ONTOGENETIC STUDIES OF SHOOT APICES OF *PHARBITIS NIL*

MORPHOGENETIC AND *IN VITRO* STUDIES OF THE SHOOT APICES OF *PHARBITIS NIL* CHOIS, STRAIN VIOLET IN RELATION TO PHOTOPERIODIC INDUCTION

by

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SCOPE AND CONTENTS:

In this investigation the methods of obtaining uniform and maximum germination of seeds and of producing contamination-free cultures of the shoot apices of *Pharbitis nil* have been examined and established.

Flowering response of vegetative seedlings of different ages has been investigated as related to photoperiodic induction. The sequence of morphological changes at the shoot apex has been examined in *Pharbitis nil*, in relation to an inductive photoperiod. The phenomenon of reversion has been investigated, by excising and culturing *in vitro* the shoot apices from intact plants, at varying periods following inductive period. Floral induction in cultured vegetative apices has been attempted.

It is shown that the intact plants respond differently in flower formation to an inductive photoperiod. The apices vary in morphological configuration and cytologic zonation, during the germination, vegetative and reproductive

stages. The in vitro studies have also shown that the flowering process once initiated, cannot be reversed until and unless a mechanism inhibits its regulatory process in the early stages of transition. The leaf is the primary site of photoinduction. Floral induction is related to the presence of a minimum leaf area.

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INTRODUCTION

It is acknowledged that in plant development, growth is an orderly process that gives rise to specific organs at specific times. In all vascular and non-vascular plants, with the first division of the fertilized egg, there is a distinction between the apex and the base. Further differentiation includes the formation of a distal region of meristematic cells in which the protoplasmic content is very dense. This region later becomes the shoot apex with a specific and distinctive organization.

The shoot apex of different species differs with regard to shape, size, mode of organ formation, and pattern of tissue differentiation (Boke, 1947; Popham, 1951; Gifford, 1954; Wardlaw, 1965). So far, seven different types of apical construction have been recognized (Wardlaw, 1957). These range from the "apical cell type" exemplified by ferns and Selaginella spp. to those in which apical initial cells are completely absent but in which embryonic cells are arranged in "histogenic" layers. Several theories have been proposed attempting to explain the extensive diversity in the structure of shoot apices of different species. These theories, named the Apical Cell theory (for cryptogams); Histogen, Tunica Corpus and Cytohistological theories (for angiosperms and gymnosperms) have been reviewed by various workers (Gifford, 1954; Esau, 1965; Romberger, 1963; Wardlaw, 1965).

The apical meristem which has been established during embryogenesis is assumed responsible for all the primary morphogenetic activity and co-ordinated development of the axis. Meristematic cells at the apex

continue to divide and to initiate leaf primordia and certain tissue systems. The ontogeny of the vegetative shoot apices of angiosperms has been extensively studied for different plant species (Boke, 1947, 1948, 1949; Popham and Chan, 1950; Gifford and Tepper, 1962; Millington and Fisk, 1956; Wetmore, Gifford and Green, 1959).

In flowering plants, with the onset of the reproductive stage, the shoot apex no longer produces vegetative structures but initiates floral organ primordia. The floral parts, from their genesis, appear to be leaves, but they are extensively modified and their position on the stem is radically different from true leaves. Though this change of the vegetative shoot apex, involving transformation to the floral habit, has been studied by various workers, only a few have related the phenomenon of floral morphogenesis to photoperiodic induction (Popham and Chan, 1952; Gifford and Tepper, 1961; Naylor, 1941; Stein and Stein, 1960; Wetmore et al., 1959). These studies indicate that photoperiodically induced shoot apices differ in size, pattern of organization, organ differentiation and mitotic activity. Hence, the less explored area of floral initiation and development, in relation to photoperiod, needs extensive investigation for different plant species.

The differentiation of the reproductive structures from the vegetative shoot is a conspicuous phase in the life cycle of a flowering plant. This change is controlled by environmental factors which interact with the genetic phenotype in a specific manner (Bonner, 1965; Zeevaart, 1964). Temperature, photoperiodism (Hillman, 1962, defines photoperiodism as a response to the duration and timing of the light and the dark condition),

intensity and quality of light, nutrition, C-N ratio and age of the plant are confirmed environmental factors which affect floral initiation. Under natural conditions and when other factors are not limiting, the two main factors which control floral initiation are temperature and photoperiod.

The effect of temperature on flower formation is either direct or indirect, the latter being identified as vernalization. The direct effect of temperature has been studied mostly on bulb plants, as reviewed by Hartsema (1961). These studies have indicated that there are categories of bulb-plants requiring appropriately different temperatures during storage and growth to effect flower development. The voluminous literature on vernalization has been adequately reviewed by various workers (Chouard, 1960; Purvis, 1961; Lang, 1965). It is clear from these accounts that in many species, flowering, especially of many biennials and perennials, is induced by low temperature. Most of the literature describes only the manifest responses. To date, nothing is understood about the biochemical processes underlying vernalization.

The effects of photoperiodism and intensity, quality and duration of light in floral initiation have been studied and summarized by Hillman (1962), Lang (1952, 1965), Salisbury (1961, 1963), and Zeevaart (1962a, 1963). When photoperiod controls reproductive development, floral initiation depends upon the formation of a mobile floral stimulus. This stimulus, formed in the leaves during the inductive photoperiod (Lang, 1965), is translocated to the shoot apex. The rate of translocation has been determined by Imamura and Takimoto (1955a) for Pharbitis nil* as 6.2

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*Some authorities also use the generic name of Pharbitis as Ipomoea.

to 9.1 centimeters per 24 hours. After the floral stimulus reaches the shoot apex, biochemical and morphological changes occur, resulting in a floral apex.

The light quality has been postulated to affect the flowering process through the involvement of a phytochrome system (Hendricks and Borthwick, 1963; Nakayama, Borthwick and Hendricks, 1960; Borthwick, 1959; Nakayama, 1958; Downs, 1956). This hypothesis proposes the presence of the pigment phytochrome in two photochemically interconvertible forms, a red absorbing form (PR), and a far red absorbing form (PF), (Hendricks and Borthwick, 1963). The conversion from the (PF) to the (PR) form takes place slowly in darkness by a thermal process. The (PF) form brings about the inhibition of induction in short day plants (SDP), that is, plants in which flower initiation is promoted by day length shorter than a particular valve differing from species to species (Hillman, 1962).

Apart from the environmental conditions effecting flowering, photoperiod sensitivity may also change with the age of the plant. Some plants, notably Pharbitis nil, (Kujirai and Imamura, 1958) and Chenopodium rubrum (Cumming, 1959), can be fully photoinduced as seedlings without any leaves. Here, full photoinductive sensitivity is attained in the cotyledons. In many other plants, the Soybean (Borthwick and Parker, 1938), Perilla (Zeevaart, 1958), Kalanchoe blossfeldiana (Harder, 1948), and Chenopodium amaranticolor (Lona, 1949), seedlings and young plants do not respond to photoinduction at all. They have to reach "ripeness to flower" or pass their "juvenile phase" before they

can be photoinduced.

In the case of Pharbitis nil, the juvenile phase lasts for 1 day after germination, then the seedlings become responsive to short days (Kujirai and Imamura, 1958). After attaining the maximal sensitivity to photoperiodic induction, which lasts for 2-4 days, the seedlings do not respond to inductive photoperiod.

Bonner (1965), Melchers (1961), and Zeevaart (1962a, 1964a) have advanced the following hypothesis regarding flowering. All the cells of higher plants possess the same genetic information but all of which is not expressed at the same time. When the vegetative bud is transformed into a floral bud in response to photoperiodic induction, the floral genes previously repressed become active and bring about the change in the developmental pattern.

Certain other phenomena are associated with the environmental control on flower bud formation. Lang (1965, p. 1380) points out, "However, once a meristem has been determined as a flower primordium, it is usually unable incapable —except perhaps at the very earliest stages—of reverting to vegetative growth." So far, no conclusive report concerning the irreversibility in development after the induction of floral primordia has been noted in the literature. This irreversibility needs further experimentation if control and ontogeny are to be adequately evaluated.

In vitro studies of the meristems without the photoperiodic factor have demonstrated their potential for growth and regeneration, when certain cultural conditions are established (Ball, 1948, 1952;

Wardlaw, 1950). Studies of a limited number of investigations on growth of excised shoot apices in tissue culture have been reported (Ball, 1948, 1952, 1960; Wetmore, 1954). Some workers (White, 1933; Loo, 1946; Hendrickson, 1954; Baldev, 1959; Raghavan and Jacobs, 1961) have induced flowering in culture in the growing explants. Recently, Golun, Yung and Lang (1962), and Tepfer, Greyson, Craid and Hindman (1963) have reported growth of detached floral buds of Cucumis sativus and Aquilegia in which floral primordia had already been well differentiated. In an abstract, Carr (1959) reported that apices excised from plants, which had been given three long dark periods, formed flower primordia, after one month of growth in vitro. The author did not state the stage of floral differentiation of the apices used in the studies, nor the stage of floral differentiation achieved after the apices were in culture for one month.

Although leaves are considered to be the principal sites of photoperiodic induction, they should not be considered as the only plant organs responsive to photoinduction. Lona (1949) reported floral induction in defoliated plants of Chenopodium amaranticolor. In Zeevaart's (1958) experiments with Perilla, some flowering was obtained when a petiole was grafted onto a non-induced one. In vitro experiments of Baldev (1959) and Raghavan and Jacobs (1961) have indicated that flowering can be induced in excised shoot tips when these are cultured and subjected to photoinductive conditions.

THE OBJECTIVES

The literature surveyed in the preceding section draws attention to four aspects of flower formation which sould be investigated.

The first is the flowering response of Pharbitis nil vegetative seedlings of different ages to an inductive photoperiod. Kujirai and Imamura (1958) and recently Marushige (1965a) have reported that vegetative seedlings of Pharbitis nil become insensitive to photoperiod, after attaining the maximal sensitivity. From the ontogenetic point of view, it is essential to know about the "juvenile" and "adult" phases of the plant as the transition, from the vegetative to reproductive stage of development, cannot be achieved until the plant has passed the "juvenile" phase. This area of investigation gives information about the period during which the "juvenile" and "adult" stages appear in the life of the plant. It also explores the causes relating to the insensitivity of the plants to photoperiodic induction, after a certain period of growth.

The second investigation examines the ontogenetic development of the vegetative and floral shoot apices of a (SDP)* Pharbitis nil, and the relation of different stages of floral development with the photoperiodic induction. The investigation extends from the time the plants germinate until all the floral organ primordia have been initiated. Morphological research on the histogenesis of floral parts (origin of different parts of the floral shoot apex) is used extensively for the interpretation of the ontogeny of the flower and differentiation of the

* Flower initiation in (SDP) is promoted by daylength shorter than a particular value, which differs from species to species.

apical meristem. In vivo morphological studies on the shoot apices are significant as a reference, for those wishing to account for specific stages observed in in vitro studies.

The third approach investigates Lang's (1965) hypothesis that in vivo photoperiodically induced apices rarely revert to the vegetative stage except perhaps at the earliest stages. This investigation proposes to determine whether apices excised and cultured from photoperiodically induced intact plants after one inductive dark period, would continue to differentiate under controlled conditions, or would revert to the vegetative conditions. This approach will give information as to whether or not the flowering process, once initiated by the floral genes (Bonner, 1965), will persist or be inhibited in the apices, either through excision or during in vitro culture. Better controls of growth and development were achieved in the present study by isolating the appropriate tissue and growing it under more controlled in vitro conditions than used by previous workers. Thus the possibility of any other system originating in other organs, and interfering with the process of floral initiation was reduced.

The aim of the fourth investigation is to determine the site of floral induction in the case of Pharbitis nil when the apices are excised and cultured in vitro and then subjected to a single inductive photoperiod. This allows for the determination of whether the apices can be induced without bearing any folded or unfolded leaves or, whether the presence of a certain amount of leaf area is an essential factor for floral induction.

The Plant Material

The plant material selected for the aforementioned investigations was a (SDP) Pharbitis nil chois, strain violet (Japanese Morning Glory). This strain is used extensively as a highly sensitive (SDP) for photoperiodic and physiological studies on floral initiation (Imamura, 1953; Kujirai and Imamura, 1958; Takimoto and Ikeda, 1959; Zeevaart, 1962b, 1964a; Bonner, Heftman, and Zeevaart, 1963).

Plants differ greatly in the length and number of photoperiods required for floral initiation. On one hand, there are plants like Xanthium pensylvanicum, Chenopodium amaranticolor, and Pharbitis nil which require a single inductive cycle; on the other hand, there are some like Glycine max and Chrysanthemum morifolium, to which a number of photoperiodic cycles must be given without interruption for flower formation. It has been reported by Lona (1950), Carr (1955), and Naylor (1961), that cyclic inductive photoperiods result in cumulative response of flowering, either in a greater number of flowers or in the earliness of flower opening. In such cases, the net results, exhibited as a flowering response, cannot be attributed solely to the effect of the inductive photoperiod, but are apparently due to the summation effects of photoinductive cycles. Thus the process of floral initiation can best be studied with those plants which require a single inductive photoperiod rather than those which flower after cyclic inductive photoperiods. The response to a single inductive photoperiod then can be interpreted only as the effect of an inductive period and is not complicated by the

additive effect of various short day photoperiods.

Basic Problems to the Investigations

In order to fulfill these objectives an essential condition was the obtaining of plants in large numbers which are uniform in growth and equivalent in age. This meant a uniform, homogeneous and maximum percentage of seed germination of Pharbitis nil seeds had to be obtained. Various workers (Takimoto and Ikeda, 1959; Kujirai and Imamura, 1958; Zeevaart, 1962b) treated Pharbitis seeds with sulphuric acid for different lengths of time in order to obtain germination. These workers neither mentioned the percentage of germination obtained, nor the condition of the germinated seedlings. Preliminary studies on seed germination using the method adopted by (Kujirai and Imamura, 1958) indicated uneven and low percentage of germination. These results indicated the need to devise a suitable method of seed treatment which would not injure the embryos and would facilitate the procurement of a homogenous sample and maximum germination, to meet the experimental requirements.

Production of normal growth under in vitro conditions is presumably dependent upon maintenance of the aseptic environment in the culture medium along with many other factors. Therefore, attention was initially diverted to development of a method of asepsis. During the preliminary experimentation, contamination by fungi and several kinds of bacteria was common and rates of contamination were high--ranging from 90-100%. These studies indicated that a method had to be developed

which would insure production of contamination-free cultures of the shoot apices without injuring the tissue.

Another basic problem of tissue culture was to select or develop a suitable medium for the in vitro culture of the excised shoot apices of Pharbitis, which would allow a satisfactory growth and development of the shoot apices. This medium could not contain ingredients which would possibly interfere with the process of floral initiation. A variety of media has been used by different workers (White, 1933; Tepfer et al., 1963; Ball, 1963) for studies of growth and development of shoot apices. All of them have essentially the basic ingredients of White's medium (White, 1943) but are modified by the addition of various trace elements, vitamins, amino acids and growth regulators. They are balanced to obtain satisfactory growth and development of the shoot apices. Preliminary studies were conducted on the growth and development of the shoot apices of Pharbitis nil in media employed by Tepfer et al. (1963) and Ball (1963). Initially, a limited amount of growth was obtained by using these media, but at later stages of growth the apices did not develop either in length or in the production of leaves. The media used contained growth regulators and various other ingredients, which could have effected growth of the apices. In order to have a satisfactory growth of the apices, a medium had to be selected so that the floral differentiation of the shoot apices could be studied without any apparent interference by the substances used in the medium.

In addition, a technique to establish the size of the apices

to be cultured which would grow and develop in in vitro conditions had to be found.

Organization of the Thesis

The second chapter has been devoted to the establishment of techniques which were considered basic to the investigation of the major areas of research. This chapter comprises the procedures, observations, and results for the techniques of seed treatment and germination, production of aseptic culture, selection of suitable growth medium and culture of the shoot apices of suitable size. The chapters of material and general methods, observations and results will conform to the major areas of research. Discussion will contain a section of seed treatment and germination followed by discussion on the four major objectives of the problem.

ESTABLISHMENT OF TECHNIQUES

A. SEED TREATMENT AND GERMINATION

Early in the present study, the seeds of Pharbitis nil were found to remain dormant for more than 8 weeks under conditions generally considered to be optimum for seed germination. In order to obtain germination of the seeds, 50 seeds were treated with concentrated sulphuric acid for 30 minutes and washed overnight in running tap water at 15°C, as described by Kujirai and Imamura (1958). Only 24 percent germinated two days after sowing and 10 percent germinated on the third day. It was thought that the low and uneven percentage of germination might be attributed to the low temperature of the washing water and also to the short length of time of treating the seeds with concentrated sulphuric acid.

In the next series of experiments, 50 seeds were treated for 45 minutes with the acid and washed overnight with running water at a temperature of 30°C. It was observed that 44 percent of the seeds germinated two days following sowing and 5 percent germinated on the third day.

These initial trials indicated that the increase in the treatment time of seeds with concentrated sulphuric acid and increase in the temperature of washing water resulted in a more even and increased percentage of seed germination. These studies suggested the need to establish a standard procedure for obtaining a uniform and maximum percentage of seed germination. Consequently, varying lengths of time of treatment of the seeds with concentrated sulphuric acid and different temperatures

at which these seeds were washed with running water were tested.

Eighteen sets of 50 seeds each were counted at random from the bulk of the seeds. Then, each batch of 3 sets was stirred for 30, 45, 60, 75, 90, and 105 minutes respectively, with concentrated sulphuric acid. Then one set was washed overnight with running water at approximately 15°C, the second set at approximately 30°C, and the remaining set at approximately 45°C. The seeds were then planted in a mixture of vermiculite and fine gravel in plastic glasses with holes drilled in the bottom to ensure adequate drainage. Glasses were covered with 4-mil. polyethylene film, before being placed in a controlled environmental chamber providing a constant temperature of 27°C and 16 hours of light and 8 hours of dark during each 24 hours. Both fluorescent and incandescent light sources of about 800 ft. c. intensity at the plant level were used. Forty-eight hours after sowing the seeds, the polyethylene film was removed. The seeds were watered daily with the Hoagland nutrient medium (Zeevaart, 1962b). Counts were made of germinated seeds showing two cotyledons above the surface of vermiculite mixture.

It is evident (Fig. 1 and Appendix 1) that when the sulphuric acid treatment was prolonged, there was a proportional increase in germination. The maximum percentage (72%) of germination was obtained with immersion in concentrated sulphuric acid for 90 minutes and overnight washing in running water at a temperature of 30°C. When acid treatment was reduced to 75 minutes, no apparent loss in germination was encountered. The percentage of germination declined when the seed

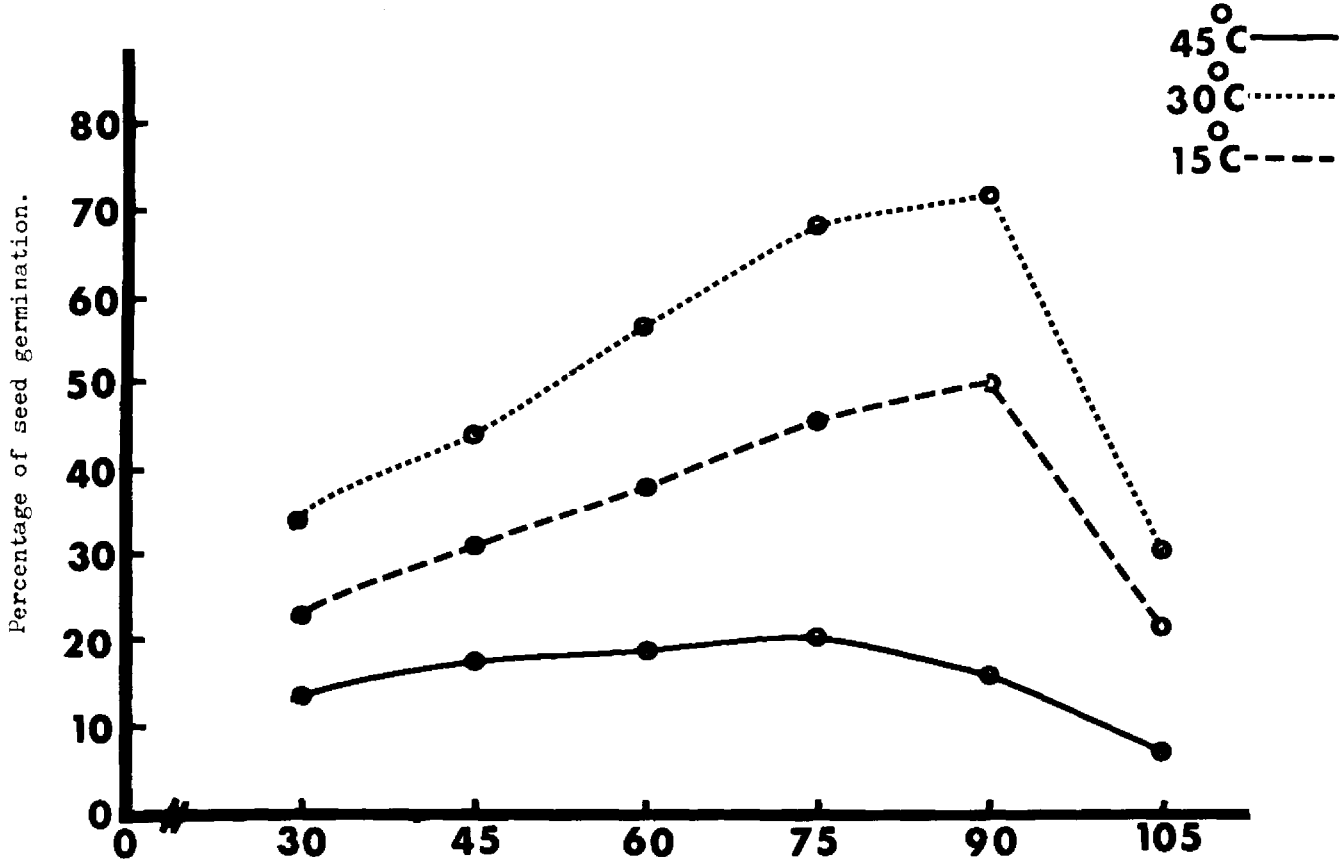

Fig. 1. The effect of variation in length of concentrated sulphuric acid treatment and temperature of water used for washing, on seed germination of Pharbitis nil.

treatment was extended to 105 minutes. In all cases, the germination was lowest when the seeds were water washed at a temperature of 45°C. Thus, a water temperature of 30°C and a sulphuric acid treatment of 90 minutes was accepted the optimum for practical purposes. This procedure was adopted for all the studies.

B. PRODUCTION OF ASEPTIC CULTURES OF SHOOT APICES

The first group of 15 apices were cultured on White's medium (White, 1943), without tissue sterilization. The apices were dissected and handled in the manner described on (page 27). A control in this case was designed to give a measure of contamination introduced during the culturing process. In order to achieve this, the control vials free of explants were handled the same way as the experimental vials which contained unsterilized apices. Contamination by fungi and several kinds of bacteria was common in these cultures and rates of contamination were high—ranging from 90-100 percent. The contamination in the control vials was comparatively low (Table I).

In order to reduce the contamination introduced from the tissue explants, different surface sterilizing agents were used. These included Chlorox as used by Tepfer et al. (1963), Pittchlor (Raghavan and and Jacobs, 1961) and Javex (Riddell, 1961). These workers had used different concentrations of the sterilizing agent and treated the apices for varying lengths of time. In order to compare the different sterilizing agents at the same level, it was decided to use one concentration of the sterilizing agents at treatment times of 5, 10, and 15 minutes. In all

TABLE I

Incidence of contamination of apices cultured without surface sterilization (Data from 15 explants).

Treatment	% of cultures contaminated
Experimental vials with unsterilized apices	93.3
Control vials with no apices	13.3

cases, the excised apices were treated with 5% sterilizing agent and rinsed with distilled sterilized water. After the treatment, the apices were handled in the manner described on (page 27). The control of these experiments consisted of a treatment in which the apices were sterilized only with distilled water.

Contamination from the explants in the control treatment is high, ranging from 86.6-100% (Table 2). The number of cases of contamination despite the kind and period of application of the sterilizing agent used, is low as compared with the control treatment. In all cases, the immersion of the apices for 15 minutes reduced contamination but also resulted in the death of tissue, which was indicated by the disorganization of the tissue which turned brown in colour, 2 days after culturing. Really there was no appreciable difference in times of 5 and 10 minutes treatment in each case. Since at least 50% of the cultures were contaminated, it was deemed necessary to find a better method of sterilization.

A wetting agent "Tween twenty" was included in the sterilizing solution with the sterilizing component. This would afford penetration through hairs present on the surface of the apices and allow more adequate contact of the sterilizing solution with the shoot surface.

This method was quite effective. The addition of 2 drops of "Tween twenty" to 100 c.c. of 5% surface sterilizing agent effectively reduced the contamination rate. Greater effectiveness was with Chlorox treatment for 5 minutes than with any of the other sterilizing agents. Treatment of the apices for 10 and 15 minutes resulted in the inhibition

TABLE 2

Incidence of contamination of apices following treatment with three sterilizing agents (Data from 15 cultures)

Sterilizing Agent (5% solution)	Time of Treatment (Minutes)	% of Cultures Contaminated	% Contamination in Control
Pittchlor	5	53.3	100.0
	10	59.9	93.3
	15	39.9	93.3
Javex	5	46.6	86.6
	10	53.3	100.0
	15	33.3	93.3
Chlorox	5	59.9	93.3
	10	53.3	93.3
	15	26.6	86.6

of growth in all cases (Table 3).

