Plant growth under controlled conditions. IV. Response of California annuals to photoperiod and temperature. Amer. Jour. Bot. 32:1-12.

98. GAUTHERET, R. J. 1947. pH et cultures de tissus végétaux. Rev. Gen. Bot. 54:5-34.

99. BONNER, J., and A. W. GALSTON. 1952. Principles of Plant Physiology. W. H. Freeman and Co., San Francisco. P. 499.

100. HENDERSON, J. H. M. (unpublished results).

101. CORRENS, C. 1904. Ein typisch spaltender Bastard zwischen einer einjährigen und einer zweijährigen Sippe des Hyoscyamus niger. Ber. deut. botan. Ges. 22:517-524.

102. GREGORY, F. G., and O. N. PURVIS. 1938. Studies in the vernalization of cereals. III. The use of anaerobic conditions in the analysis of the vernalising effect of low temperature during germination. Ann. Bot., N.S. 2:753-764.

103. ———, and ———. 1936. Devernalization of winter rye by high temperature. Nature 138:1013-1014.

104. PURVIS, O. N., and F. G. GREGORY. 1952. Studies in the vernalization of cereals. XII. The reversibility by high temperature of the vernalized condition in Petkus winter rye. Ann. Bot., N.S. 16:1-21.

105. STOUT, M. 1946. Relation of temperature to reproduction is sugar beets. J. Agr. Research 72:49-68.

106. LANG, A., and G. MELCHERS. 1947. Vernalisation und Devernalisation bei einer zweijährigen Pflanze. Zeit. f. Naturforsch. 2b:444-449.

107. PURVIS, O. N. 1947. Studies in the vernalisation of cereals. X. The effect of depletion of carbohydrates on the growth and vernalisation of excised embryos. Ann. Bot., N.S. 11:269-283.

108. LANG. A. 1951. Untersuchungen über das Kältebedürfnis von zweijährigem Hyoscyamus niger. Der Zuchter 21:241-243.

109. PURVIS, O. N., and F. G. GREGORY. 1937. Studies in the vernalisation of cereals. I. A comparative study of vernalisation of winter rye by low temperature and by short days. Ann. Bot., N.S. 1:569-592.

110. LANG, A. (unpublished results).

111. PURVIS, O. N. 1940. Vernalisation of fragments of embryo tissue. Nature 145:462.

112. CURTIS, O. F., and H. T. CHANG. 1930. The relative effectiveness of the temperature of the crown as contrasted with that of the rest of the plant upon the flowering of celery plants. Amer. Jour. Bot. 17:1047-1048.

113. PURVIS, O. N. 1944. Studies in the vernalisation of cereals. VIII. The role of carbohydrate and nitrogen supply in the vernalisation of excised embryos of Petkus winter rye. Ann. Bot., N.S. 8:285-314.

114. ———. 1948. Studies in the vernalisation of cereals. XI. The effect of the date of sowing and of excising the embryo upon the responses of Petkus winter rye to different periods of vernalisation treatment. Ann. Bot., N.S. 12:183-206.

115. GREGORY, F. G., and O. N. PURVIS. 1938. Studies in the vernalisation of cereals. II. The vernalisation of excised mature embryos, and of developing ears. Ann. Bot., N.S., 2:237-251.

116. von DENFFER, D. 1941. Über die Wechselbeziehungen zwischen Stickstoffbedürfnis und photoperiodischer Reaktion bei einigen Lang- und Kurztagspflanzen. Planta 31:418-477.

117. CHOUARD, P., and P. POIGNANT. 1951. Recherches préliminaires sur la vernalisation en présence d'inhibiteurs de germination et de respiration. Compt. Rend. Acad. Sci., Paris 232:103.