In order to still reduce the rate of contamination, another method was tried. This entailed rinsing the apices in 70% ethanol for 15 seconds before immersion in the sterilizing solution. Contamination-free cultures were obtained in all but one case (out of 15 cultures, a result which is within the range of acceptable limits). Thus, aseptic cultures for *in vitro* studies of shoot apices were obtained by subjecting the apices to 70% ethanol for 15 seconds, rinsing twice with distilled sterilized water, immersing then in 5% Chlorox solution to which 2 drops of "Tween twenty" had been added for 5 minutes and rinsing with distilled sterilized water.

C. SELECTION OF THE MEDIUM

The initial experiments consisted of comparing the growth and development of the excised and sterilized shoot apices of *Pharbitis nil*, in media employed by Tepfer *et al.* (1963) and Ball (1963). The apices attained a limited growth for the first 4 days but later on, no visible growth and development were observed until 4 weeks after culturing. As both media contained growth regulators and coconut milk, it was postulated that these ingredients might have an inhibitory effect on the growth and development of *Pharbitis* apices. Consequently, other media which did not contain ingredients which might be interfering with the process of floral differentiation were tested. White's (1943) medium was tried for growth and development of the apices. The shoot elongation and production of leaves were better in White's medium as compared with

TABLE 3

Incidence of contamination of apices following treatment with three sterilizing agents (with addition of 2 drops of "Tween twenty") (Data from 15 cultures)

Sterilizing Agent	Time of Treatment (Minutes)	% of Cultures Contaminated
Pittchlor	5	33.3
	10	13.3
	15	6.6
Javex	5	26.6
	10	13.3
	15	13.3
Chlorox	5	19.9
	10	13.3
	15	6.6

that of Ball (1963) and Tepfer et al. (1963). Taking into consideration the major objectives of the present study, the better growth yield and the simplicity of White's medium made it most suitable for growth of the apices in the present investigations.

D. CULTURE OF THE APICAL BUDS

When apices lacking visible leaf primordia were excised and cultured, there was no evidence of growth and development. But when apices bearing two leaf primordia were cultured, growth and differentiation occurred. Growth was manifested as elongation and as increase in leaf number and leaf area. Root initials proliferated from the base of the apices into the medium. Thus, in this study, apices with two leaf primordia were used.

MATERIAL AND GENERAL METHODS

The seeds of Pharbitis nil chois, strain violet (Japanese Morning Glory) were initially supplied by Dr. J. A. D. Zeevaart and then multiplied in the Research Green House of McMaster University. All the seeds were obtained from self-pollinated plants in order to ensure the genetic purity of the plant material.

FLOWERING RESPONSE OF VEGETATIVE SEEDLINGS OF DIFFERENT AGES TO AN INDUCTIVE DARK PERIOD

Appropriately treated seeds were allowed to germinate under (LD) conditions (16 hours of light and 8 hours of dark). One inductive dark period of 16 hours was imposed daily on a set of 5 seedlings from the time of germination until the seedlings were 20 days old. After the inductive period, the plants were placed under (LD) conditions for observations of flower formation which indicates the response of the vegetative seedlings to an inductive dark period. Hence, flower counts were made to relate the effect of the dark period with the age of the seedlings. The mean was expressed as the number of flowers per plant.

In order to further explore the results of the forementioned experiment (described in the next chapter), the cotyledons of 4-day old seedlings were removed. After the treatment, the plants were kept under (LD) conditions for growth and development. From this pool of vegetative seedlings (with no cotyledons), 20 plants were selected at random after every three days. Out of these, leaf areas of 10 plants were determined with the help of a planimeter, directly from actual measurements of the

leaves. Three measurements were made and the mean of these taken as a representative datum. The remaining 10 plants were subjected to an inductive dark period of 16 hours and then returned to (LD) conditions after the treatment for observations on flower formation.

The results suggested the need to investigate further the implications of the data in (Fig. 3 and Appendix VI). Specifically, was the lack in flowering response of the older seedlings attributable only to effects of the mature cotyledons or was it due to the influence of young developing leaves in which the metabolic rate is faster? In order to investigate this aspect, the expanded and young growing leaves of a set of 5 seedlings at different ages were removed, but cotyledons retained, before the inductive dark period. These seedlings were then returned to (LD) conditions for observations on flower formation.

ONTOGENY OF THE SHOOT APICES

To record the early stages of ontogeny the seeds were put in petri dishes containing vermiculite moistened with Hoagland solution, while for later stages they were sown individually in plastic glasses. They were then placed under (LD) conditions (16 hours light and 8 hours dark) in a controlled environmental chamber at a temperature of 27°C. The seeds germinated giving two cotyledons above the surface of the vermiculite, 72 hours after sowing. The germinated seedlings were then transferred to the other controlled environmental chamber with the same (LD) conditions and a temperature of 23°C.

Ten apices were collected at 24-hour intervals after sprouting,

until the seedlings were 14 days old. In this way, samples could be taken during seed germination and vegetative stages of the apices. Mature leaves were removed from the apices and the apices were then fixed in Navashin's fixative (Jensen, 1962). In order to remove the trapped air on the hairs on most of the buds and to increase the penetration of the fixative, the apices were placed in a specially designed vacuum apparatus (Whitlake, 1942). The apices were left in the fixative for 48 hours. They were then washed with running water for 48 hours, dehydrated with a tertiary butyl alcohol series as described by Johansen (1940), and embedded in paraffin with a melting point of 61°C (Appendix II). Five apices were sectioned at a thickness of 8 μ longitudinally and the other five, horizontally. The sections were stained with the triple stain, safranin, Delafield's hematoxylin and anilin blue (Popham, Johnson, and Chan, 1948), the procedure of which was modified to balance for the plant material. The complete staining procedure has been described in (Appendix III). The slides were mounted in Permount with No. 1 coverslips.

Four-day old seedlings (i.e. 96 hours after germination or 168 hours after sowing), were ready for floral induction. At this time, the cotyledons were fully expanded and the epicotyl was elongating. Seedlings were selected for uniformity of cotyledons, length of the epicotyl and the size of the plant. A single dark period of 16 hours given to the 4-day old seedlings will give optimum results as measured by total number of floral buds induced per plant (Zeevaart, 1962b). As such in the present investigation, the dark period was imposed on the 4-day old seedlings.

In this investigation, only the terminal floral bud was studied.

Seedlings selected for induction were transferred to another controlled environmental chamber, for one long dark period of 16 hours and at a temperature of 27°C to allow for full effectiveness of the photoperiod (Zeevaart, 1962b). The plants were returned then to the (LD) chamber (16 hours light and 8 hours dark) with a temperature of 23°C. Seedlings selected for vegetative control were left in the (LD) chamber with no inductive photoperiod.

Ten apices were collected at each 24-hour interval after the photoperiodic induction until ovules were initiated in the ovary of the floral apex and the stigma was being formed on the distal end of the style. These apices were fixed in Navashin's fixative and processed in a manner described in the earlier part. Five apices were sectioned longitudinally, and the other five, horizontally. In each of the 5 cross-sectioned apices, the median section was selected and the number of floral primordia were counted. The mean of the count of the 5 apices gave the representative datum for the particular stage of growth which was then related to the time of photoperiodic induction.

The diameter of the apices was measured at the level of the axil of the first leaf, in a median longitudinal section. For measurements of the height, an arbitrary horizontal base line was drawn from the axil of the first leaf. From the centre of this line, the height was measured vertically up the tip of the apex.

IN VITRO STUDIES OF THE SHOOT APICES:

General Procedure for Selection, Excision and Preparation of the Shoot Apices for in vitro Culture:

Tissues for culture were excised from vigorously growing 4-day old seedlings. In order to ensure uniform results for any one experiment, the apical buds were selected from seedlings of the same age which were equal in height and had cotyledons approximately of the same size. Because of the difficulty in determining without damage the exact number of leaves in the apices, controls were collected, sections prepared and studied microscopically for number of leaf primordia and structure of the apex. With these precautions, uniformly growing cultures were routinely obtained.

For in vitro culture the apices were aseptically prepared according to the procedure developed in the Establishment of the Techniques. The apices, excised from the seedlings with sterile razor blades, were surface-sterilized first with 70% ethanol for 15 seconds, rinsed twice with redistilled sterile water, again sterilized for 5 minutes with 5% Chlorox to which 2 drops of "Tween twenty" had been added, and then rinsed 4 times with redistilled, sterilized water. Usually apices of tissues larger than actually required for culturing were initially sterilized. The purpose of this was to avoid absorption at points too close to the delicate parts of the apex, which could be affected by the sterilizing agent. After sterilization and rinsing, the apices were placed between pads of sterilized filter papers in petri dishes to remove excess moisture.

The apices were dissected under a Zeiss dissecting microscope

in an ultra violet light transfer room. The stage of the microscope was repeatedly washed with 70% ethanol and with 437 ppm. solution of "Ioclide Germicide"[1]. The instruments used for dissecting the tissues were first sterilized with 1% "C. R. 1. Germicide"[2] for 15 minutes and then washed with redistilled sterilized water. The second sterilization of the instruments was done by autoclaving for 20 minutes at a pressure of 18 lbs./sq. inch. The extra tissues were removed aseptically with the help of microscalpels (made by attaching slivers of stainless steel razor blades to stainless steel needles), watchmaker's forceps No. 4, and microneedles.

Apices were cultured in test tubes 95 x 25 m.m. containing 20 ml. of the medium. Under these conditions, in all but a few discarded cases, the cultures remained sterile.

Preparation of the Medium:

The ingredients used for the medium are listed in Appendix IV. The medium was solidified by the addition of 0.75% agar. Sterilization of the medium was carried out by autoclaving the inorganic salts, glucose and the agar, for 15 minutes at 16 lbs./sq. inch. The organic compounds which are thermolabile were sterilized by filtering through a Morton bacteria filter apparatus (Morton, 1944). The two components were mixed

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1. Ioclide Germicide is an Iodophor concentrate and is manufactured by Clay-Adam Inc., New York.

2. C. R. 1. Germicide has been developed by Clay-Adam Inc., New York, for cold disinfection of surgical instruments.

aseptically and 20 ml. were dispensed, with an automatic pipette, in each sterilized test tube, which was plugged with a disposable plug.

Maintenance of the Cultures:

Unless otherwise stated, the cultures were maintained in a growth chamber under LD conditions. The growth chamber provided a temperature of 23°C. The light intensity was 800 ft. c. and the relative humidity, 70%. The cultures were transferred to a fresh medium every four weeks from the time of culturing the apices.

In vitro Culture of the Floral Apices after Different Periods of the End of Short Day:

Four-day old seedlings were selected for uniformity and were given an inductive dark period of 16 hours in the growth chamber at a temperature of 27°C. This dark period allows the Morning Glory seedlings to transform the vegetative structure of their apices into floral structure.

Ten apices were excised from the main plant before the beginning and after 0, 6, 12, and 24 hours following the end of the inductive dark period. These apices were sterilized and prepared for in vitro culture in White's medium, as described under the general procedures. Thereafter, ten apices were cultured at 24-hour periods until 8 days from the end of the dark period. A control series consisting of five apices, was collected from the seedlings at the time of inoculation. The cultures were maintained for 4 weeks in a growth chamber which provided

(LD) conditions at a temperature of 23°C.

Morphological changes in the apices were observed daily. The apices, after 2 and 4 weeks of growth in in vitro conditions, were fixed in Navaschin's fixative and prepared for microscopic examination.

Floral Induction in the Cultured Vegetative Apices:

Four-day old seedlings grown under (LD) conditions were selected for uniformity with regard to size of the cotyledons, height and age of the plant. Apices from these selected seedlings were excised, surface sterilized and prepared for in vitro cultures, by the method already described. A control series of 10 apices was collected at random for determination of the number of leaf primordia and for the structure of the apex.

After culture all the apices were placed in (LD) conditions in the growth chamber at a temperature of 23°C. Ten apices were selected at random, after every 72 hours of growth, given an inductive dark period of 16 hours and then placed under (LD) conditions. Before imposing the dark period a control series of 5 cultured apices was collected. Areas of the unfolded leaves of the control were recorded. The leaf areas of the unfolded leaves were measured either directly from actual size of the leaves with the help of a planimeter, or with a Zeiss micrometer grid from the magnified images obtained with the dissecting microscope.

Samples of 5 apices were collected, after 28 days from the end of the dark period and the apices fixed in Navashin's fixative and

prepared for microscopic examination. The number of days required for the reproductive change of the vegetative structure was recorded and correlated with leaf area at the time of the inductive dark period.

OBSERVATIONS AND RESULTS

ORGANOGRAPHY

About 3 days after planting, seedlings show a well-developed hypocotyl and an epicotyl concealed by overlapping petioles of two cotyledons. The cotyledons are photosensitive and respond to a photoperiod. After 6 days, alternately arranged leaves appear from the developing epicotyl. The seedlings remain vegetative when grown under (LD) photoperiodic conditions and behave as twining plants with leaves deeply cordate and broadly ovate to suborbicular in outline. After one (SD) 4-day old seedlings produce 4 or 5 flowers. Each flower consists of two bracts, five sepals, five petals and five stamens. The petals are fused to give a trumpet-like appearance. The pistil consists of three carpels fused along their margins. The ovary has three locules, each bearing two ovules with axile placentation.

FLOWERING RESPONSE OF VEGETATIVE SEEDLINGS OF DIFFERENT AGES TO AN INDUCTIVE DARK PERIOD

Seedlings of different ages, with attached cotyledons, respond differently to a single inductive dark period of 16 hours (Fig. 2; Appendix V). Immediately after germination they are photoperiodically insensitive but a day after germination, they respond to the inductive (SD) photoperiod. The maximum response for flowering is obtained at the 4-day stage when the seedlings are controlled as to photoperiod. Flowering response declines when a (SD) period is applied to 5-day old seedlings

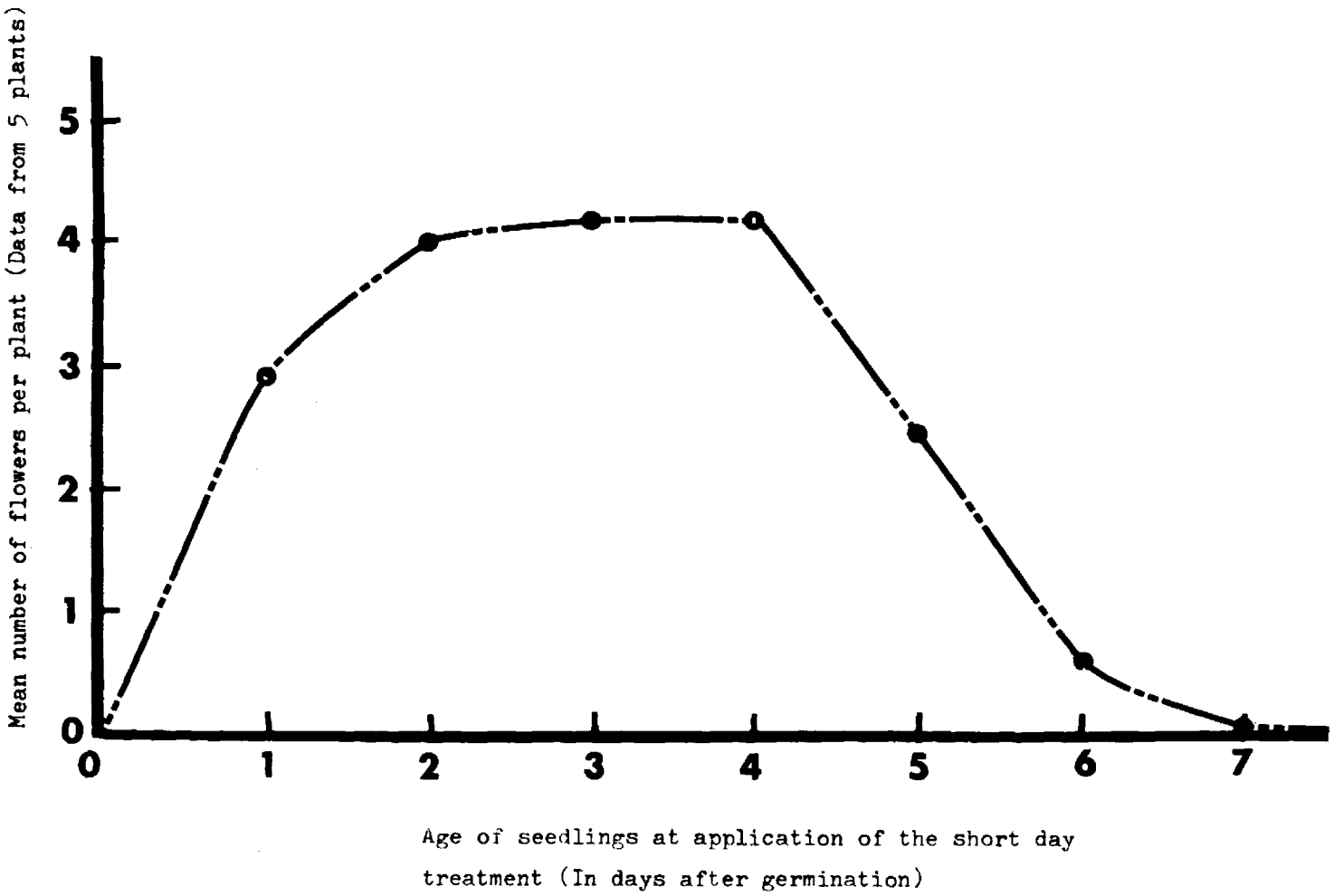

Fig. 2. Effect of one short day (16 hours) on flowering of seedlings of different ages.

and the photoperiod is ineffective when the seedlings are 7 or more days old.

In another series of experiments Imamura and Takimoto (1955a, 1956a, b), have induced flowering by giving dark period to the leaves of Pharbitis seedlings, whose cotyledons have been removed. In order to confirm these results under the environmental conditions of the present investigation, the cotyledons of 4-day old seedlings were removed, allowed to develop leaves, and subjected to an inductive dark period.

The results (Fig. 3; Appendix VI) indicate that there is a direct increase in the development of leaf area, with increase in the age of the plant. The plants with a leaf area of less than 4.2 cm^2 before the inductive dark period did not flower, while 70% of those with leaf area of 4.2 cm^2 exhibited flowering. The maximum number of flowering plants were obtained from those in which mean leaf area of 8.6, 15.4, 19.5, and 24.1 cm^2 per plant was developed before inductive dark period. A decline in flowering response was obtained when the seedlings were 24 or more days old.

The foregoing results suggested the need to investigate further the implications of the data presented in fig. 2. Particularly, was the absence of flowering of seedlings of 7 or more days in age, due only to the presence of mature cotyledons or was it due to the effect of young developing leaves in which the metabolic rate is faster or is it due to the changes in the apical bud which is not able to express the floral stimulus? In order to investigate this aspect, the seedlings of different

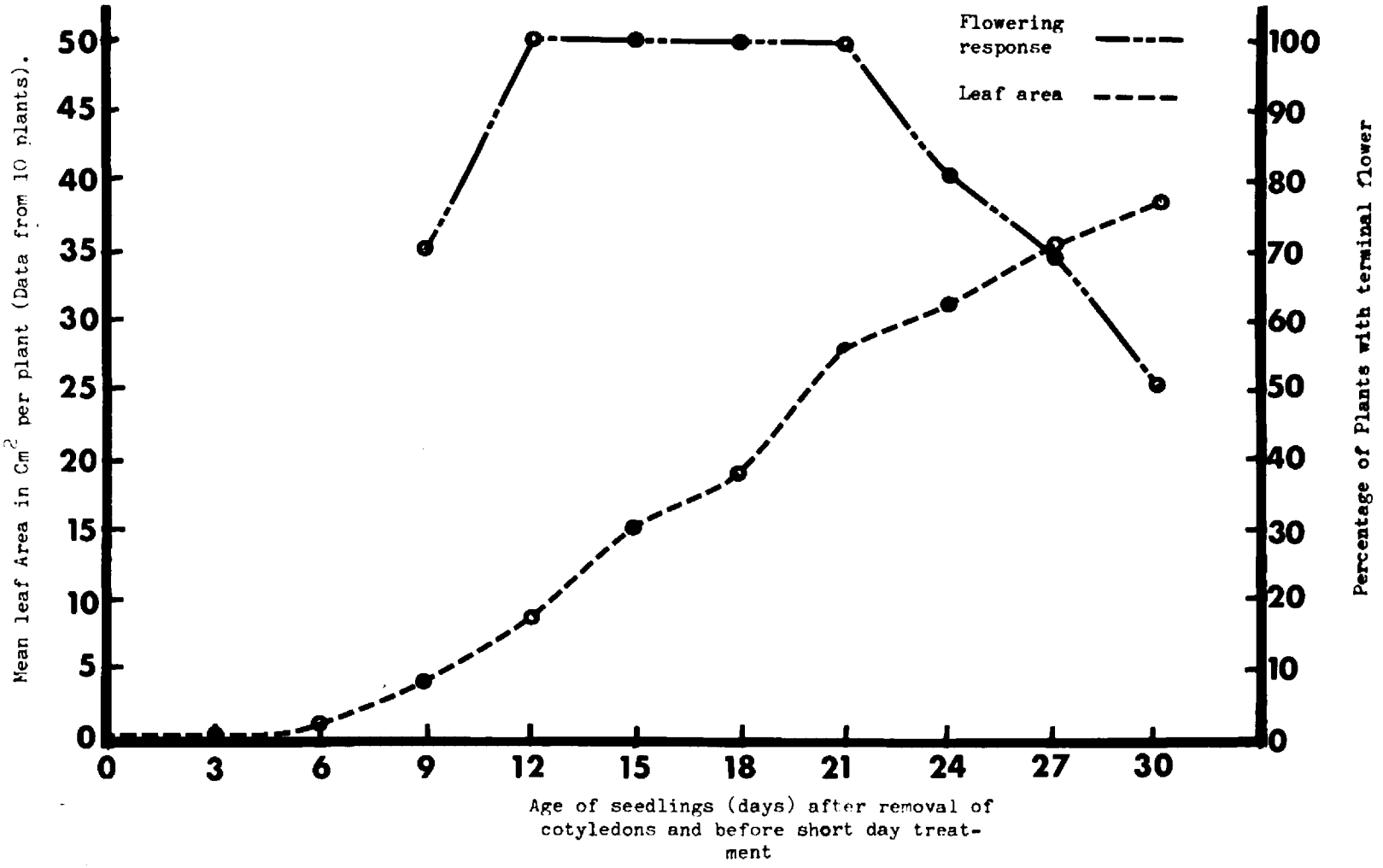

Fig. 3. Flowering response in relation to leaf area developed in intact plants after removal of cotyledons.

ages were defoliated (cotyledons retained) before the inductive dark period.

The results (Fig. 4; Appendix VII) indicate that the pattern of flowering response is the same as obtained in plants with attached cotyledons and developing leaves.

ONTOGENY OF THE SHOOT APICES

The ontogeny of the shoot apices will be described in accordance with the tunica-corpus theory (Schmidt, 1924) and the system recognized for cytohistological zonation described by Majumdar (1942) and Philipson (1947, 1949).

Vegetative Shoot Apex

Descriptive morphology for the shoot apex of Pharbitis nil is conveyed schematically in Fig. 5. The meristem shows a two layered tunica (T1 and T2) and a corpus which constitutes the central core and consists of a central zone, a peripheral zone, and a rib meristem. The tunica contributes to surface growth and the corpus to growth in volume. In the tunica, the plane of cell division is predominantly anticlinal; in the corpus, it may be periclinal, oblique or anticlinal.

Both the tunica layers of Pharbitis nil consist of small darkly stained, thin walled cells with dense cytoplasm, except at the summit of the dome shaped apex where this uniformity is lacking. A group of initial cells at the extreme tip of the dome is easily recognized by their large size, vacuolated cytoplasm, large nuclei, and thick cell walls. These

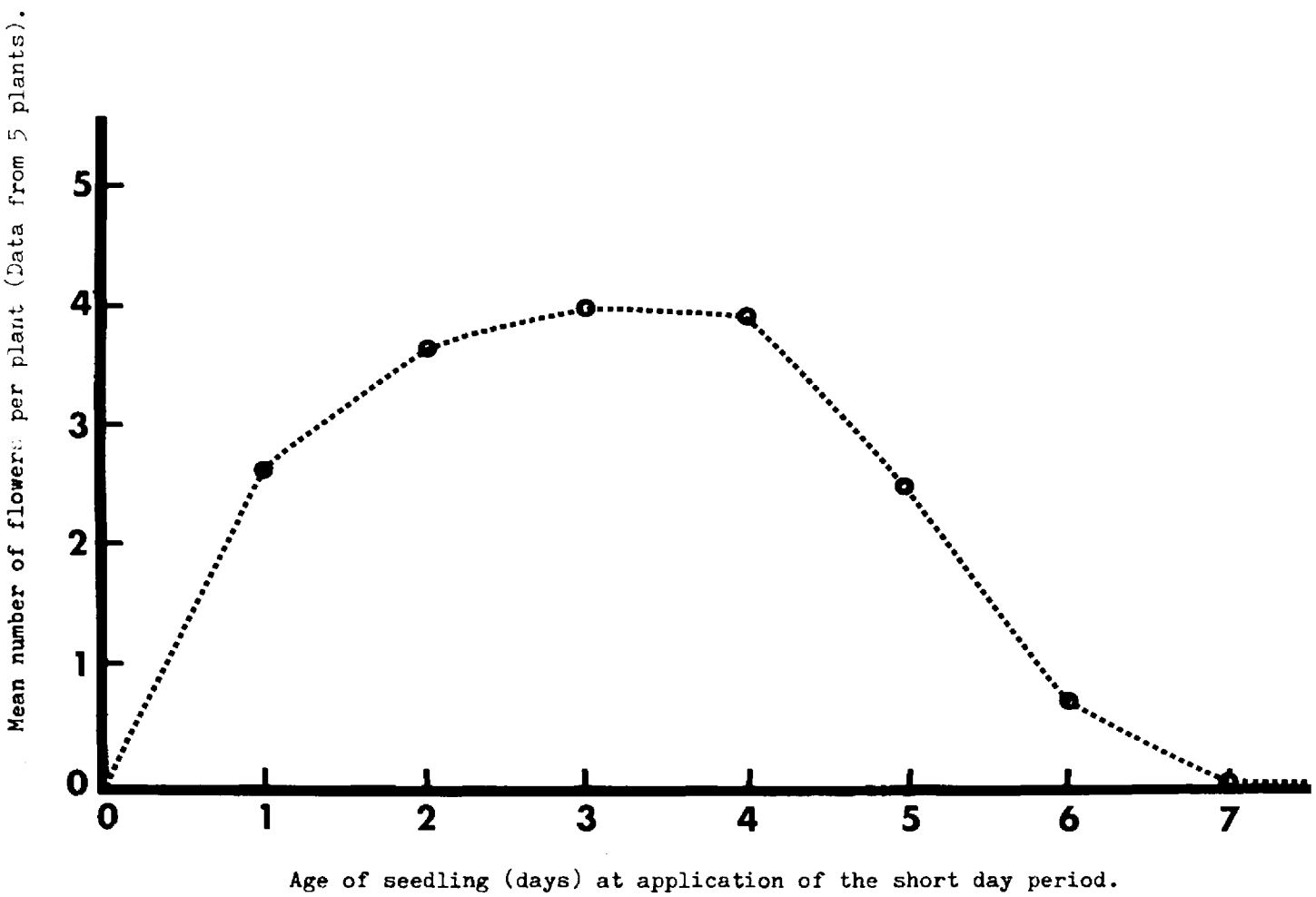

Fig. 4. Effect of one short day (16 hours) on flowering of seedlings of Pharbitis nil of different ages with leaves removed.

tunica initial cells are marked in Plate (IVA).

Unlike the cells of the tunica, those of the corpus are irregularly arranged as the result of divisions in varying planes. The central zone is the core of the corpus and consists of cells similar to the tunica initial cells. These cells are always in contact with the tunica initial cells, causing both zones (considered together) to appear as a central cup shaped zone (Plates IVA, VA, VIB). In Plates (I, II, and III), the central zone is in the process of differentiation but is markedly delineated in Plates (IVA, VA, and VIB). The cells of this zone are lightly stained and their walls are unevenly thickened. Althought mitotic figures are infrequent in this zone, they do occur, especially towards the periphery of this zone (Plates IIIB, IVA, VA). As a result of cell division, derivatives are contributed laterally and below. The central zone is surrounded by smaller cells with denser content, which are 3-4 layers thick. This band of cells forms a transition tissue. Towards the periphery of the transition tissue lies the peripheral zone.

The peripheral zone lies at the flanks of the apex (Fig. 5). It consists of relatively small, dark stained, thin-walled cells with dense content, aligned in regular rows, which radiate from the transition tissue towards the periphery of the apex. Periclinal and anticlinal divisions take place in this zone (Plates IIB, VA). The derived cells then participate in the formation of the peripheral meristem, which in turn contributes cells to the cortex and to the procambial tissue of the shoot and the leaves.

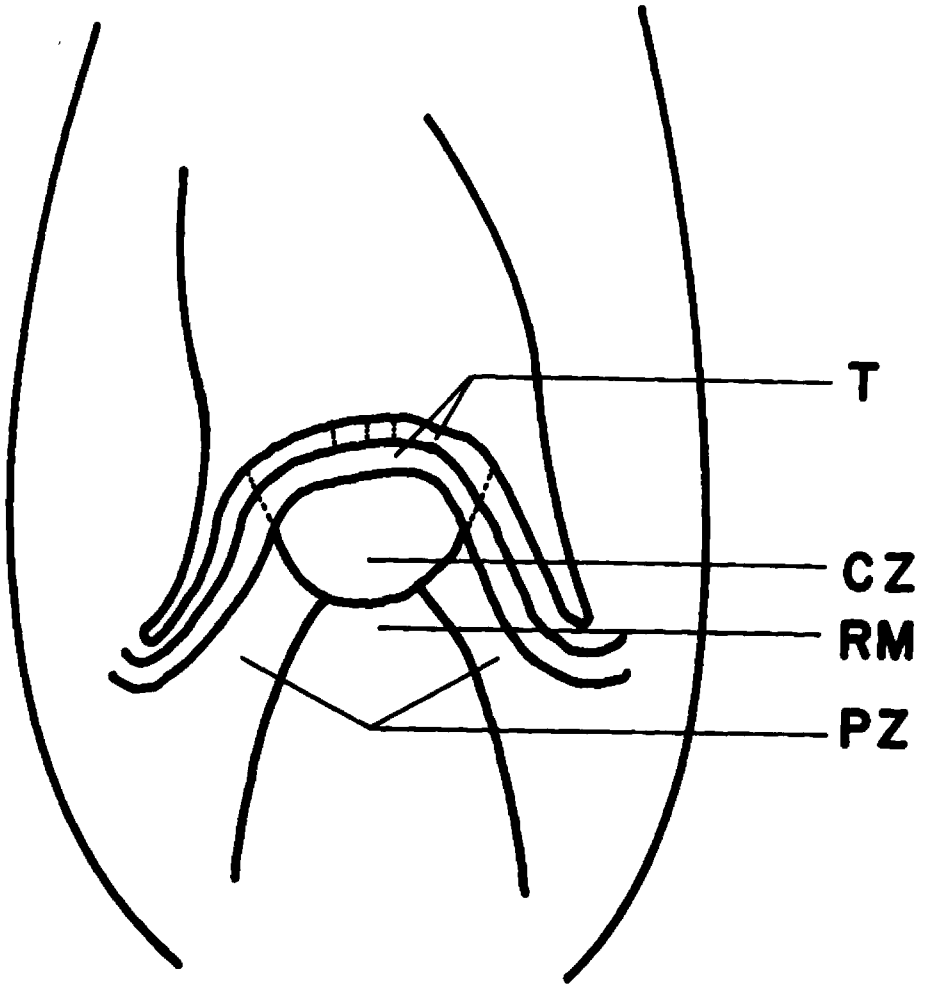

Fig. 5. Diagrammatic representation of the median longitudinal section of the vegetative shoot apex of Pharbitis nil during the active period of growth. The apex shows cytohistological zonation, biseriate tunica. The superimposed initial cells of tunica are situated at the summit of the apex. Central zone (CZ), tunica layers (T), peripheral zone (PZ) and the rib meristem (RM) are illustrated.

The rib meristem originates at the base of the transition tissue. It is formed of highly vacuolated, uniformly thick-walled cells more or less rectangular in shape. The cells of this zone are large in size with dense content and darkly stained nuclei. The intensity of staining in the nuclei decreases in the older portion. Transverse cell divisions occur (Plate VIIA) and the cells are arranged in regular files which are poorly differentiated.

Appropriately treated seeds attain maximum size and germinate with the emergence of the radicle at the micropyle. The apex below the folded cotyledonary petioles measures 292 μ in width and 39 μ in height at the axil of the petioles. At this stage only one leaf primordium has appeared (Plate IA, IB). The apical meristem consists of one tunica layer and its cells are different in shape and size than those of the underlying layers (Plate IIA). Peripheral and rib meristem zones are easily recognized. All the cells of the various zones of the apex have dense contents. The granular appearance of the cytoplasm probably is due to the presence of fat globules and other reserve food material equally dispersed throughout the tissues.

Following sowing, when the seeds are in the process of germination, the apex is at a distal position. This change occurs within the first day. The leaf primordium initiated on the first day has enlarged and the second leaf primordium has appeared as a leaf buttress. A uniseriate tunica is characteristic for this stage of growth. The cytohistological zones have started to differentiate (Plate IIB). The granular character of the cytoplasm has disappeared from the cells of both the tunica and the

central zone. The cells of the peripheral and rib meristem zones have retained granular character but the amount of granulation has decreased.

On the second day after sowing two cotyledons emerge. At this stage of development, the two leaf primordia have been differentiated (Plate IIIA). The granular character of the cell content in the peripheral and rib meristem zones has disappeared. The cytohistological zonation has become delineated (Plate IIIB). The apical meristem shows a single layered tunica. In the second layer below the tunica initials, the cells are large and have large, lightly stained nuclei (Plate IIIB). The cells are probably the initials of the corpus.

The apices of 1, 2, 3, and 4-day old seedlings have no marked difference in zonation, when compared with each other (Plates IV, V). A uniseriate tunica still persists. There are fluctuations in the width of the apices, probably due to the different ages of the seedlings. Leaf primordia originate at the rate of one leaf every 24 hours.

In the apices of 5, 6, 7, and 8-day old seedlings, the cells of the central zone are smaller in size than those of younger seedlings (Plates VI, VII). The apices are now rounded and cytohistological zonation has been established. A biseriate tunica has appeared by the time the seedlings are 5 days old (Plate VIA). Anticlinal divisions in T2 are common, thus establishing a biseriate tunica layer. A few large cells occur at the summit of the apex.

The cells of the different zones from the apices of 9, 10, 11, and 12-day old seedlings become relatively uniform in appearance when stained (Plates VII, VIII, IX). The apices assume the characteristic dome

shape. In the older seedlings, staining is intensified in the apices, in contrast to the condition for younger seedlings (Plate X). The leaf primordia appear approximately at 24-hour intervals.

Reproductive Shoot Apex

The results described in the preceding section indicate that the response to flowering is optimum, when the 4-day old seedlings are subjected to one inductive dark period of 16 hours at a temperature of 27°C. Therefore, in order to obtain maximum induction and differentiation, the dark period was imposed on the 4-day old seedlings.

To establish a reference useful in recognizing the ontogenetic stage at which the reproduction character is initiated, comparison was made between the inductive experimental material and the vegetative material. The apices of the seedlings before the dark period are similar in zonation and cytohistological pattern to the 4-day old seedlings (Plate VB).

After the end of one inductive dark period, the morphological zonation and the cytohistological pattern remain the same as before the dark period.

Between the first and second day after the end of the dark period, the morphological configuration of the floral apex does not change (Plate XIA, XIB) from that of the previous days. The first recognizable sign of the floral transition becomes evident on the second day, when an increase in the mitotic activity of the apex is observed (Plate XIB). The primordia initiated in the peripheral zone resemble the leaf primordia but later

differentiate into floral bracts. One bract is initiated per day after the dark period is imposed. In the case of Pharbitis, only two floral bracts are initiated.

The first change of the structure of the apex becomes visible on the third to the fourth day following the dark period when the sepals arise. There is an increase in the number of cells of the apex which now appears as a low, broad dome (Plate XIIA, B). The central zone is not discernible at this time. On the third day the outer sepal primordia are initiated and on the fourth day, the inner second whorl of the sepal primordia differentiates.

The apex remains broad and dome shaped. The central and peripheral zones now appear as one outer zone, while the rib meristem forms an inner zone. In the outer zone, the cells have dense cytoplasm and light staining nuclei. The cells of the inner zone have thin cytoplasm and dark staining nuclei. Both the zones are clearly visible in Plate (XIIIA). Floral organs are initiated in the outer zone and the inner zone forms the pith-like parenchyma of the receptacle. By the end of the fourth day, the initiation of the five sepals is complete.

On the fifth day, the floral apex enlarges rapidly in width and has flattened (Plate XIIIB). The nuclei in the outer zone become small and stain lightly. Alternating primordia of petals and stamens appear so soon after sepal initiation that they seldom are seen alone in a section. After initiation the stamen primordia develop more rapidly than those of petals. This arrangement is clearer at a later stage (Plate XIVB).

On the sixth day after the dark period and following the initiation of petals and stamens, the apex enlarges in diameter and flattens. During the period of enlargement, the marginal cells of the floral meristem grow at a somewhat faster rate than those in the center resulting in a low circular ridge, the carpel primordium which surrounds a circular depression (Plate XIVA). Continuity of this meristematic ring was established by the study of serial sections and by examination of dissected fresh specimens (Plate XIVA, B).

At this stage, the petal and stamen primordia initiated a day earlier, differentiate and become more distinct. At the early stages, the petals are completely free (Plate XIVB shows a conspicous space between adjacent petal margins).

On the seventh and eighth days, the differentiation of floral primordia continues. Margins of the petal primordia are still free. The stamens differentiate to form four microsporangia, though this demarcation is not always clear until the eighth day (Plate XVB). The carpel primordia enlarge and the meristematic depression becomes accentuated in longitudinal section (Plate XVA).

Fusion of the petal margins begins on the ninth day following the dark period (Plate XVIB). The stamens differentiate and four sporogenous cell groups are clearly delineated by the tenth day. A single layered tapetum has developed (Plate XVIIB).

The carpel primordia converge until, at a later stage, their tips touch. Marginal growth occurs on the lower portion of each primordium in such a way that the margins curve ventrally (Plate XVIA,

XIXB). By differentiation of the inner zone of the remnant apex, the placenta is initiated and this process accounts for the remainder of the floral apex.

Between the eleventh and twelfth days following the dark period, the petal primordia gradually fuse. The stamens show pollen mother cells (Plates XVIII, XIX, XX), and the tapetal layer is well formed. Stamens and petals are fusing (Plate XVIIIB).

The carpel primordia have enlarged and their tips are contiguous though not fused. They are of different lengths and in a transverse section taken distally (Plate XXB) seem to be on separate whorls. At a more proximal transverse level (Plate XXA), a single ring is observed. The lower marginal portion of each carpel primordium proliferates, the margins meet, become reflexed towards the mid rib and produce two ovules (Plate XIXB)

From the thirteenth to fifteenth days, the petal primordia fuse, forming a regular ring; during ontogeny, the corolla tube is formed by the union of the bases of the petals. With the growth of the microsporangia, the cells of the inner wall collapse and only two layers remain around the pollen mother cells. Two ovules are developing at the base of each carpel (Plate XXB); the carpels have completely fused to form a ring (Plate XXIA).

Between the sixteenth and eighteenth days, the microspores have developed into mature pollen grains with exines (Plate XXIIB). The ovules are well developed. A transverse section shows that the carpels have become fully fused (Plate XXIIA). The style extends longitudinally and by the twentieth day, secretory cells form the epidermis of the stigma.

IN VITRO STUDIES OF THE SHOOT APICES

In Vitro Culture of the Floral Apices after Different Periods of the End of Short Day

In the present investigation, the different stages of floral development of the apices, before culturing, were identical with the ontogenetic stages of reproductive apices described in the preceding section.

The experiments indicate that apices cultured without the inductive dark period developed and remained vegetative in structure and organization. This result is identical with the observations recorded with intact plants as already described. The average number of leaf primordia increased to 3.2 after 2 weeks of *in vitro* growth and finally to 4.0 after 4 weeks. The apex was reduced in width and maintained a clear cup-shaped central zone (Plate XXIVA).

The results of the initiation of different floral organ primordia from the apices excised and cultured at various periods following an inductive photoperiod have been tabulated in Table 4.

The apices cultured immediately following, and at least 6 hours after the dark period, remained vegetative after 4 weeks of *in vitro* growth. There was no difference in structure and zonation of these apices as compared with those cultured without the dark period (Plate XXIVB). The results obtained on culturing the excised apices immediately following the dark period were the same as those obtained with intact plants whose cotyledons were removed immediately after the end of the dark period (Table 5). However, when apices were excised and cultured 6

TABLE 4

In vitro growth of apices excised and cultured at various periods following an inductive dark period

Interval between photo-induction and culturing of the excised apices	Interval between the *in vitro* culture and examination of the excised shoot apices (In weeks)	Floral organ primordia developed + = Presence, - = Absence				
		Sepals	Petals	Stamens	Carpels	Ovules
0 hours	2	-	-	-	-	-
	4	-	-	-	-	-
6 hours	2	-	-	-	-	-
	4	-	-	-	-	-
12 hours	2	+	-	-	-	-
	4	+	-	-	-	-
1 day	2	+	-	-	-	-
	4	+	-	-	-	-
2 days	2	+	+	-	-	-
	4	+	+	+	+	-
3 days	2	+	+	+	-	-
	4	+	+	+	+	-
4 days	2	+	+	+	-	-
	4	+	+	+	+	-
5 days	2	+	+	+	-	-
	4	+	+	+	+	-
6 days	2	+	+	+	-	-
	4	+	+	+	+	-
7 days	2	+	+	+	+	-
	4	+	+	+	+	-
8 days	2	+	+	+	+	+
	4	+	+	+	+	+

TABLE 5

Effect on the terminal apex of Pharbitis nil of removing the cotyledons at various times following one inductive dark period of 16 hours. Data from 15 plants. (Total)

No. of Hours Following the Dark Period	0	2	4	6	8	10	12	24	36	48
No. of Plants with Terminal Floral Apex	0	0	13	15	15	15	15	15	15	15

hours after the end of the dark period, they remained vegetative while the apices of intact plants having the cotyledons severed 6 hours after the end of the dark period developed normal floral primordia. It was observed (Table 5) that for in vivo conditions, plants whose cotyledons were removed 4 hours or more after the end of the inductive dark period, developed normal floral primordia at the terminal apex.

The apices excised and cultured from intact plants at 12 hours or later after the end of the dark period initiated different floral organ primordia after 2 and 4 weeks of in vitro growth (Table 4). The different stages of floral initiation after in vitro growth have been shown in Plates (XXV, XXVI, XXVII, XXVIII, XXIX, XXX).

The preceding results have indicated that in the case of Pharbitis nil chois, strain violet, the apices excised and cultured from intact plants, 6 hours after the end of the inductive dark period, remained vegetative while the controls with the intact plants developed normal floral primordia. The apices excised and cultured 12 hours or later, after the end of the inductive dark period, developed and differentiated floral organ primordia.

Floral Induction in Cultured Vegetative Apices

The results presented in Fig. 6 (Appendix VIII) indicate that in the case of Pharbitis nil, the excised apices without any folded and unfolded leaves but bearing two leaf primordia, visible only under a microscope, grew and developed in leaf area. When these apices were

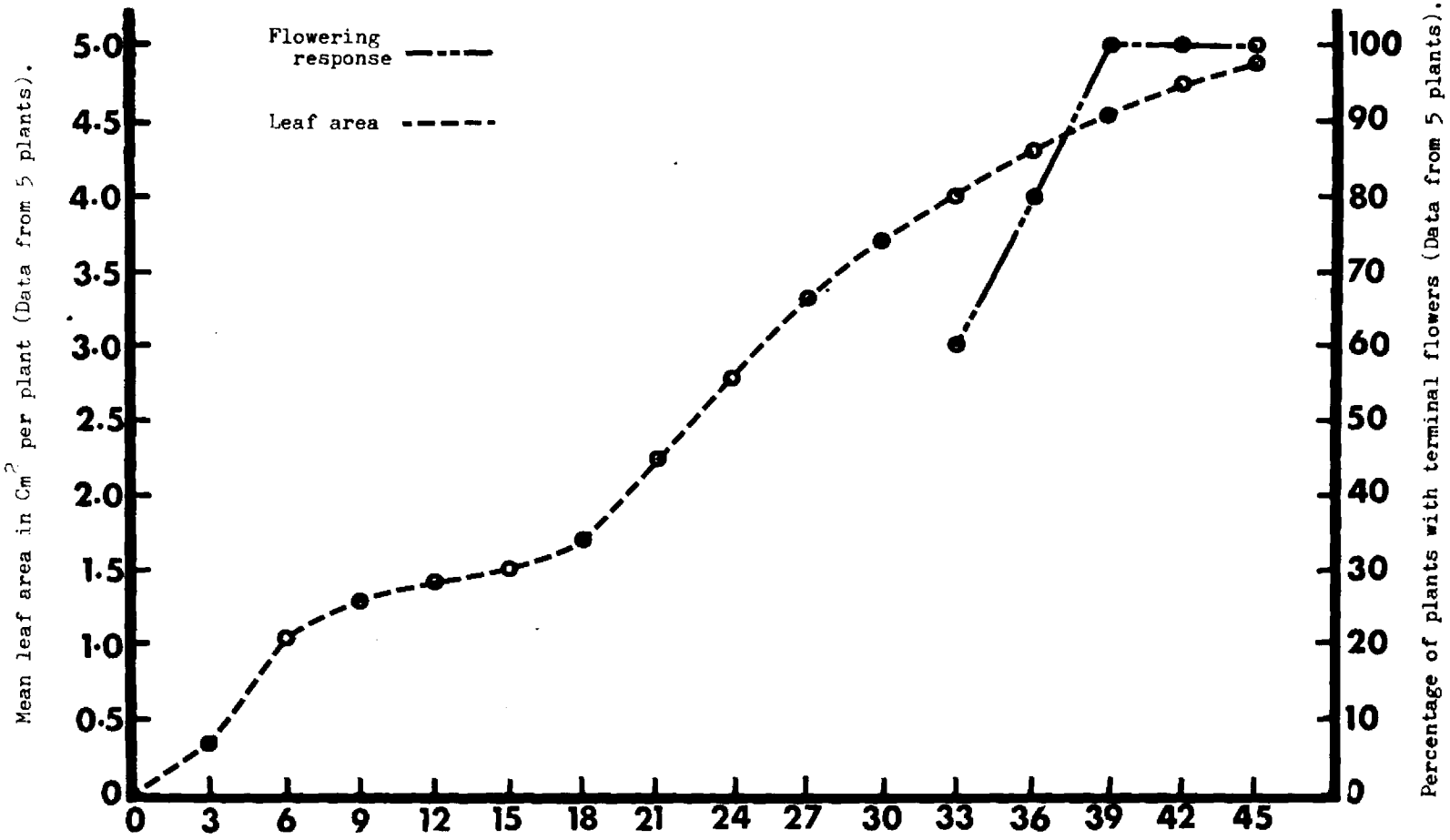

Fig. 6. Flowering response of Pharbitis nil in relation to leaf area and age of the apex (days) after in vitro culture.

subjected to an inductive dark period, no evidence of floral primordia was noted for as long as 33 days after the end of the dark period. Floral development was observed in cases which developed a leaf area of 4.0 cm^2 or more, before the inductive dark period was imposed. Maximum flower formation was observed in cases which developed a leaf area of 4.5 cm^2 or more.

DISCUSSION

TREATMENT AND GERMINATION OF SEEDS

It is generally accepted that a common cause of seed dormancy is the presence of a hard seed coat, which may be impermeable to water and gases, or may mechanically constrain the embryo (Mayer and Poljakoff-Mayber, 1963; Crocker and Barton, 1953; Barton, 1965). Various methods have been suggested for breaking this type of dormancy. One of the common methods is to treat the seeds with concentrated sulphuric acid for periods which vary in length of time and then to wash the seeds with running water.

In the case of Pharbitis nil, various reports (Takimoto and Ikeda, 1959; Kujirai and Imamura, 1958; Zeevaart, 1962b; Marushige, 1965a) indicate that the seeds were treated with concentrated sulphuric acid within a range of 20-45 minutes and then washed in running water overnight. Present studies indicate that a treatment time of 30 minutes is not adequate to produce an even, homogeneous and maximum germination. It is possible that the seed coat is not chemically decomposed during this 30 minute period of treatment, so that the seeds are unable to germinate properly. A 90-minute treatment does not cause injury to the embryo and at the same time it promotes best results insuring for a high and desirable percentage of germination. Treatment of more than 90 minutes may be causing injury to the embryo resulting in a low percentage of germination.

The low rate of metabolism notable among dry seeds, is probably due to the low percentage of water content (Mayer and Poljakoff-Mayber,

1963). As soon as the seed coat becomes permeable and is hydrated, a marked increase in the rate of respiration and breakdown of reserve materials in the seed are observed (Barton, 1965). The rate of metabolism of seeds is influenced not only by the endogenous conditions but also by a number of external factors such as the presence of moisture around the seeds, temperature, oxygen and carbon dioxide concentration in the atmosphere, and light. It is known that with an increase in temperature, the respiration rate of plant tissues also increases. The Q10 of respiration for plant tissues has been reported by Mayer, Anderson and Bohning (1960) to be between 2.0 and 2.5, within the temperature range of 10°C to 30°C. With the increase in respiration at higher temperature, the metabolic activity of the plant tissues also increases and this leads to a greater utilization of stored foods. From the results of the present investigation, it seems that the respiration metabolism approaches a maximum rate of approximately 30°C; this presumably allows hydrolysis of the stored foods to proceed at a faster rate resulting in uniform and maximum germination. At 45°C, the temperature of water may be considered as detrimental to the growth of the embryo.

Other factors effecting seed germination were not investigated, but it is possible that the chemical treatment and washing of the seeds at higher temperature also may have induced changes, perhaps in permeability to gases, sensitivity to light, and content of inhibitory substances. Whatever the physiological pattern may be, the results herein demonstrate the significance of treatment of the seeds with concentrated

sulphuric acid and of temperature in the process of germination and on the procurement of the experimental samples required.

FLOWERING RESPONSE OF VEGETATIVE SEEDLINGS OF DIFFERENT AGES TO AN INDUCTIVE DARK PERIOD

From the results, it can be concluded that the Pharbitis nil seedlings are in the "juvenile" stage only for one day following germination when they are photoperiodically insensitive. After one day of growth, the seedlings can be considered as being in the "adult" stage when they respond to photoinduction; here full photoinductive sensitivity is attained in the cotyledons.

The results presented in Figs. 2 and 3 indicate that 7 or more days old Pharbitis seedlings with attached cotyledons are insensitive to photoperiod but seedlings of the same age without cotyledons respond to a photoinductive period. These results suggest three possibilities for the lack of flower formation in older seedlings with attached cotyledons. Firstly, that with seedlings older than 7 days, the apex is not able to receive or express the floral stimulus translocated from the cotyledons. Secondly, it may be due to the presence of older cotyledons and finally, it may be attributed to the presence of young developing leaves which in some cases interfere with the translocation of the floral stimulus to the apex. The first possibility is inadmissable in view of the results presented in Fig. 3, in which the apex was receptive to the floral stimulus in seedlings between 9-21 days old. Floral induction in older seedlings of Pharbitis by Imamura and Takimoto

(1955a) also supports this view. The results of Fig. 4 were seedlings were defoliated (cotyledons retained) before (SD) photoperiod, eliminate the third possibility. This leads to the conclusion that in the case of Pharbitis, the flowering response is dependent upon the age of the cotyledons or of the leaves. The presence of either of these matured organs will inhibit flower formation. The results of various workers (Kujirai and Imamura, 1958; Khudairi and Hamner, 1954) have shown that the relative sensitivity to photoperiodic induction, of Pharbitis cotyledons and Xanthium leaves, increases with the age of the plant and reaches a maximum when the organs in question are fully expanded. A reversal occurs after this point and the sensitivity decreases with the increase in the age of the cotyledons or of the leaves.

The response to flowering, for old seedlings with matured intact cotyledons and young expanding leaves, can be attributed to the presence of one or more products of an inhibiting reaction. If these do exist, it may be possible that these are produced in the old cotyledons and their final expression leads to an anti-floral stimulus, which may suppress the floral expression of the seedlings. The explanation of this inhibitory flowering response will contribute greatly to existing knowledge of floral physiology.

ONTOGENY OF THE SHOOT APICES

During the course of the present investigation, Marushige (1965a, b) published related ontogenetic studies on Pharbitis nil chois,

strain violet. His investigation differed from ours in terms of photoperiodic treatment of the reproductive apices but was similar in relation to treatment of the vegetative apices. Discussion of similarities and differences, between the results of the present study and Marushige's (1965a, b) may be useful in understanding certain aspects of the ontogeny.

Marushige did not attempt to study the growth and development of the organs involved with reproduction, in relation to the photoinductive period. On the assumption that these organs have ontogenetic significance and may have influence in the life history of the flower, the author considered that environmental factors present during growth may lead to abnormal development of the reproductive organs and thus are likely to render the floral organs incapable of fertilization. A knowledge of the relationship of the development of the reproductive organs, with the photoperiodic induction seemed relevant, in order to ascertain for the specified stages required for the in vitro studies. Thus it is important to know their in vivo development.

Vegetative and Reproductive Apices

It is known that various zones of the apex assume different growth activities resulting in the development of the tissues present in the shoots and the leaves of the plants. Buvat (1952, 1955) postulated a different growth activity by the various zones of the apex and thus proposed a different zonation scheme for angiosperms. He characterized the most active zone, the peripheral and sub-terminal as anneau

initial, the tunica and the central zones as méristème d'attente, and the rib meristem as méristème médullaire. In the vegetative apex, the méristème d'attente is in an inactive state. If the apex becomes reproductive, the méristème d'attente becomes most active, whereas the anneau initial and méristème médullaire become quiescent.

From the present studies, it was clear that in the vegetative apices, there were fewer cell divisions in the central zone than in the surrounding peripheral zone or in the rib meristem. Inactive zones in both the shoot and root apex have been described by investigators (Buvat, 1955: Lance, 1958; Clowes, 1958, 1961). In root apex, the quiescent centre (Inactive Zone) has an extremely low cell division rate as well as a low concentration of protein, RNA and DNA when compared with other parts of the root (Jensen, 1958). Although the quiescent centre of the root is analogous to the central zone of the shoot apex (Clowes, 1961), the results described in this study do not agree that the central zone is completely inactive. Sorokin (1956a, b) using histochemical methods, has reported that the central zone is more active than the peripheral zone, when considered in relation to concentration of dehydrogenases and oxidases in mitochondria of the cells of the central zone. Results of the present work and the parallel studies of Sunderland, Heyes and Brown (1956, 1957); Gifford (1954); Boke (1947); Popham and Chan (1952); and Marushige (1965a) suggest that the central zone considered by Buvat (1952) to be in an inactive state in vegetative apices, appears actually to be an active zone. This zone, thought by Buvat as the source of the meristems from which arise inflorescences

and flowers, cannot be so interpreted by the present investigation. Instead, the entire apical meristem becomes involved. Ultimately, as floral induction proceeds, more and more of the peripheral region of the apex produces floral organ primordia.

The morphological changes occurring at the apex, following an inductive dark period, result in the initiation of different floral organ primordia, at different periods. Following a single inductive photoperiod, the first recognizable sign of floral transition becomes evident on the second day, with a general increase in the mitotic activity of the apex. On the third and fourth days, the sepals are initiated and the apex appears as a low broad dome. Petals and stamens initiate simultaneously on the fifth day when the apex enlarges in width and is flattened. On the sixth day after the end of the dark period, the carpel primordia originate and the apices show a low circular ridge. Between the eleventh and twelfth days following the dark period, ovules are produced. During the sixteenth and eighteenth days, the pollen grains develop and the style extends. Between the nineteenth and twentieth days, the secretory cells have formed the epidermis of the stigma. Marushige (1965b) described the same relationship of the origin of different primordia of the floral organs following cyclic inductive dark periods. From these results it can be postulated that in the case of Pharbitis nil chois, strain violet, there is no difference in floral organ initiation of sepals, petals, stamens and carpels, when the seedlings are subjected to a single inductive dark period or to a cyclic inductive dark period. The flowering response due to cyclic photoperiods,

however, may become evident in later stages of floral development.

It has been reported by Lona (1950), Carr (1955) and Naylor (1961), that cyclic inductive photoperiods result in cumulative response of flowering, either in a greater number of flowers or in the earliness of flower opening. This suggests that the results of Marushige (1965b) on initiation of floral organ primordia under cyclic inductive photoperiods should be attributed to the cumulative effects of photoperiods resulting in early floral organ initiation. But the results of Marushige's investigation on initiation of floral organ primordia are identical to those of the present studies in which initiation of floral organ primordia was investigated following a single inductive photoperiod.

From the identical results on initiation of floral organ primordia under two different photoinductive conditions, it can be postulated that the inner threshold condition essential for floral initiation, once achieved by one photoinductive period is not affected by multiple photoinductive periods. Therefore, the initiation of the floral organ primordia, in plants requiring a single photoinductive period of flowering should be identical, on subjection to one or more inductive photoperiods. At the present, the literature does not report any such comparative studies. It is hoped that future research on other plants would indicate if the initiation of floral primordia differs in response to single or cyclic inductive photoperiods.

IN VITRO STUDIES OF THE SHOOT APICES

In Vitro Culture of the Floral Apices after Different Periods of the End of Short Day

The results described earlier (P. 46) suggest that when the apices are excised and cultured 6 hours after the end of the dark period, they do not possess the potential to initiate floral primordia. Since the biochemistry of the transition to flowering is still unknown, no definite mechanism can be proposed to account for the reversal observed at the early flower initiating stage. It is possible, however, that this reversal may be related in terms of (a) modification in the supply of substances available to the apical meristem, while the apices are cultured *in vitro*, (b) elimination of regulatory effects attributable to older leaves (in the case of *Pharbitis*, cotyledons), on floral initiation and (c) lack of mitotic divisions at the apical meristem which may be due to the combined effects of (a) and (b).

One of the prerequisites for the expression of the floral stimulus is the presence of actively dividing cells at the apex (Zeevaart, 1962b; Bonner *et al.*, 1963). It is possible that the floral stimulus, translocated from the leaves, does not come in contact with actively dividing cells at the apex, is not able to express itself and becomes dissipated. Only vegetative development ensues. Separation from organic connection with the main plant and the effects of conditions (a) and (b) postulated above, may result in low mitotic activity in apices excised from intact plants for *in vitro* culture. Kujirai and

Imamura (1958) and Zeevaart (1962b) have reported that if the floral stimulus does not find an actively dividing bud, it apparently disappears in a period of about 48 hours.

In vivo reversion of the floral or inflorescence apices to the vegetative conditions seldom has been reported. Generally, it is accepted that the change of the apical meristem from a vegetative to a floral phase is almost always irreversible (Cutter, 1965). Evans (1960), Thomas (1961) and Wycherley (1954) have reported in vivo reversion of the floral apices. This reversion occurred in those plants which had been maintained in inductive day lengths for too short a period, or occasionally in flowering plants subsequently placed in non-inductive conditions. These treatments, however, do not always induce reversion (Lance, 1957). Wardlaw (1963), working with Petasites hybridus under in vivo conditions, reported inducing reversion in the early transition stages by using surgical treatment. As the plant material was collected from the field, growing under natural environmental conditions, it is not certain by what photoperiodic threshold conditions these apices were induced.

The present work and the forementioned studies, indicate that in vivo the flowering process, once initiated, cannot be reversed until and unless a mechanism as yet unknown inhibits the regulatory process for the interaction of the floral stimulus at the apex in the early stages of transition.

It is acknowledged that complete understanding of the control of the process of flowering will have important implications. The

present study suggests, that to some extent, control of the mechanism of flowering can be exercised. This should be possible in the initial stages, just after the photoperiodic induction, when the floral apex still may revert to the vegetative condition. Floral initiation, may be controlled by inhibiting the regulatory process or processes for floral formation at the apex. This inhibition of the regulatory process may be exercised by surgical treatment of the apex, or by treatment of the apex with some growth inhibitors such as 5-Fluorouracil, 5-Fluorodeoxyuridine and the inhibitors of steriod biosynthesis, used by Zeevaart (1962b), and Bonner et al. (1963). Such treatments of the apices result in inhibition of flowering.

Floral Induction in Cultured Vegetative Apices

The results presented in the preceding section indicate that in the case of cultured apices of Pharbitis nil, the leaf is the primary site of photoperiodic induction. When the apex was cultured with two leaf primordia and subjected to an inductive photoperiod, it remained vegetative throughout the period of growth.

Baldev (1960) grew 1-2 cms. long stem tips of Cuscuta reflexa in sterile culture, and subjected them to various periods of darkness. They flowered in continuous darkness or with a daily period of 14 hours of darkness or more, behaving as typical (SD) plants. It has been claimed (Baldev, 1960) that the bud itself is sensitive to photoinduction. Since Cuscuta is a parasite with greatly reduced scales which perform the functions of a leaf, such as photosynthesis, the possibility that the

scales and the more mature stem tissues might be sensitive to photoinduction cannot be excluded.

While studying floral induction in cultured apical buds of Perilla, Raghavan (1961) reported that the apex and the leaf primordia could participate in perception of the floral stimulus. It is difficult to accept the evidence resulting in Raghavan's hypothesis; since leaves are considered to be the major sites which are sensitive to photoinduction. Recently, Raghavan (in White, 1965; p. 303) commenting on his previous (Raghavan, 1961) results, stated that the presence of leaf areas was associated with floral induction with Perilla apices. He has mentioned that at the time of culture, 0.17 cm^2 of leaf area were present on the bud. This, to the writer, suggests that the induction was more likely perceived through the leaves.

Baldev and Raghavan have been able to induce flowering in apices with reduced leaf area. In the case of Pharbitis, the necessity for attaining a minimum leaf area for photoperiodic induction has been established. Taking into account the nature of the scales in Cuscuta and the presence of leaf area in Perilla; the results obtained with Pharbitis do not contradict those of Baldev and Raghavan.

The foregoing discussion and the present data, confirm the classical hypothesis that leaves are the principal organs responsive to photoinduction, and that a definite relationship exists between leaf area and photoinduction.

CONCLUSIONS AND SUMMARY

Morphological investigations were initiated to reveal four aspects of flower formation of a short day plant Pharbitis nil chois, strain violet (Japanese Morning Glory).

The first of these is the flowering response of the vegetative seedlings of different ages to an inductive photoperiod. The second area of investigation examines the ontogenetic development of the vegetative and floral shoot apices of Pharbitis nil, and the relation of different stages of floral development with the photoperiodic induction. The investigation applies from time of germination to when floral organ primordia are initiated following a single inductive photoperiod. The third approach purposes to determine whether apices excised and cultured from photoperiodically induced intact plants after one inductive dark period, would continue to differentiate under controlled conditions, or would revert to the vegetative conditions. The aim of the fourth investigation is to determine the site of floral induction in the case of Pharbitis nil when the apices are excised and cultured in vitro and then subjected to a single inductive photoperiod.

Apart from the four major objectives of the investigations, the methods of obtaining even and maximum germination of Pharbitis seeds, and of production of contamination-free cultures of the shoot apices without injuring the tissue, have been examined and established. The results recorded and general conclusions are listed below:

1. Even and maximum seed germination, necessary to secure experimental samples equivalent in age and ontogenetic stage, was obtained by

treating the seeds with concentrated sulphuric acid for 70-90 minutes and afterwards washing overnight in running water at a temperature of 30°C.

2. Aseptic cultures for *in vitro* studies of the shoot apices of *Pharbitis nil* were secured by surface sterilizing the apices first with 70% ethanol for 15 seconds, rinsing twice with redistilled sterile water, again sterilizing for 5 minutes with 5% Chlorox solution to which 2 drops of "Tween twenty" had been added and then rinsing 4 times with redistilled, sterilized water.
3. The cotyledons of the seedlings of *Pharbitis nil*, on germination are not photosensitive, but a day later, they respond to a photoinductive period. The seedlings of different ages respond differently in floral development to a single dark period. The maximum response for flowering is obtained at the 4-day stage when the seedlings are controlled as to photoperiod. It has been indicated that the lack in flowering response of the seedlings with increase in age is due to the insensitivity of the cotyledons or the leaves which may be producing flower inhibiting substances.
4. The shoot apex of *Pharbitis nil* exhibits a cytohistological zonation which embraces a biseriate tunica, central zone, peripheral zone and a rib meristem. These zones are concerned with the development of the apex by exhibiting different growth activities, leading to the establishment of the tissues present in shoots of the plants.

At the time of germination, cytohistological zonation is not clearly demarcated, although cells of the apex have dense contents and granular cytoplasm. With increase in age, the zonation becomes

delineated and the granular appearance of the cytoplasm disappears. Leaf primordia originate at the rate of one leaf every 24 hours. In older seedlings, staining is intensified in the apices, in contrast to the condition for younger seedlings.

5. Following induction, the first recognizable sign of floral transition becomes evident on the second day, with a general increase in the mitotic activity of the apex. On the third and fourth days, the sepals are initiated and the apex appears as a low broad dome. Petals and stamens initiate simultaneously on the fifth day when the apex enlarges in width and is flattened. On the sixth day, after the end of the dark period, the carpel primordia originate and the apices show a low circular ridge. Between the eleventh and twelfth days following the dark period, ovules are produced. During the sixteenth and eighteenth days, the pollen grains develop and the style extends. Between the nineteenth and twentieth days, the secretory cells have formed the epidermis of the stigma.
6. In Pharbitis nil, the apices excised and cultured from intact plants 6 hours after the end of the inductive dark period, remained vegetative while those excised and cultured 12 hours and after developed and differentiated floral organ primordia.
7. In the case of cultured vegetative apices, the leaf is the primary site of photoinduction. Floral induction is related to a minimum leaf area of 4.0 cm^2.

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APPENDICES

Appendix I

Seed germination of *Pharbitis nil* with variations in length of concentrated sulphuric acid treatment and temperature of the water used for washing

Treatment with concentrated sulphuric acid (Minutes)	Temperature of washing water (°C)	No. of seeds treated	No. of seeds germinated	% of seeds germinated
30	15	50	12	24
	30	50	18	36
	45	50	7	14
45	15	50	16	32
	30	50	22	44
	45	50	9	18
60	15	50	19	38
	30	50	28	56
	45	50	9	18
75	15	50	23	46
	30	50	34	68
	45	50	11	22
90	15	50	25	50
	30	50	36	72
	45	50	8	16
105	15	50	11	22
	30	50	15	30
	45	50	4	8

Appendix II

Paraffin method for dehydration and embedding of the *Pharbitis nil* apices

Fixed apices washed in running water	48 hours
5% ethanol	2 hours
11% ethanol	2 hours
18% ethanol	2 hours
30% ethanol	2 hours
50% solution of Tertiary Butyl Alcohol (TBA)	2 hours
70% solution of TBA	overnight
85% solution of TBA	2 hours
95% solution of TBA	2 hours
100% solution of TBA	2 hours
Pure TBA	2 hours
Pure TBA	overnight
Pure TBA	2 hours
Pure TBA + Paraffin oil (50:50)	1 hour
Pour the contents in a vial which is 3/4 full of solidified Paraffin wax, place the vial in 60°C oven. Remove stopper from the vial.	2-3 hours
Repeat the above procedure 2 times.	2-3 hours each
Replace with *tissuemat* with a melting point of 61°C.	
Imbed.	
Section.	
Fix ribbon sections to slide with Mayer albumin fixative.	

Appendix III

The triple stain of safranine, Delafield's hematoxylin and anilin blue for staining the sections of the shoot apices of *Pharbitis nil*

Fixative:- Navashin's fixative

Solutions:-

Safranin O.

Dissolve 4 gms. of Safranin O. in 200 cc. of methyl cellosolve. When the solution is complete, add 100 cc. each of 95% ethyl alcohol and distilled water, followed by 4 gm. sodium acetate and 8 cc. formalin.

Delafield's hematoxylin

This stain is available in solution form (ready to use) from Fisher Scientific Co.

Anilin blue

Prepare a saturated solution of anilin blue stain in methyl cellosolve. Dilute the saturated solution with methyl cellosolve in equal volumes.

Rinse solution

Methyl salicylate	25%
Xylene	33%
Absolute ethyl alcohol	42%

Clearing solution

Methyl salicylate	2 parts
Xylene	1 part
Absolute ethyl alcohol	1 part

Staining Procedure:

1. Bring slides to 50% ethyl alcohol.
2. Stain in 1% Safranin O for 24 hours.
3. * Dip in tap water.
4. Dip in Delafield's hematoxylin for 5 minutes.
5. Dip in tap water.
6. Dip in acidified water (1 drop conc. Hcl in 200 ml. water) to remove excess hematoxylin.
7. Wash in running water for 15 minutes.
8. Dip in each of the following: 30%, 50%, 70%, 95%, and absolute ethyl alcohol.
9. Dip in solution of anilin blue for 1 minute.
10. Dip in absolute ethyl alcohol to remove excess of anilin blue from the sections and from the slides.
11. Dip in a rinse solution.
12. Dip in a clearing solution and leave for 2 minutes.
13. Dip in a mixture of 90% Xylene and 10% absolute ethyl alcohol.
14. Dip successively into dishes of Xylene and leave until ready to proceed.
15. Mount in "Permount".

Results:

Cell wall.....dark purple
Cytoplasm.....purple
Nuclei.....reddish purple
Nucleoli and Chromosomes.....brilliant red

* The term "dip" in the procedure should be interpreted to mean "raise and lower the slide rapidly 10 or 12 times".

Appendix IV

Ingredients used in White's culture medium for the growth of *Pharbitis nil* apices

Ingredient	Milligrams per liter
KCl	65
KNO_3	80
$Ca(NO_3)_2\ 4H_2O$	300
$MgSo_4\ 7H_2O$	720
$NaSo_4$	200
$NaH_2Po_4\ H_2O$	16.5
$Fe_2(SO_4)_3$	2.5
$ZnSo_4\ 7H_2O$	3
H_3BO_3	1.5
KI	0.75
$Cu\ SO_4\ 5H_2O$	0.001
MoO_3	0.0001
Sucrose	20,000

Appendix V

Effect of one short day (16 hours) on flowering of seedlings of different ages

Age of the seedlings (Days) after germination and before the S. D. Treatment	Total number of flowers on 5 plants	Mean number of flowers per plant
0(at germination)	0	0
1 day	13	2.6
2 days	19	3.8
3 days	21	4.2
4 days	21	4.2
5 days	12	2.4
6 days	2	0.4
7 days	0	0
8 days	0	0
9 days	0	0
10 days	0	0
11 days	0	0
12 days	0	0
13 days	0	0
14 days	0	0
15 days	0	0

Appendix VI

Flower response in relation to leaf area developed in intact plants after removal of cotyledons

Age of seedlings (days) after removal of cotyledons and before S. D. treatment	Leaf area in cm^2		No. of plants with terminal flower bud (Total of 10 plants)	Percent of plants with terminal flower
	Total of 10 plants	Mean per plant		
0 (at removal of cotyledons)	-	-	0	0
3	-	-	0	0
6	10.8	1.1	0	0
9	41.9	4.2	7	70
12	85.6	8.5	10	100
15	154.6	15.5	10	100
18	185.4	18.5	10	100
21	285.7	28.5	10	100
24	310.3	31.0	8	80
27	355.2	35.5	7	70
30	384.7	38.5	5	50

Appendix VII

Effect of one short day (16 hours) on flowering of seedlings of Pharbitis nil of different ages with leaves removed

Age of seedlings (Days) after germination and before S. D. treatment	Total number of flowers on 5 plants	Mean number of flowers per plant
0 (at germination)	0	0
1	14	2.8
2	19	3.8
3	20	4.0
4	20	4.0
5	13	2.6
6	4	0.8
7	0	0
8	0	0
9	0	0
10	0	0
11	0	0
12	0	0
13	0	0
14	0	0
15	0	0

Appendix VIII

Flowering response in relation to leaf area and age of the apex after *in vitro* culture

Age of the apex (days) after *in vitro* culture and before S. D. treatment	Leaf area developed (cm^2)		No. of seedlings with terminal apex initiating floral organ primordia (Total of 5 seedlings)	Percent of seedlings with terminal floral apex
	Total of 5 plants	Mean per plant		
0 (at time of culturing)	-	-	0	0
3	2.0	0.4	0	0
6	5.7	1.1	0	0
9	6.6	1.3	0	0
12	7.1	1.4	0	0
15	7.7	1.5	0	0
18	8.8	1.7	0	0
21	11.5	2.3	0	0
24	14.2	2.8	0	0
27	16.5	3.3	0	0
30	18.6	3.7	0	0
33	20.4	4.0	3	60
36	21.7	4.3	4	80
39	22.5	4.5	5	100
42	24.3	4.8	5	100
45	24.4	4.9	5	100

PLATES

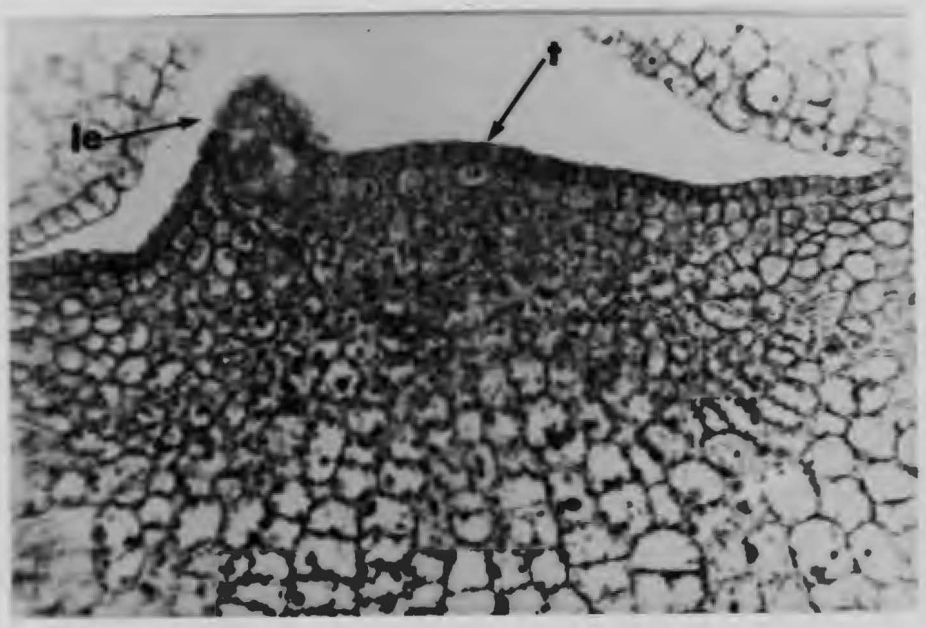
KEY TO PLATE ABBREVIATIONS

AM	apical meristem
CZ	central zone
Ca	carpel primordia
Cam	carpel margins
IZ	inner zone
lb	leaf buttress
le	leaf primordia
md	mitotic divisions
OZ	outer zone
ov	ovules
PZ	peripheral zone
pmc	pollen mother cells
pg	pollen grains
pe	petal primordia
RM	rib meristem
se	sepal primordia
st	stamen primordia
S	stigma
sty	style
t	tunica
ta	tapetum

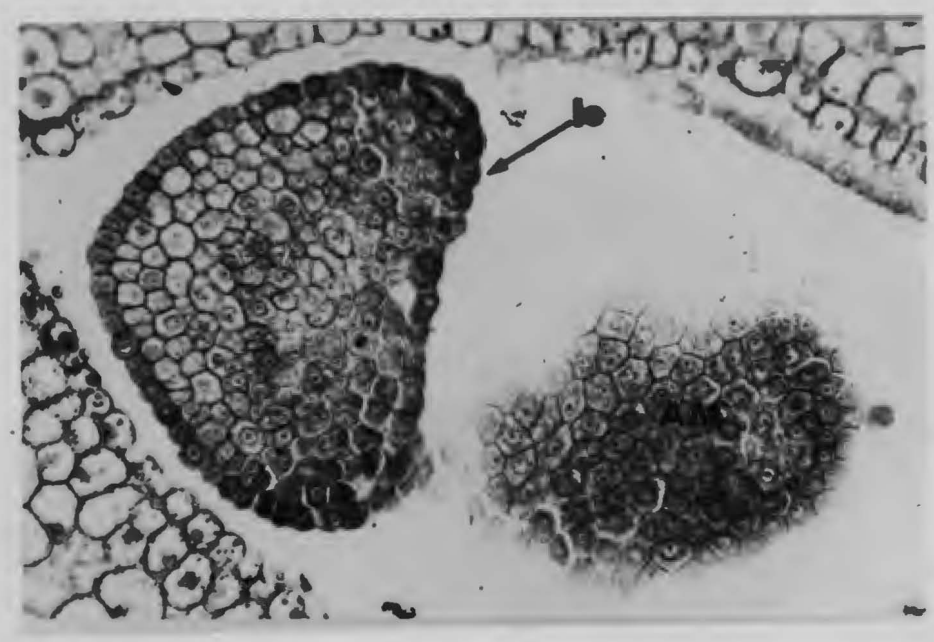
A. Median longitudinal section of the apex of Pharbitis nil taken at the time of sprouting of seeds. The apex shows the initiation of one leaf primordium and a single tunica layer (x 250).

B. Transverse section of the apex of Pharbitis nil at sprouting of the seed phase, taken at the tunica level, showing one leaf primordium and the apical meristem (x 275).

PLATE I



A

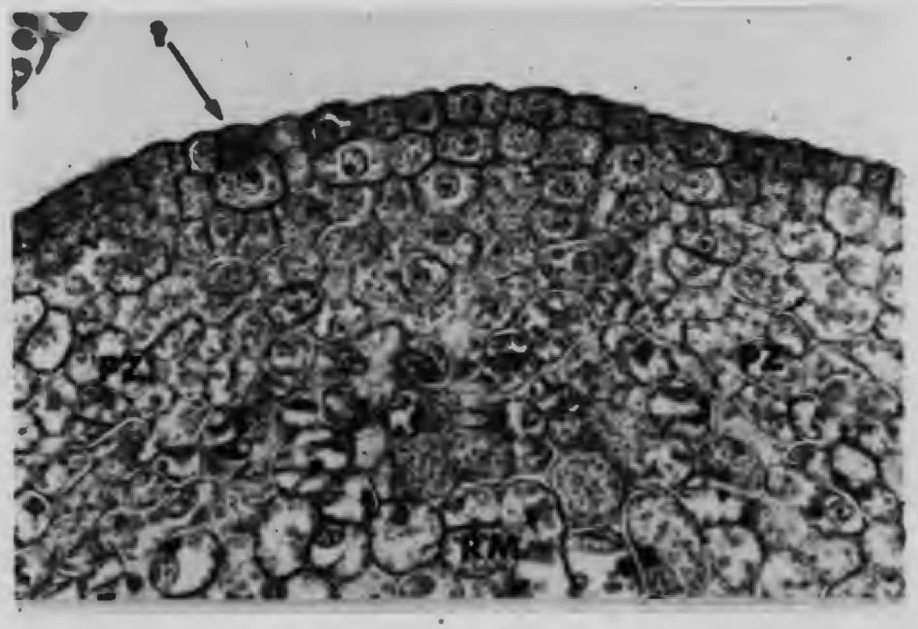


B

A. Median longitudinal section of the apex of Pharbitis nil taken at the time of sprouting of seeds. The apex shows a uniseriate tunica. The central, peripheral, and the rib meristem zones are poorly differentiated. The content of the cells of the apex has a granular appearance probably due to the presence of stored materials (x 450).

B. Median longitudinal section of the apex of Pharbitis nil 24 hours after sprouting showing the initiation of a leaf primordia as a leaf buttress. Zonation is poorly delineated. Stored material from the cells of the apex has disappeared but some is present in the rib meristem. In the peripheral zone, mitotic divisions are frequent (x 380).

PLATE II



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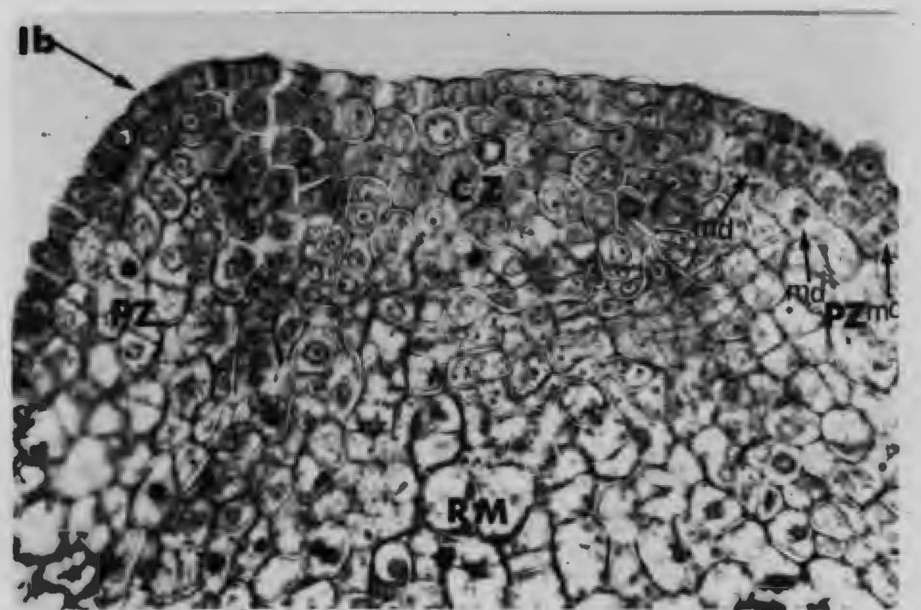
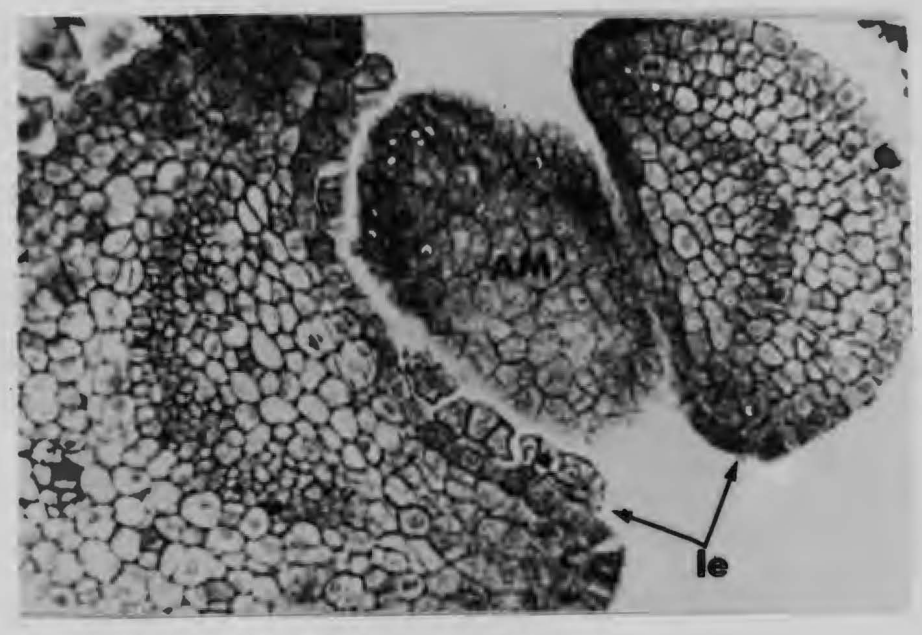


B

A. Transverse section of the apex of Pharbitis nil taken at the time of germination (2 days after sprouting of the seed), showing initiation of the two leaf primordia and the apical meristem (x 250).

B. Median longitudinal section of the apex of Pharbitis nil at seed germination, showing the zonation. The conspicuous large cells at the summit of the corpus as indicated by an arrow, probably are the initials of the Corpus (x 380).

PLATE III



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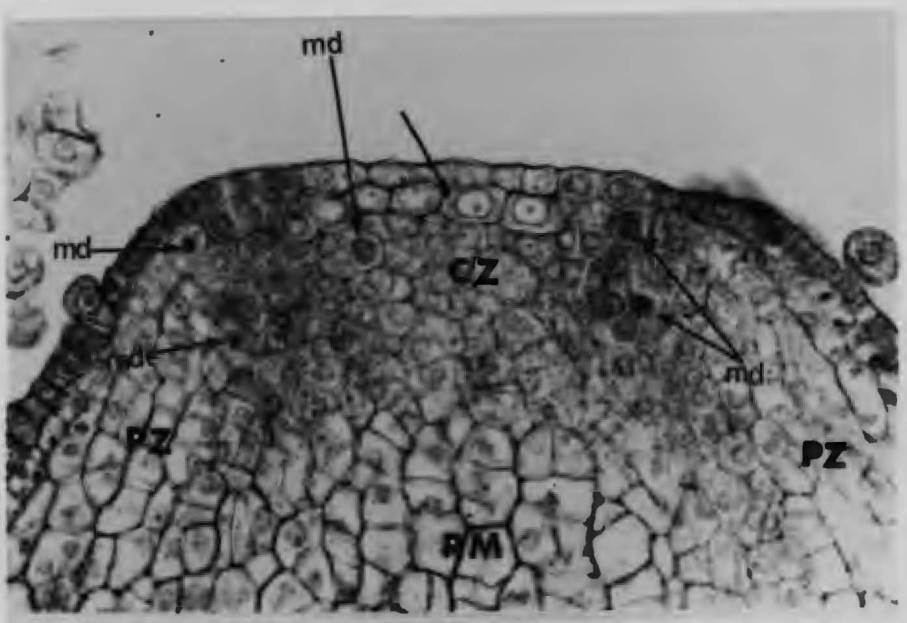
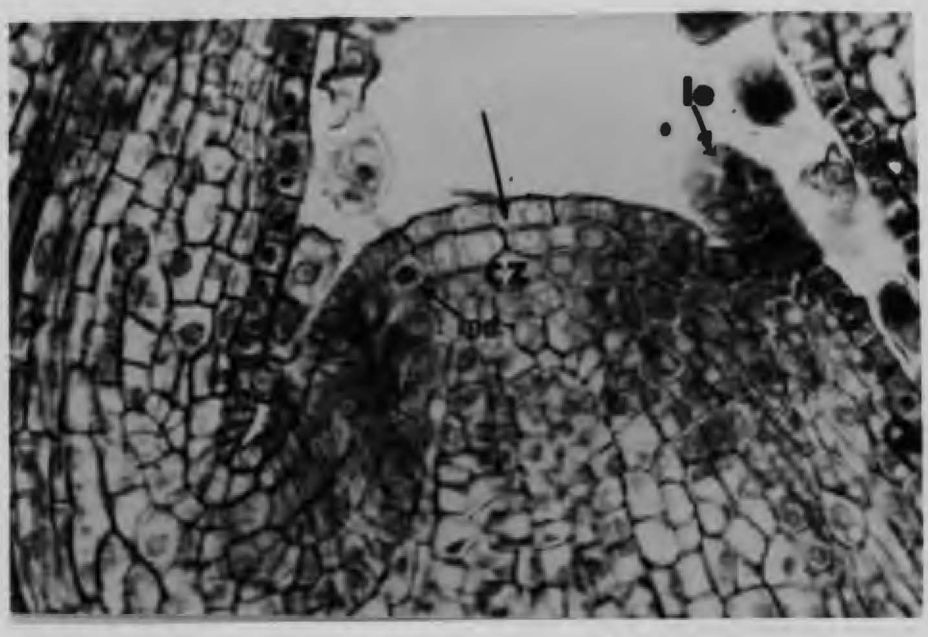


B

Median longitudinal sections of the apices from 1, 2, 2, and 4-day old seedlings respectively showing the cup shaped central zone with lightly stained nuclei. Large cells at the summit of the central zone are evident in all the phases. Mitotic divisions are frequent in the peripheral zone (Plate V A). Divisions in the peripheral cells of central zone are also visible (Plates IV A and V A). As indicated by an arrow, a group of initial cells of the tunica is easily recognized by their large size, vacuolated cytoplasm and large nuclei (Plate IV A).

Plates IV and V (x 380).

PLATE IV



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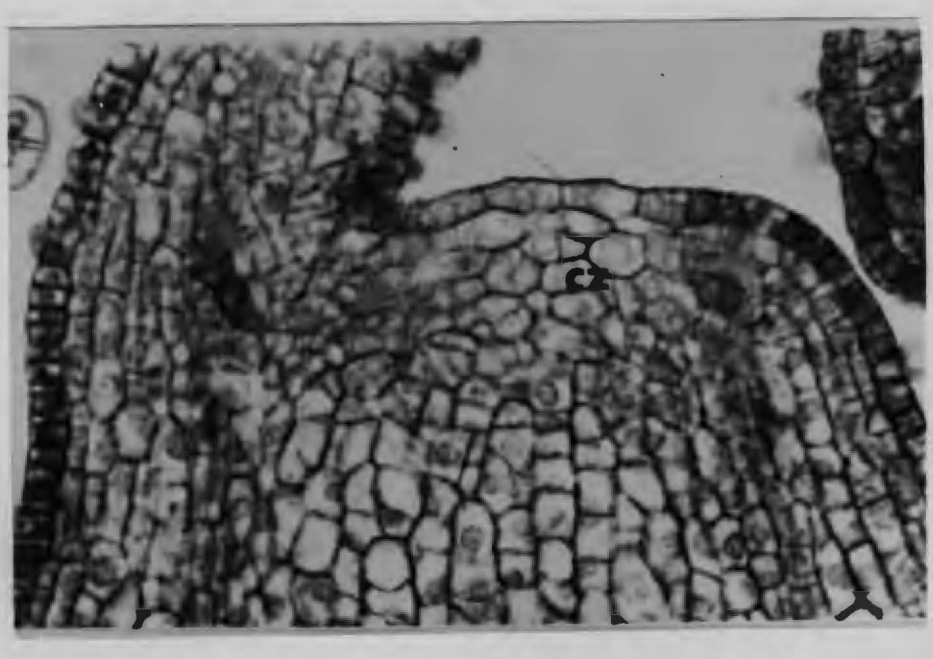
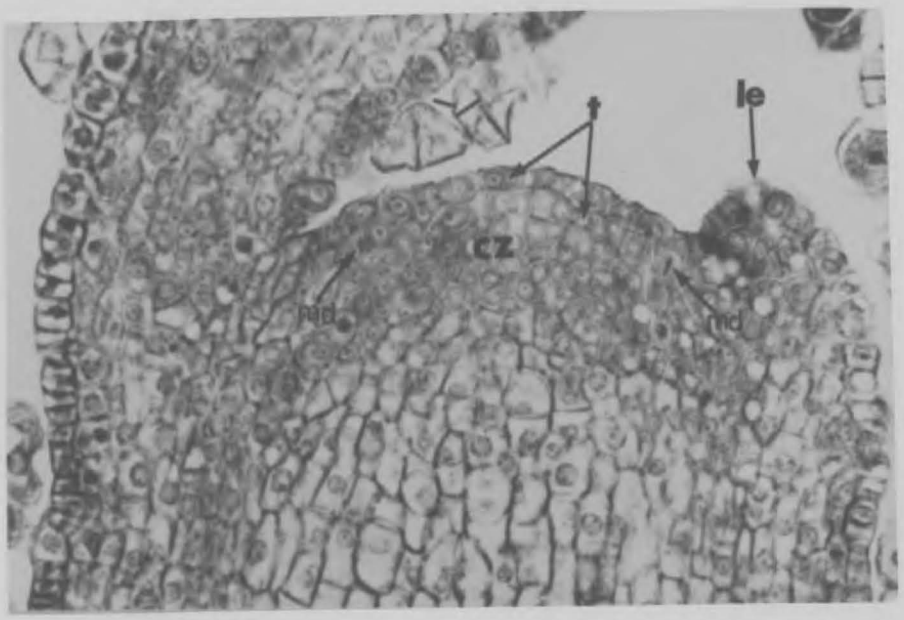


B

Median longitudinal section of the apices of 5, 6, 7, and 8-day old seedlings respectively. In the 5-day old seedling, the tunica becomes two layered. Although T2 is poorly differentiated, anticlinal divisions are evident (Plate VI A). There is reduction in the cell size of the central zone when contrasted with cell size on the apices of the younger seedlings (Plates IV, V). As indicated by an arrow, a few large cells are present at the summit of the central zone (Plate VI B). Transverse divisions are visible in the rib meristem zone (Plate VII A). Divisions in the peripheral zone are evident (Plate VII B).

Plates VI and VII (x 380).

PLATE VI

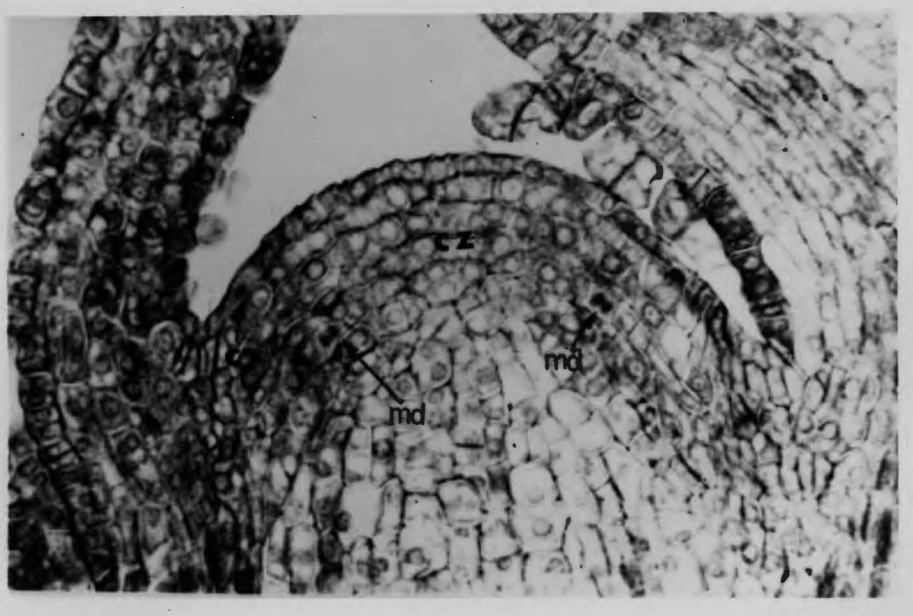


A

B

PLATE VII

A

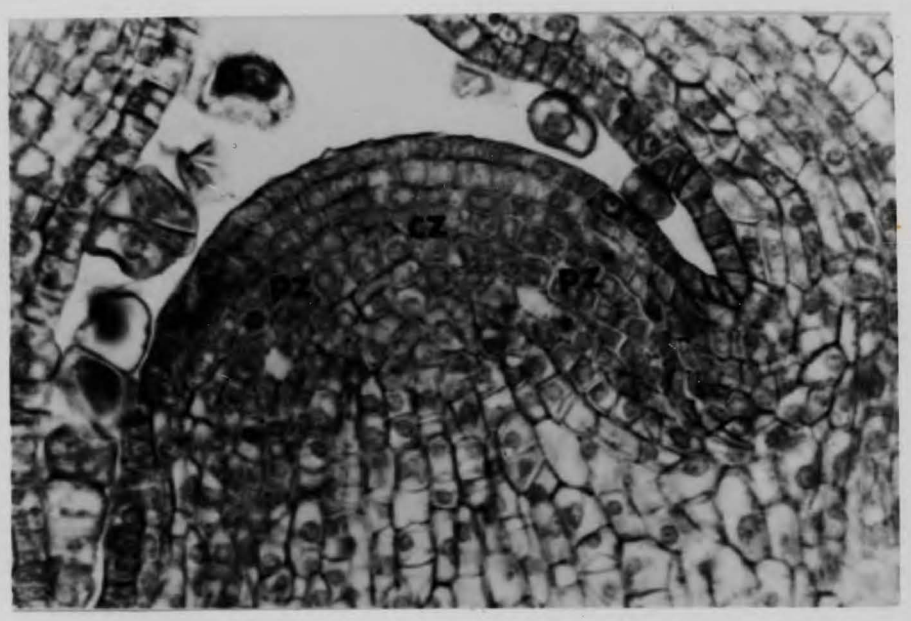


B

Median longitudinal sections of the apices of 9, 10, 11, and 12-day old seedlings respectively, showing the dome shaped appearance of the apex. The staining intensity is relatively uniform in the central and peripheral zones. The size of the cells of central and peripheral zones is almost the same.

Plates VIII and IX (x 380).

PLATE VIII



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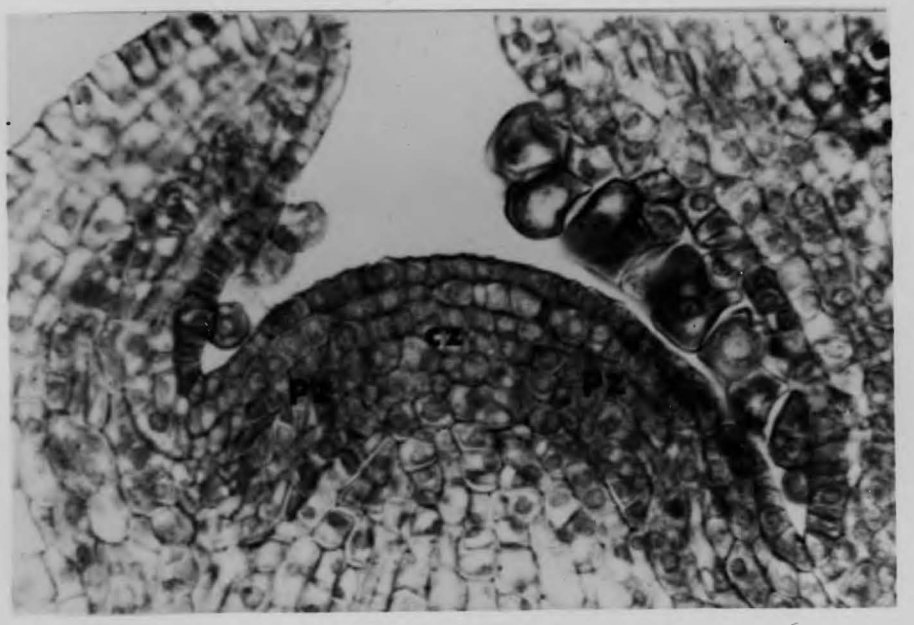
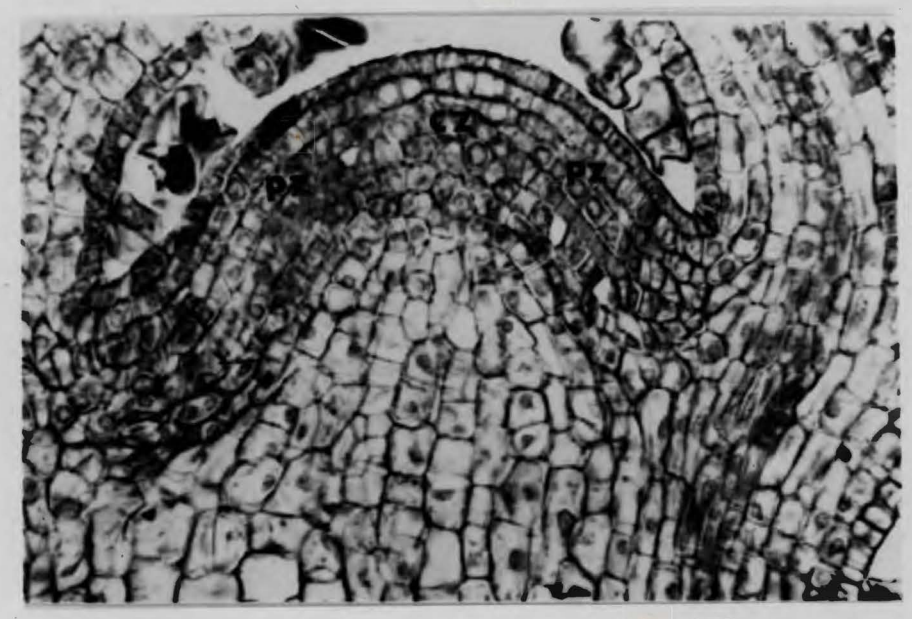


B

PLATE IX



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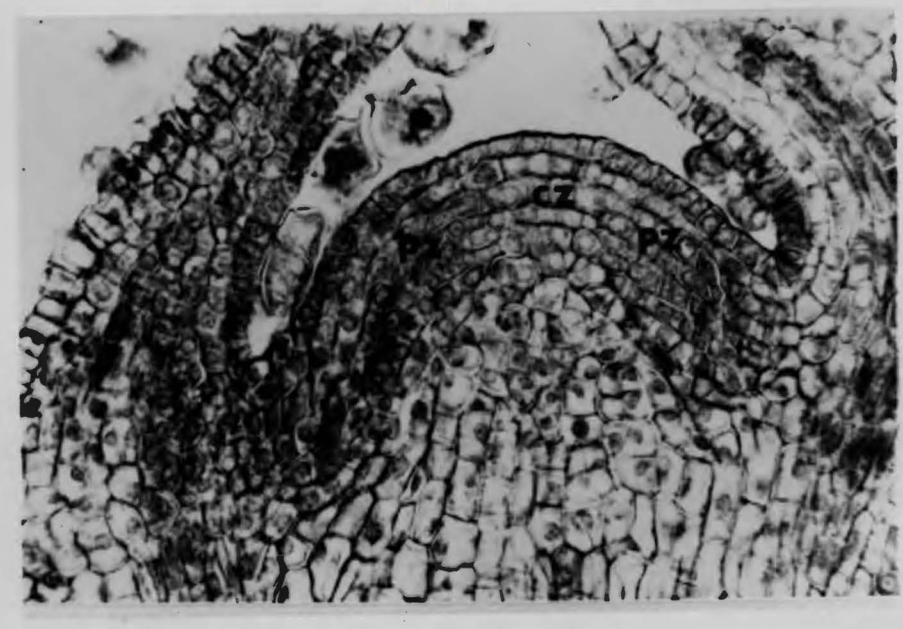
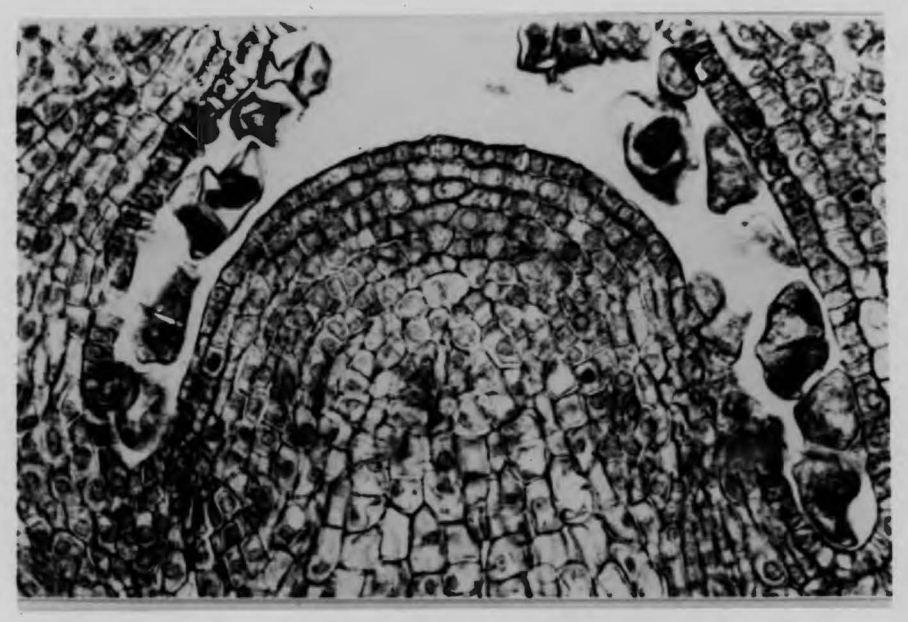


B

Median longitudinal sections of the apices of 13, 14-day old seedlings repectively showing the increase height of the apices (x 380).

PLATE X



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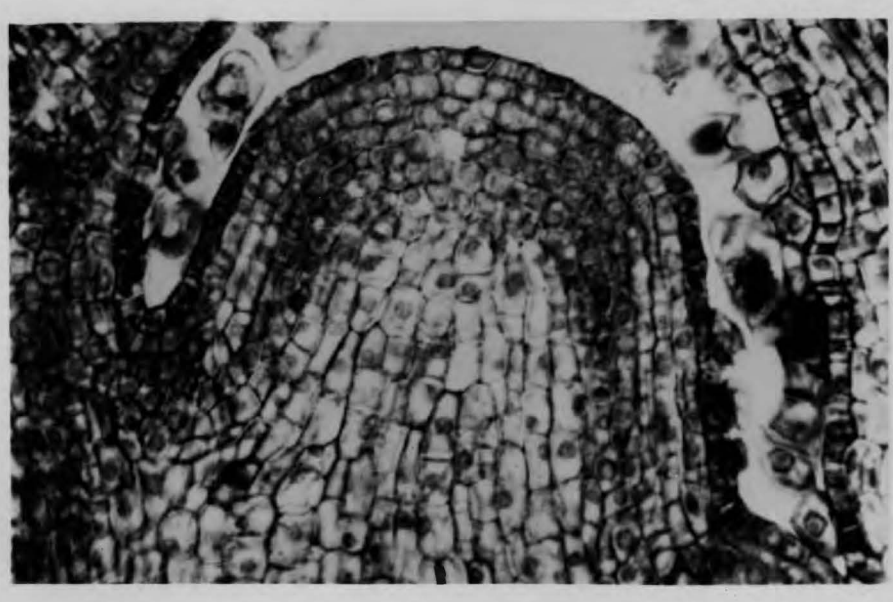


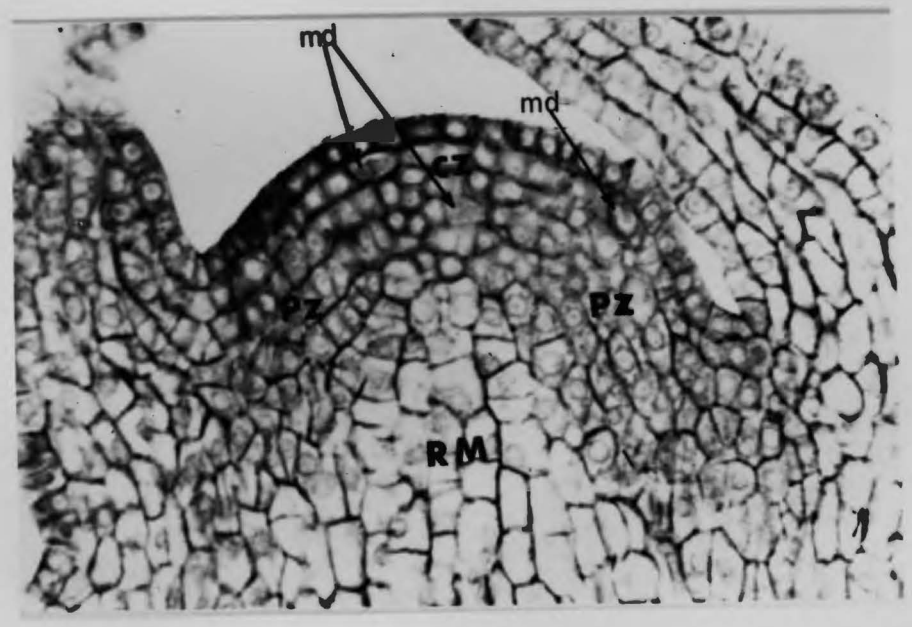
B

A. Median longitudinal section of the apex of Pharbitis nil taken one day following the inductive dark period. The apex shows morphological configuration similar to that of vegetative apices (x 380).

B. Median longitudinal section of the apex of Pharbitis nil showing vegetative like zonation, 2 days following dark period (x 380).

PLATE XI

A



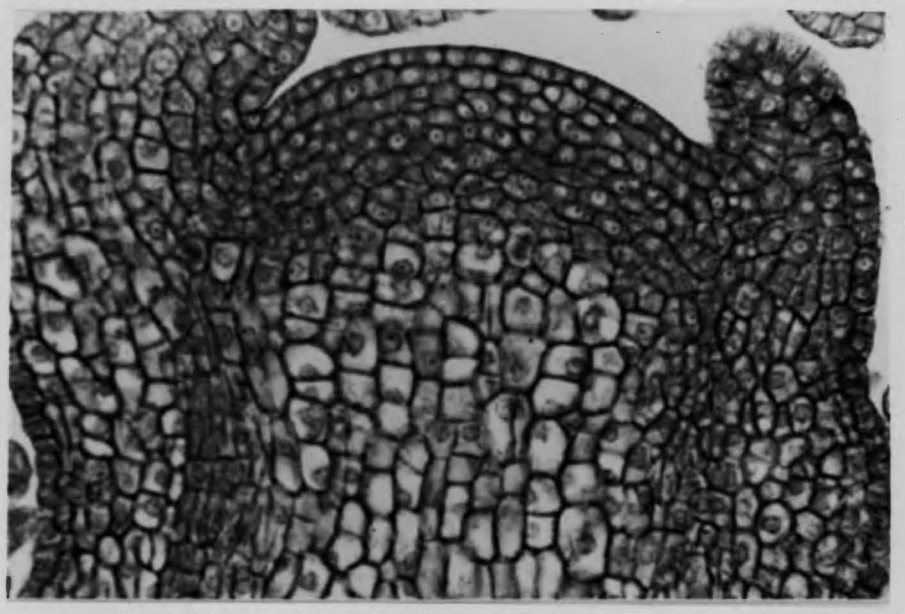
B

Median longitudinal sections of Pharbitis nil taken 3 and 4 days respectively, following the dark period showing the apices as low, broad and dome shaped. At this stage of development, the apices show initiation of sepal primordia.

A. Taken 3 days following the dark period (x 310).

B. Taken 4 days following the dark period (x 270).

PLATE XII



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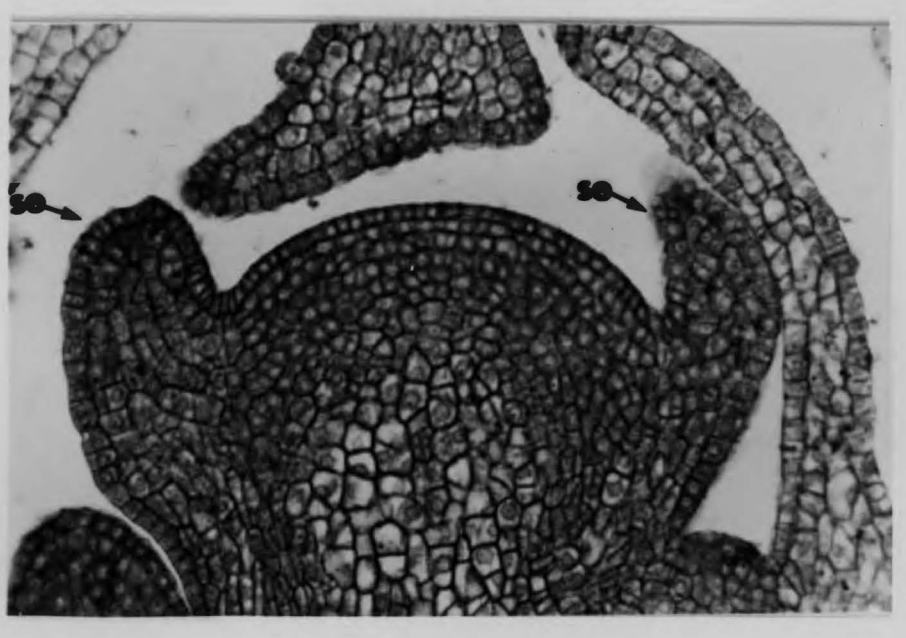
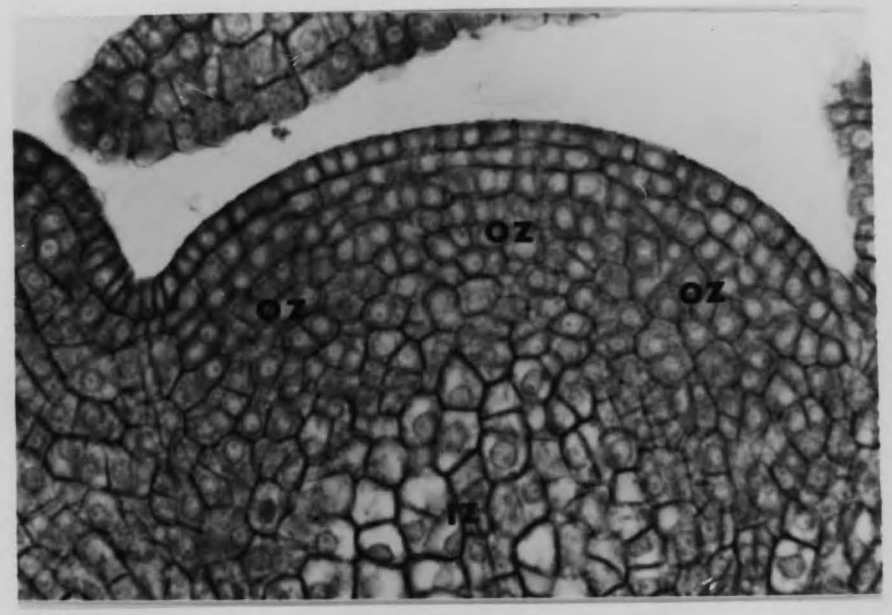


B

A. Median longitudinal section of Pharbitis nil, 4 days following the dark period, showing the outer and inner zones of the apex. The outer zone is derived from the central and peripheral zones and the inner zone has arisen from the rib meristem (x 450).

B. Median longitudinal section of the apex of Pharbitis nil taken 5 days following dark period. The apex has expanded laterally and is flat. The petal and stamen primordia (visible in this section) are initiated at the same time (x 260).

PLATE XIII



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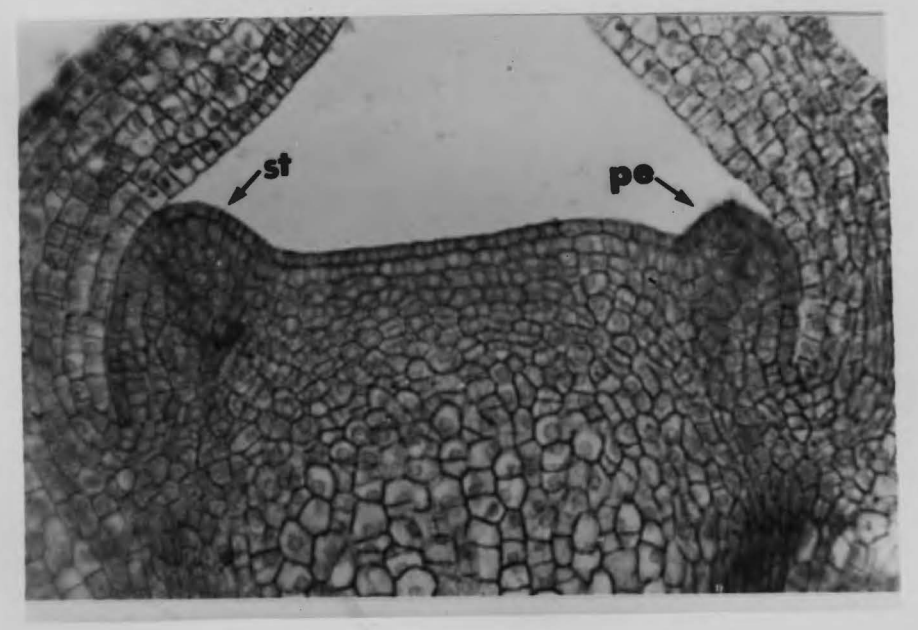
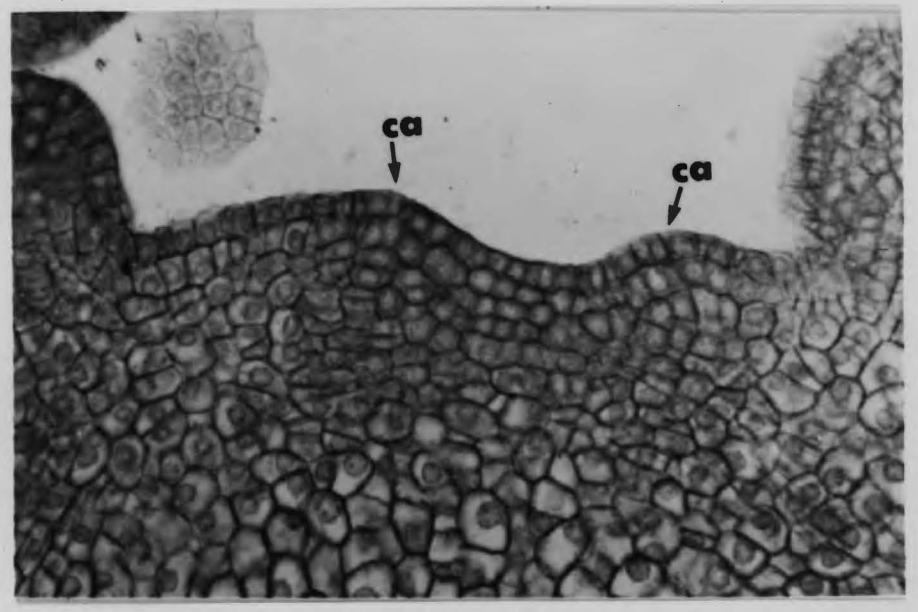


B

A. Median longitudinal section of the apex of Pharbitis nil taken 6 days following the dark period. The apex shows the initiation of carpel primordia as indicated by a low circular depression (x 450).

B. Transverse section of a 6-day old apex, showing the well differentiated petal and stamen primordia which alternate. At this stage, the adjacent petal margins are separated. Carpels form a continuous circular ridge surrounding the depression (x 260).

PLATE XIV



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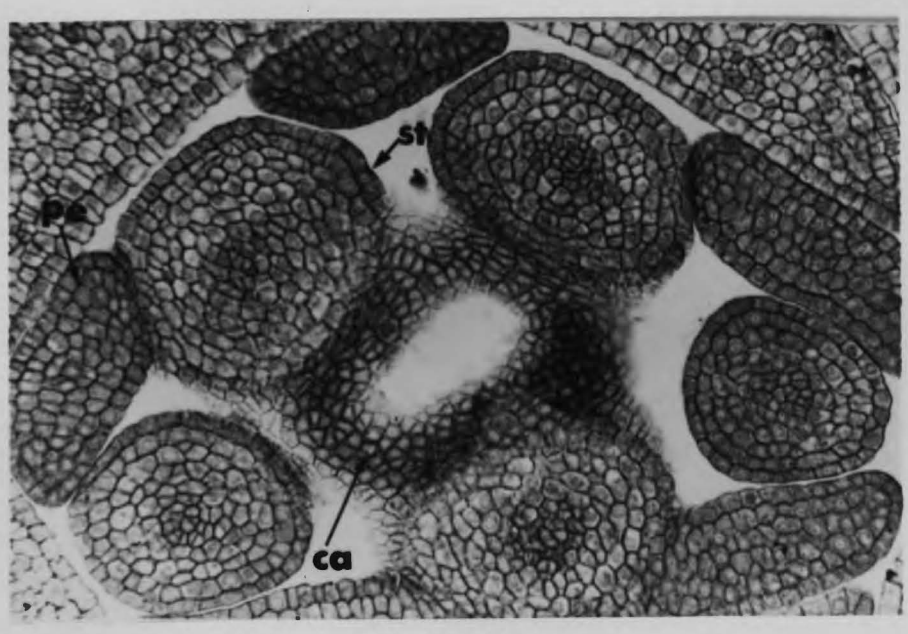


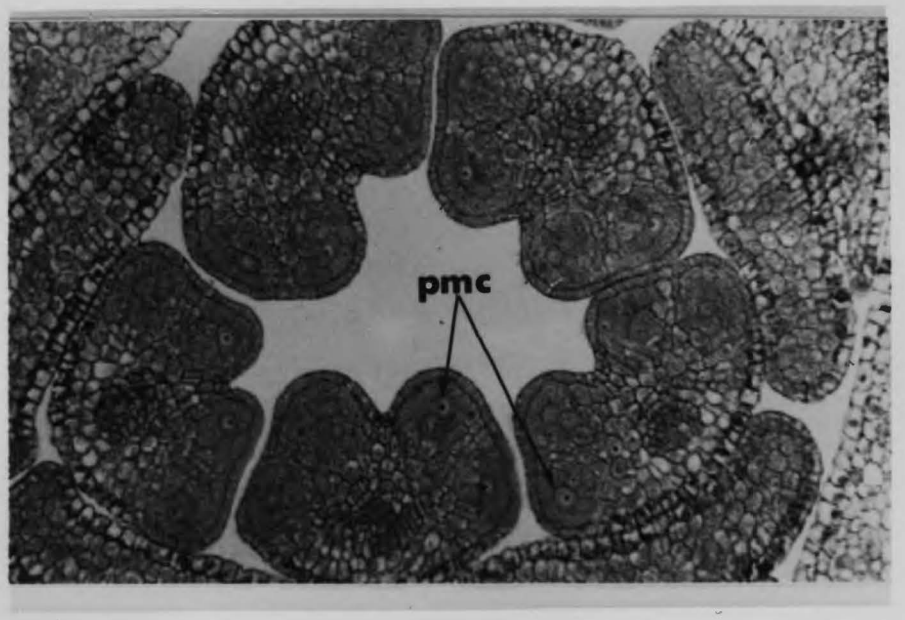
B

A. Median longitudinal section of Pharbitis nil 8 days following the dark period. The apex shows floral primordia (x 110).

B. Transverse section of an 8-day old floral apex of Pharbitis, showing four microsporangial zones and developing large pollen mother cells in the staminal tissue (x 270).

PLATE XV

A

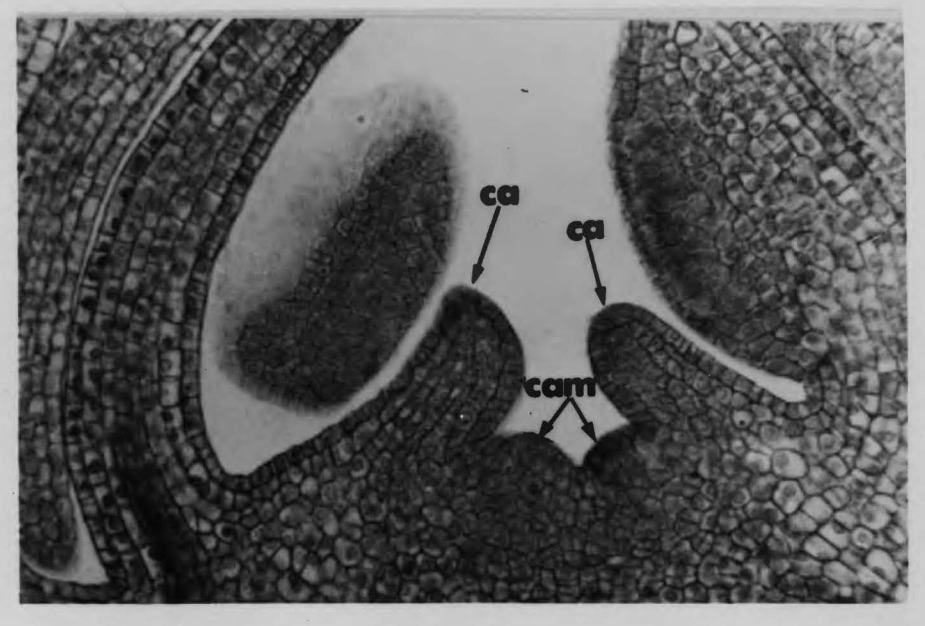


B

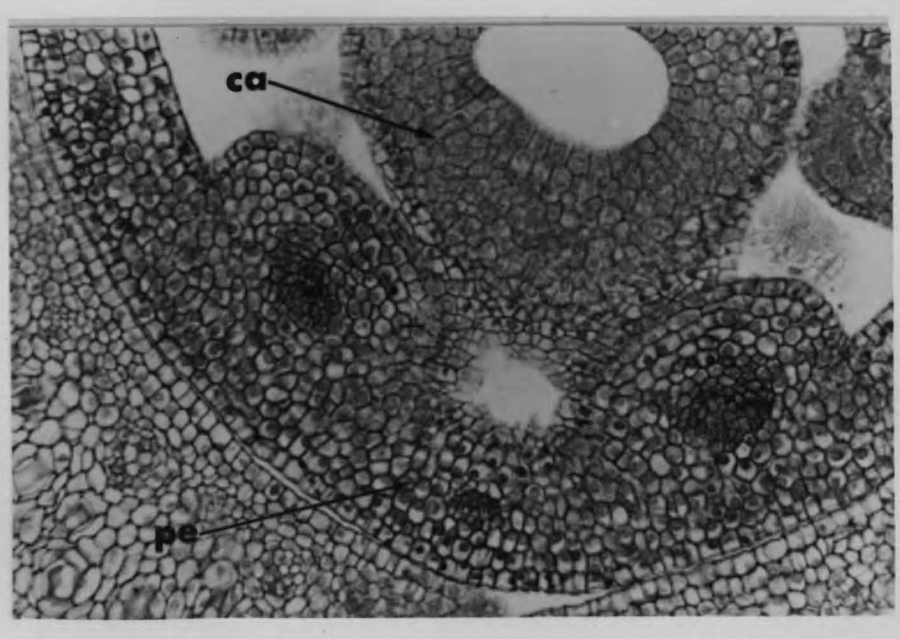
A. Median longitudinal section of the floral apex of Pharbitis nil 9 days following the dark period. The apex shows carpel primordia and the development of the margin of the carpel (x 270).

B. Transverse section of a 9-day old floral apex of Pharbitis nil revealing the fusion of the petals and the regular ring of carpel primordia (x 260).

PLATE XVI



A

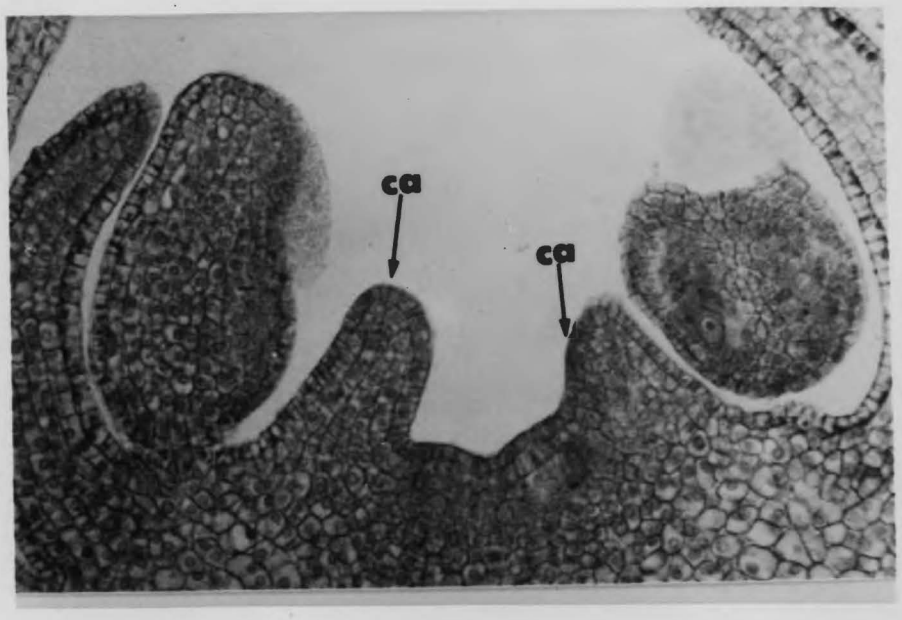


B

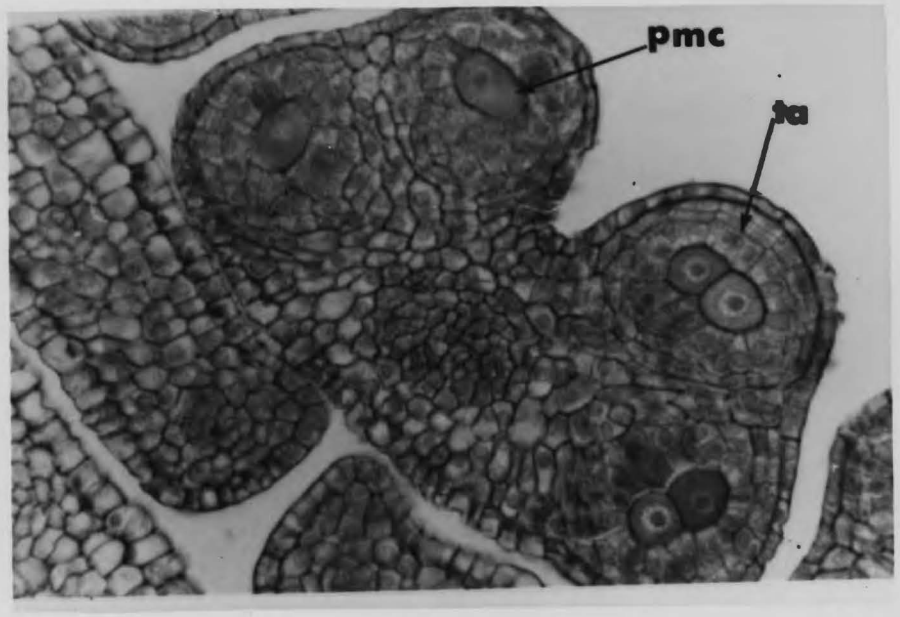
A. Median longitudinal section of the apex of Pharbitis nil 10 days following the dark period. The apex shows further development of the carpels (x 260).

B. Transverse section of the stamen from a 10-day old seedling showing four pollen mother cell groups with a well developed tapetal layer (x 680).

PLATE XVII



A

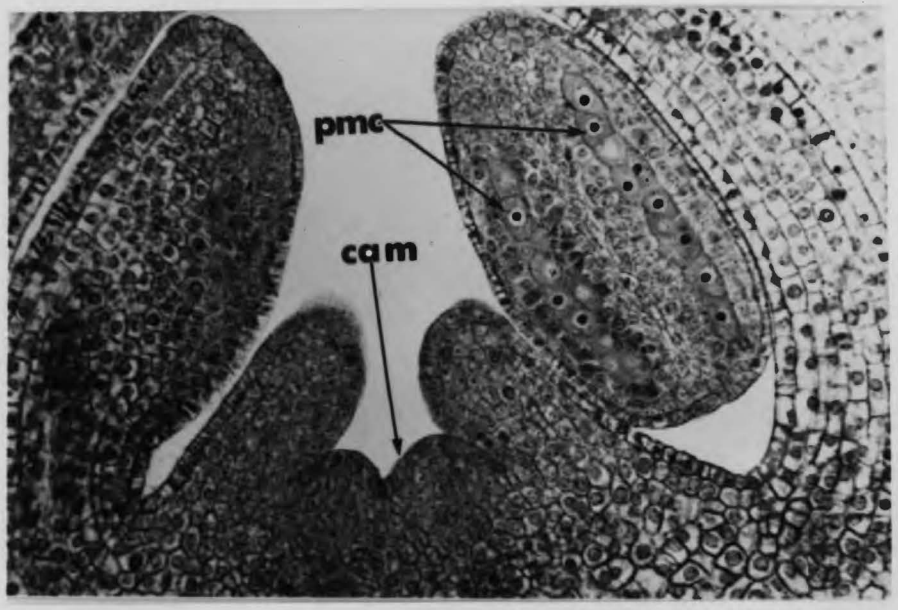


B

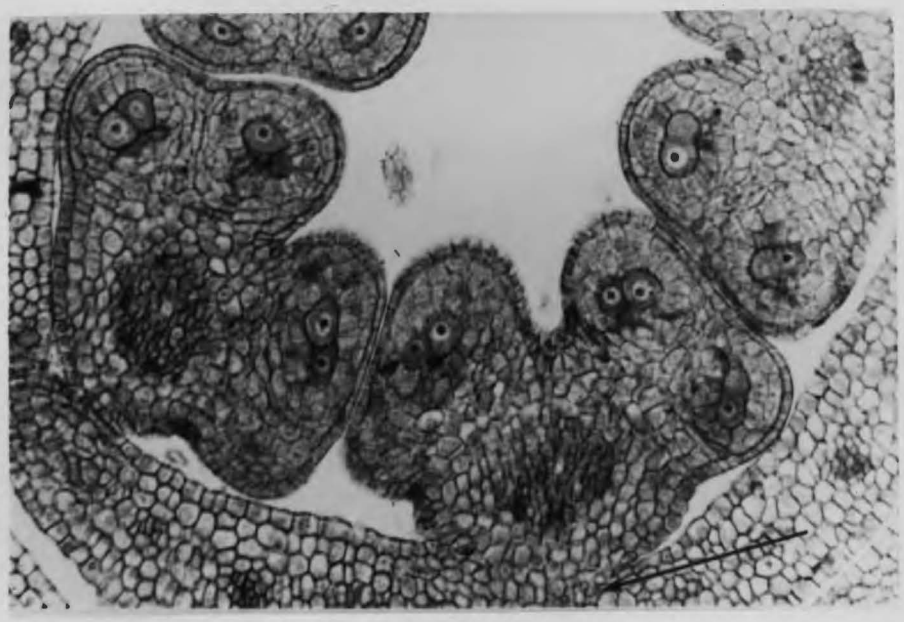
A. Median longitudinal section of the apex of Pharbitis nil, 11 days following the dark period. The apex shows the pollen mother cells arranged longitudinally in the anther. Carpel formation has commenced and development of the carpel margins is evident (x 270).

B. Transverse section of Pharbitis nil 11 days following the dark period, at the level of stamen-petal fusion. The apex shows the fusion of the stamen primordia with the petal primordia as indicated by an arrow. Fusion of the petal margins is well advanced (x 250).

PLATE XVIII



A

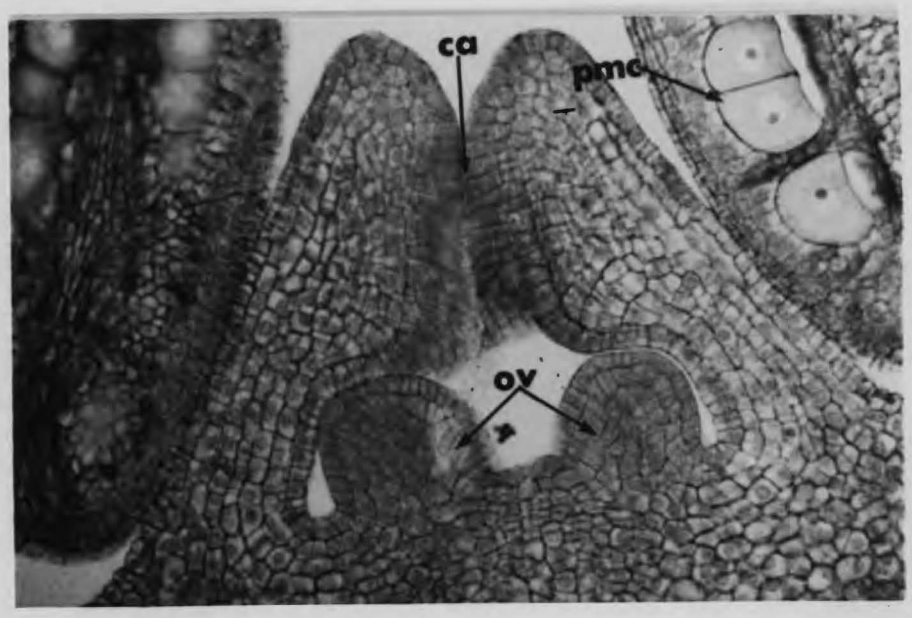


B

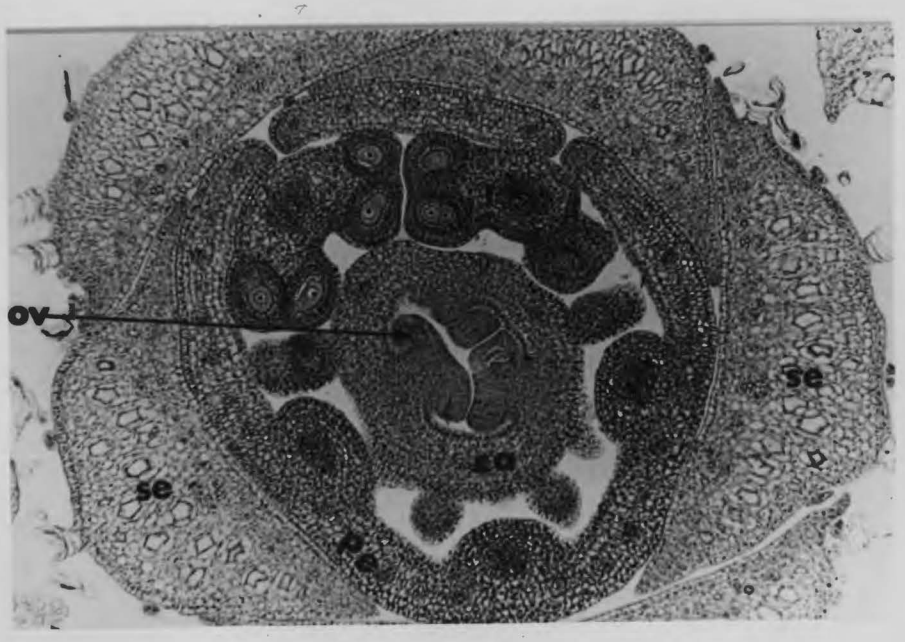
A. Median longitudinal section of the apex of Pharbitis nil 12 days following the dark period. The apex shows union of the carpel primordia and ovule formation in the placental region. Pollen mother cells are visible (x 270).

B. Transverse section of the apex of Pharbitis nil 12 days following the dark period. The apex shows the nearly complete fusion of petals. The circular ridge of the carpel primordia has formed and ovules have developed from the placental region (x 110).

PLATE XIX



A

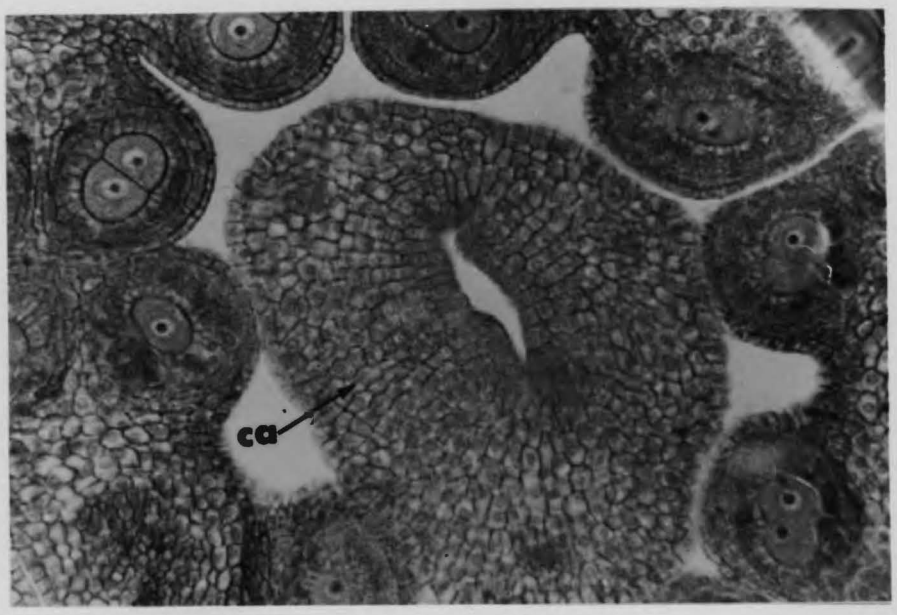


B

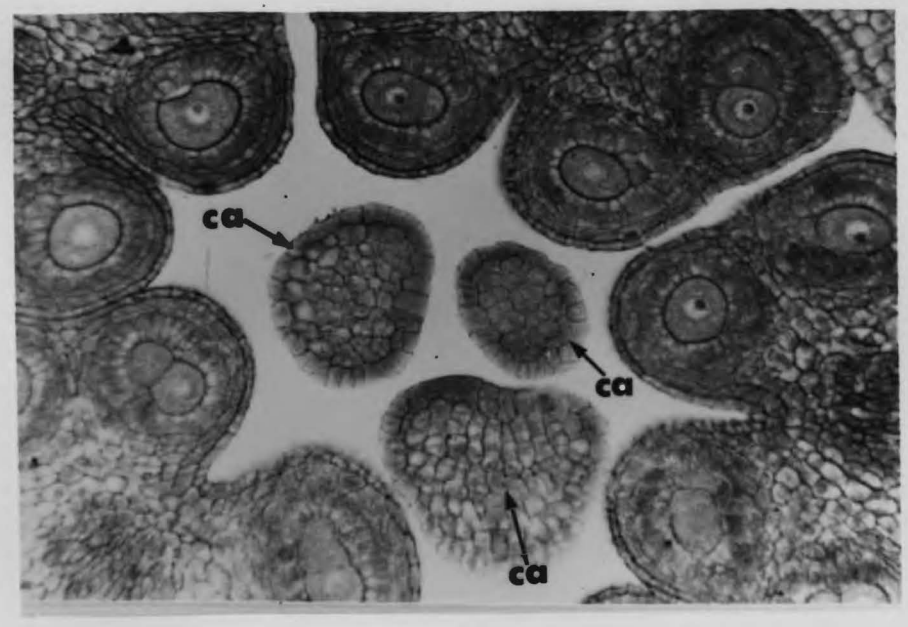
A. Transverse section of the apex of Pharbitis nil taken after 12 days of floral growth, at the median level of the carpels. The apex exhibits fusion of the carpels giving it a circular appearance; but fusion is incomplete towards the centre (x 280).

B. Transverse section of the apex of Pharbitis nil taken after 12 days of floral growth, at the position of the stigma. The carpel primordia are of different length and their margins are not fused (x 250).

PLATE XX



A

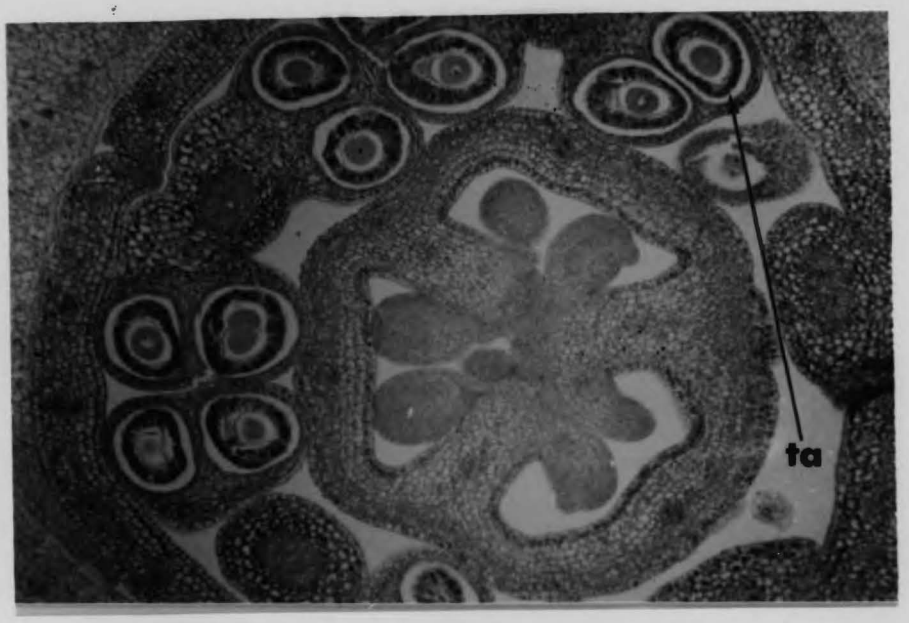


B

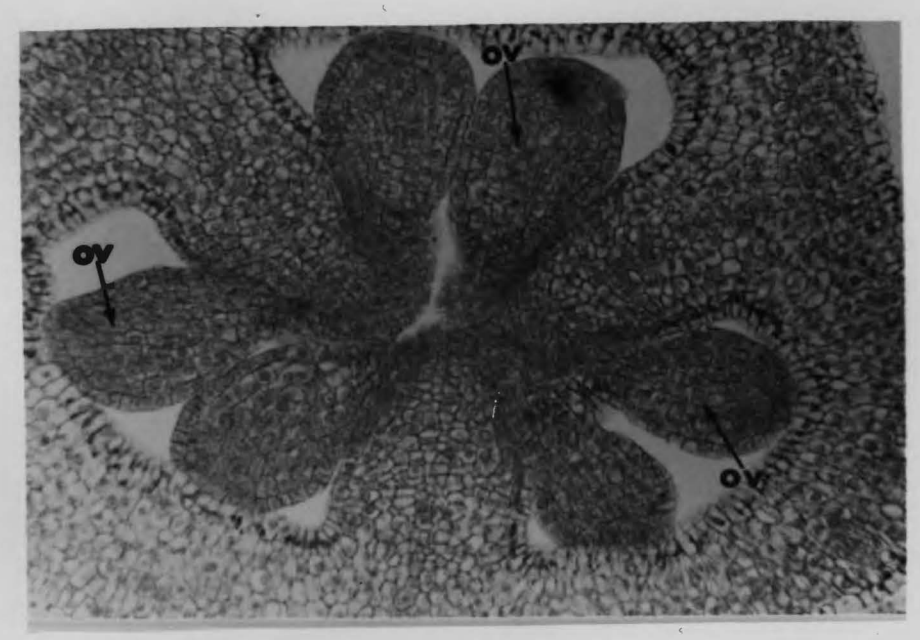
A. Transverse section of the apex of Pharbitis nil after 14 days of floral growth, showing a three loculed ovary with axile placentation. As indicated by an arrow the tapetal layer of the pollen mother cells has disintegrated and the cells have assumed the appearance of plasmodial masses (x 280).

B. Transverse section of a 16-day old floral apex of Pharbitis nil showing well developed ovules (x 250).

PLATE XXI



A

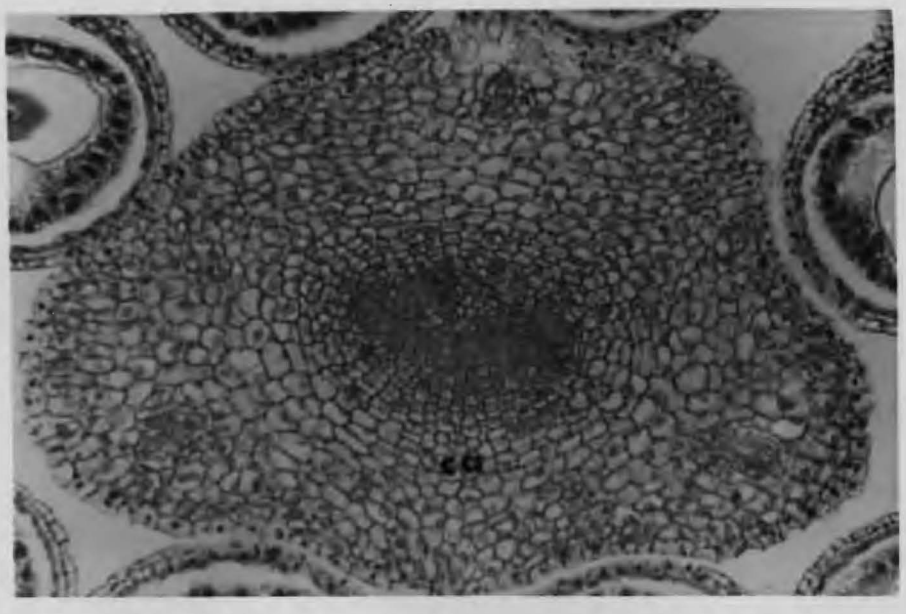


B

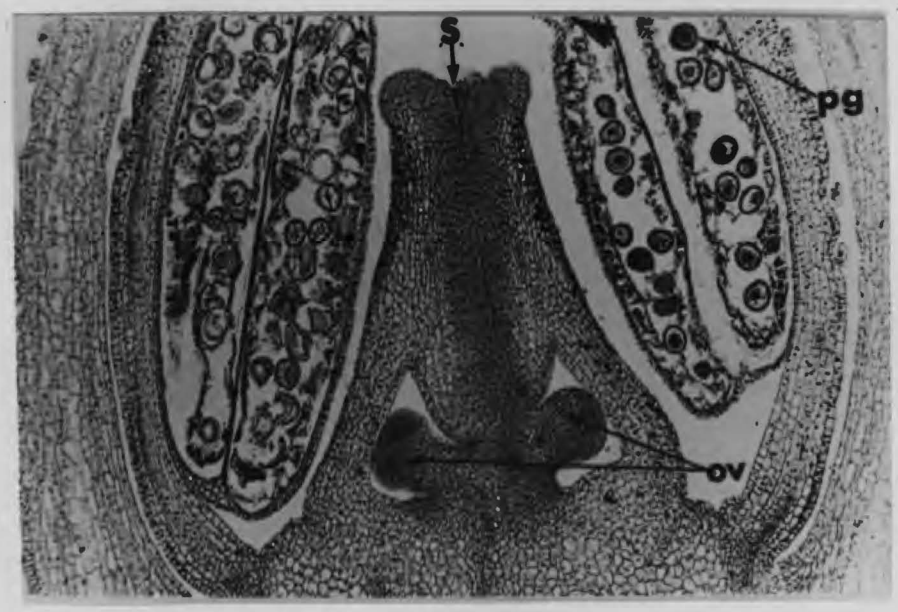
A. Transverse section of a 16-day old floral apex of Pharbitis nil at the median level of the carpels, showing their complete fusion (x 270).

B. Median longitudinal section of the apex of Pharbitis nil after 18 days of floral growth. Mature pollen grains with axines are visible. Ovules are seen in the locules of the ovary. The carpels have fused and the stigma has proliferated at the distal end (x 110).

PLATE XXII



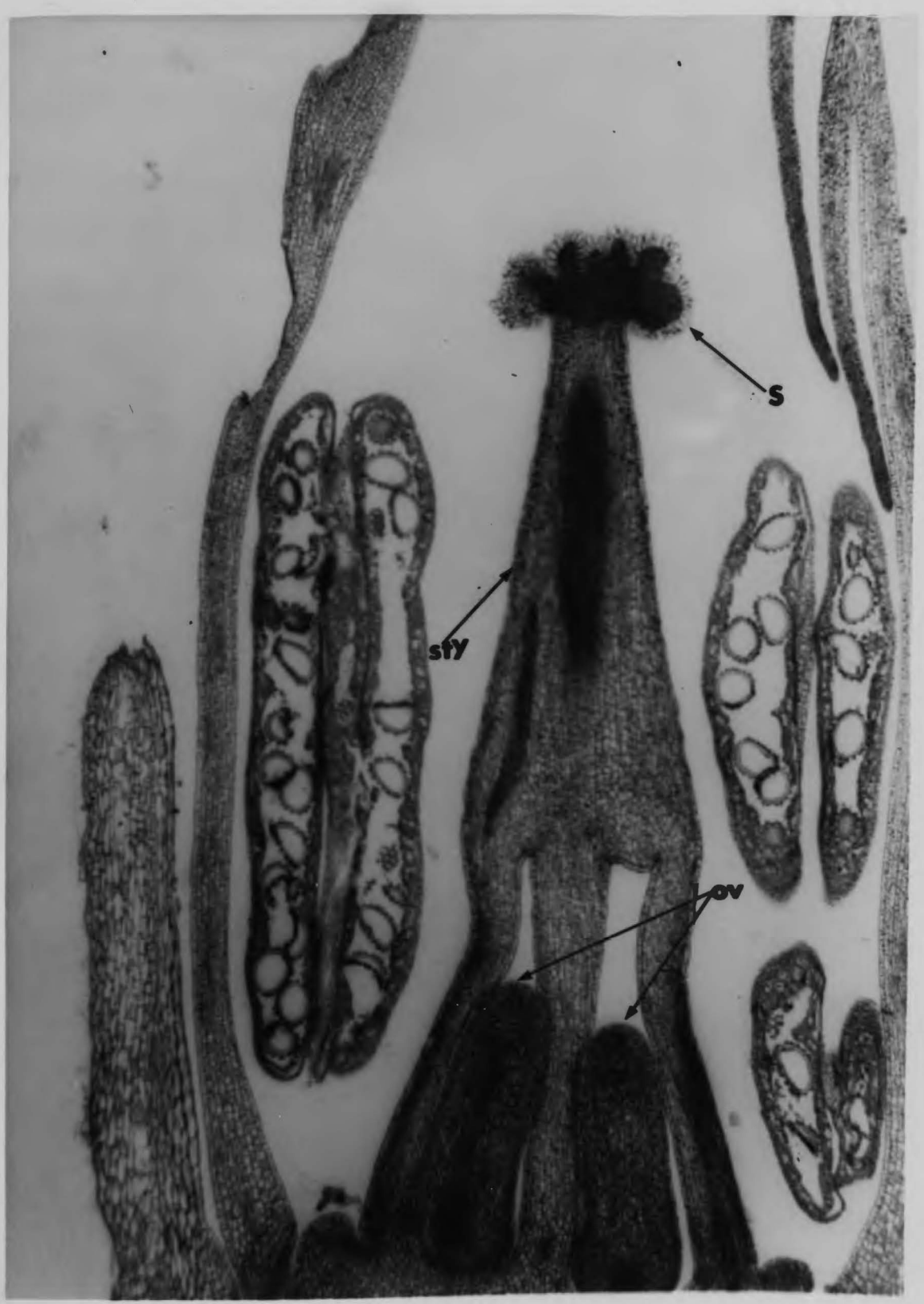
A



B

Median longitudinal section from a 20-day old floral bud of *Pharbitis nil* showing the enlongated style and two ovules. The stigma is shown with well developed secretory cells (x 85).

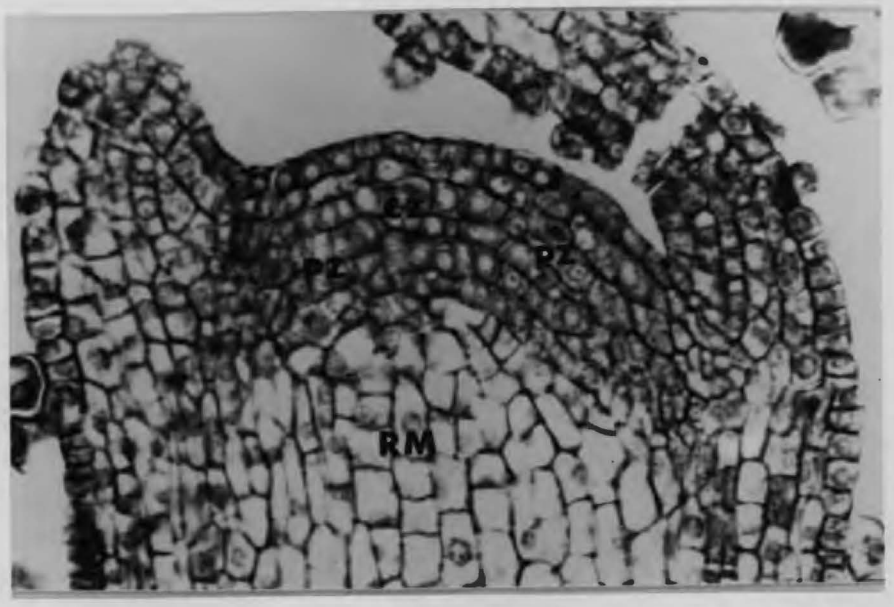
PLATE XXIII



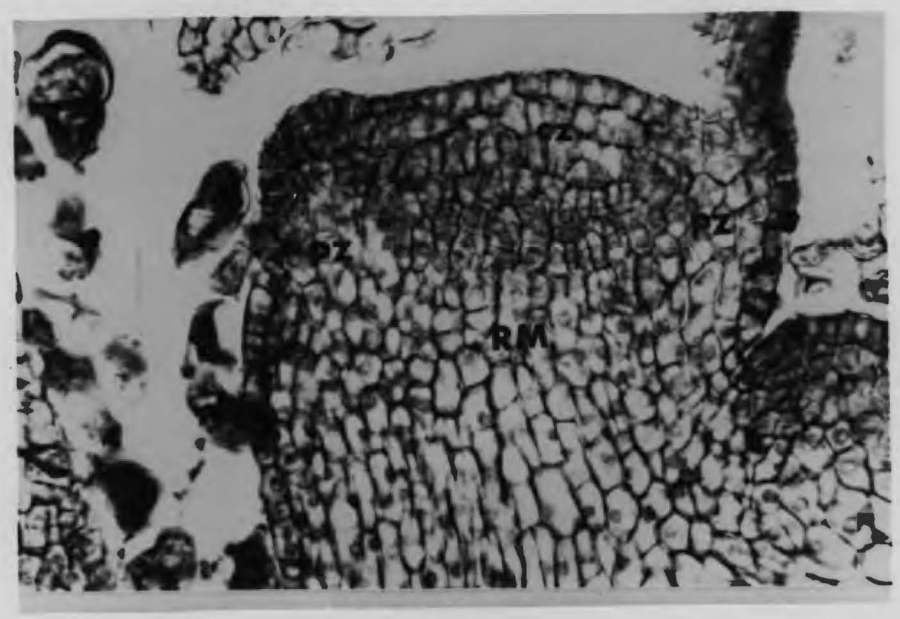
A. Median longitudinal section of the apex of Pharbitis nil, excised and cultured before the inductive period and examined after 4 weeks of in vitro growth. Note the central, peripheral and rib meristem zones. The apex has been reduced in width compared to that of an apex from a 4-day old vegetative seedling (Plate V B) (x 400).

B. Median longitudinal section of the apex of Pharbitis nil, excised and cultured 6 hours after the end of the dark period and examined after 4 weeks of growth. The apex shows the cup shaped central zone, peripheral and rib meristem, characteristic of the vegetative shoot apex. The apex is low and narrow (x 400).

PLATE XXIV



A

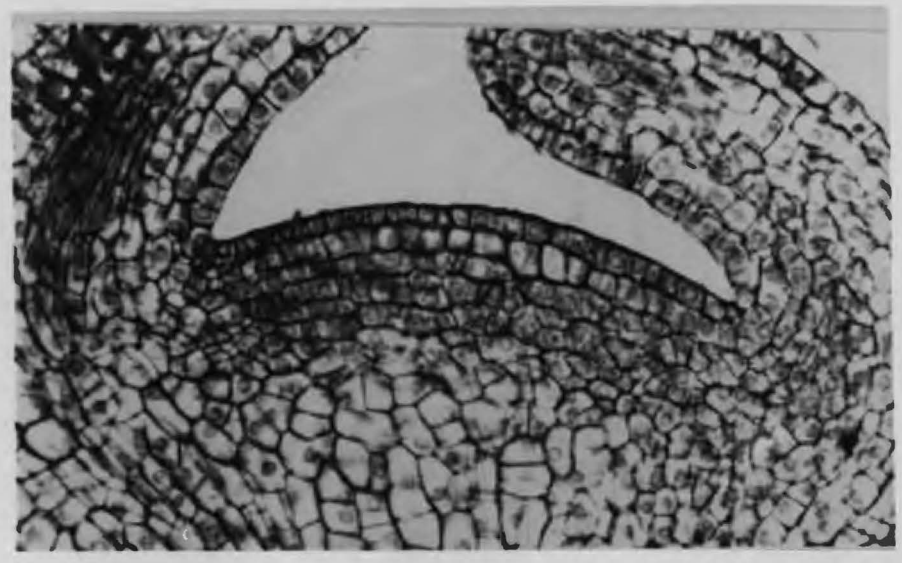


B

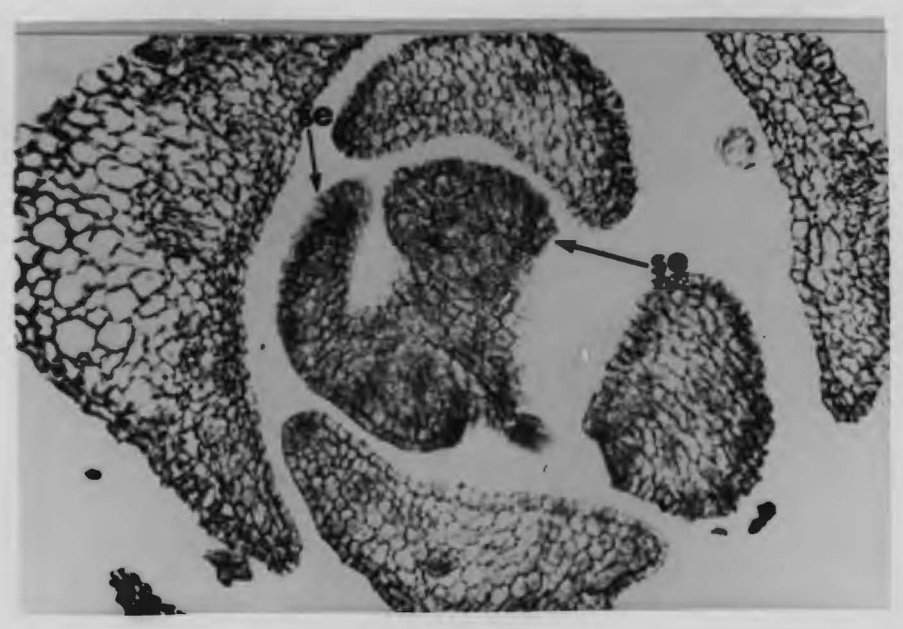
A. Median longitudinal section of the apex of Pharbitis nil, excised and cultured in vitro 12 hours after the end of the dark period and examined after 4 weeks of growth. The apex has enlarged laterally and appears as a low broad dome. The zonation pattern is diffuse (x 400).

B. Transverse section of the floral apex of Pharbitis nil, excised and cultured in vitro 1 day after the end of the dark period and examined after 4 weeks of growth. The apex shows initiation of the sepal primordia (x 260).

PLATE XXV



A

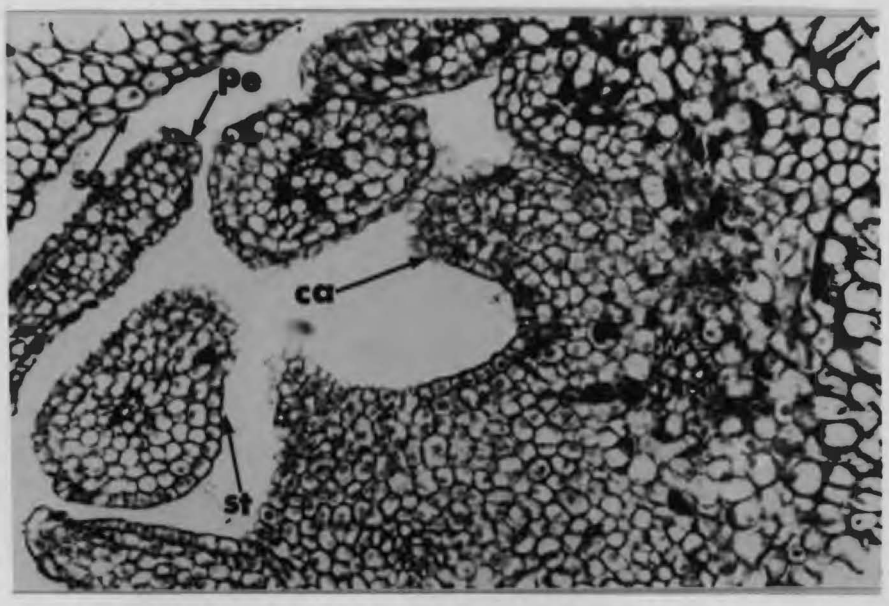


B

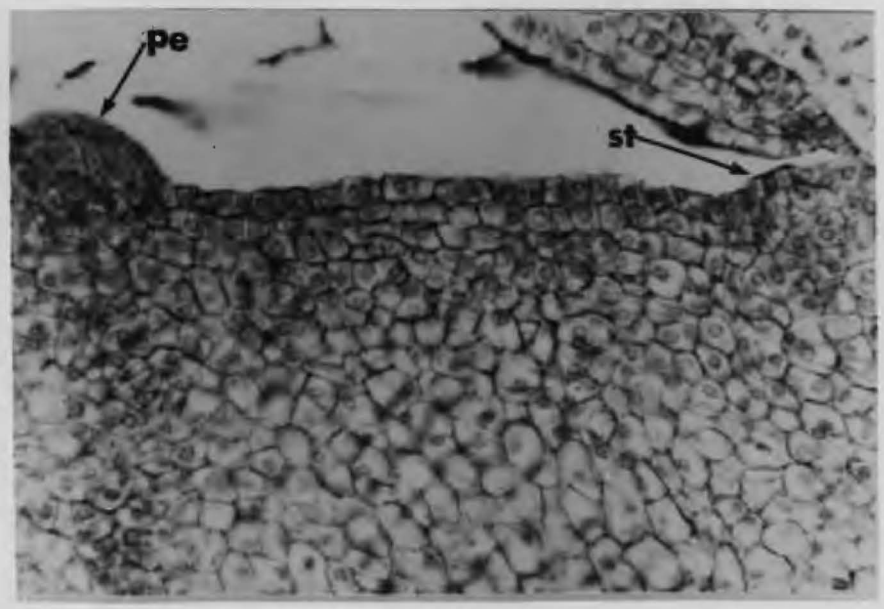
A. Transverse section of the floral apex of Pharbitis nil, excised and cultured 2 days after the end of the dark period and examined after 4 weeks of in vitro growth. The apex shows development of sepals, petals, stamens and carpels (x 330).

B. Median longitudinal section of the apex of Pharbitis nil, excised and cultured 3 days after the end of the dark period and examined 2 weeks after culturing. The apex shows the development of stamen and petal primordia (x 570).

PLATE XXVI



A

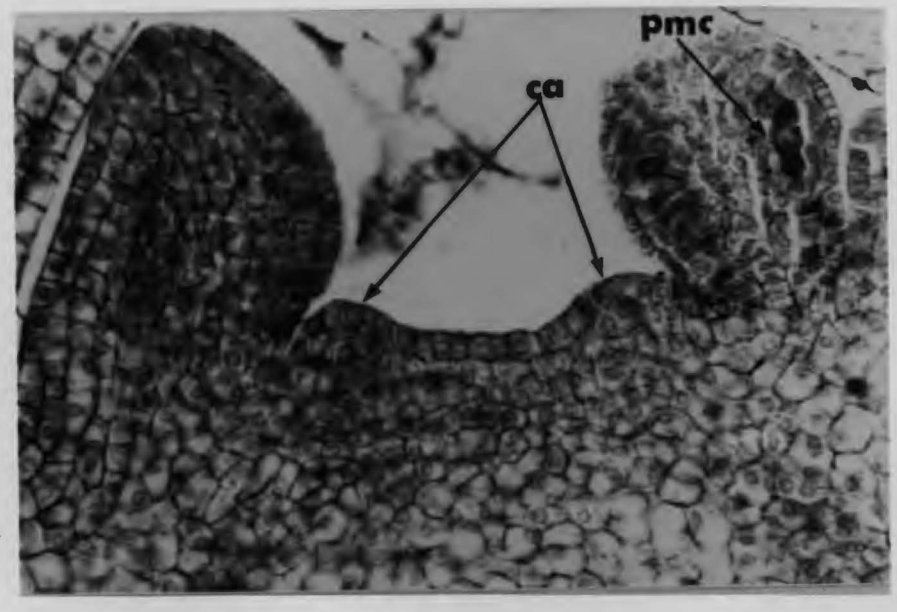


B

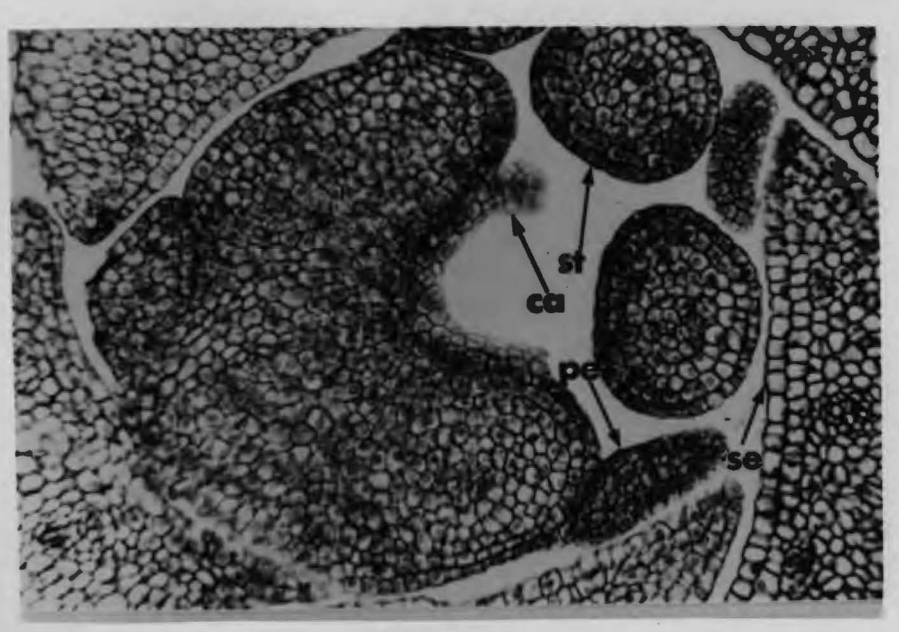
A. Median longitudinal section of the apex of Pharbitis nil, excised and cultured 3 days after the end of the dark period and examined 4 weeks after culturing. Note the development of carpel primordia and microspore mother cells in the stamens (x 440).

B. Transverse section of the floral apex of Pharbitis nil excised and cultured 5 days after the end of the dark period and examined 2 weeks after culturing. The apex shows development of sepals, petals, stamens, and initiation of the carpel primordia (x 260).

PLATE XXVII



A



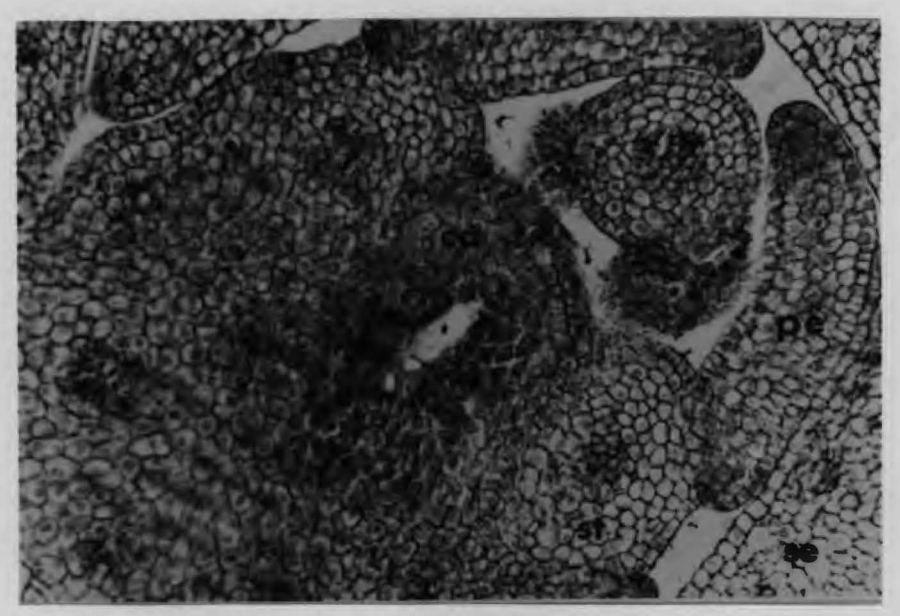
B

Transverse section of the floral apex of Pharbitis nil, excised and cultured 5 days after the end of the dark period and examined after 4 weeks of in vitro growth.

A. The apex shows a ring formed of carpels; the petals are free at the margins (x 270).

B. A section taken at the anther level showing the microspore mother cell groups and a surrounding tapetal layer (x 260).

PLATE XXVIII



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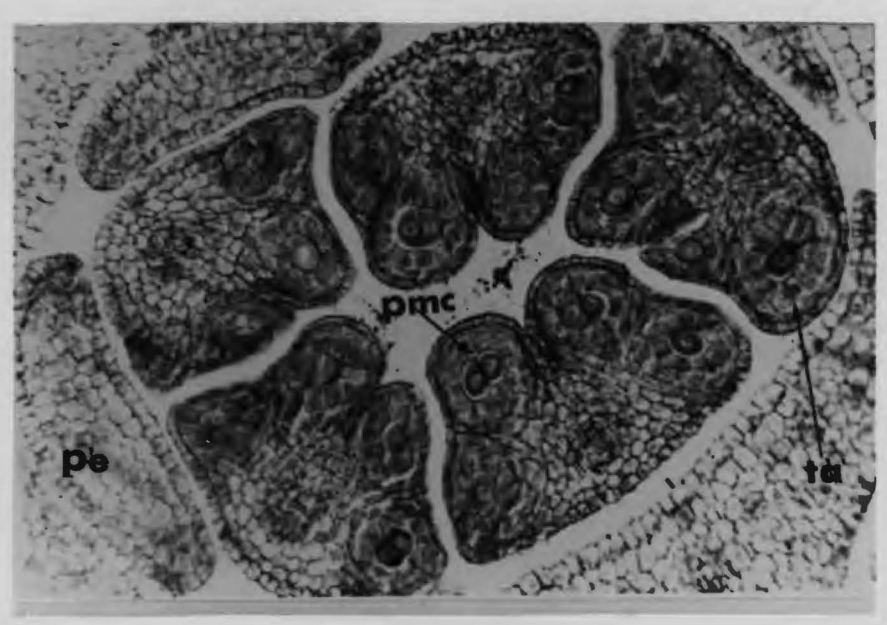
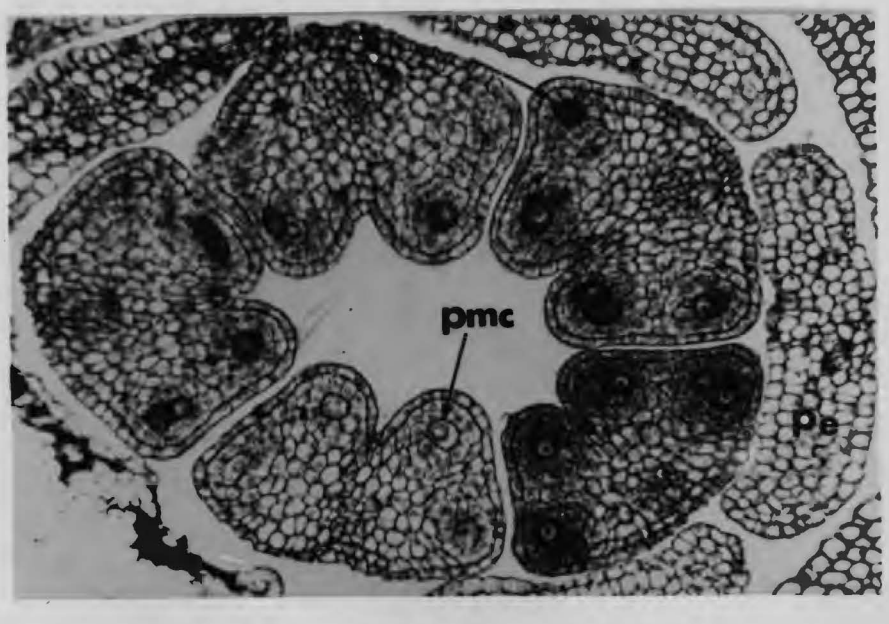


B

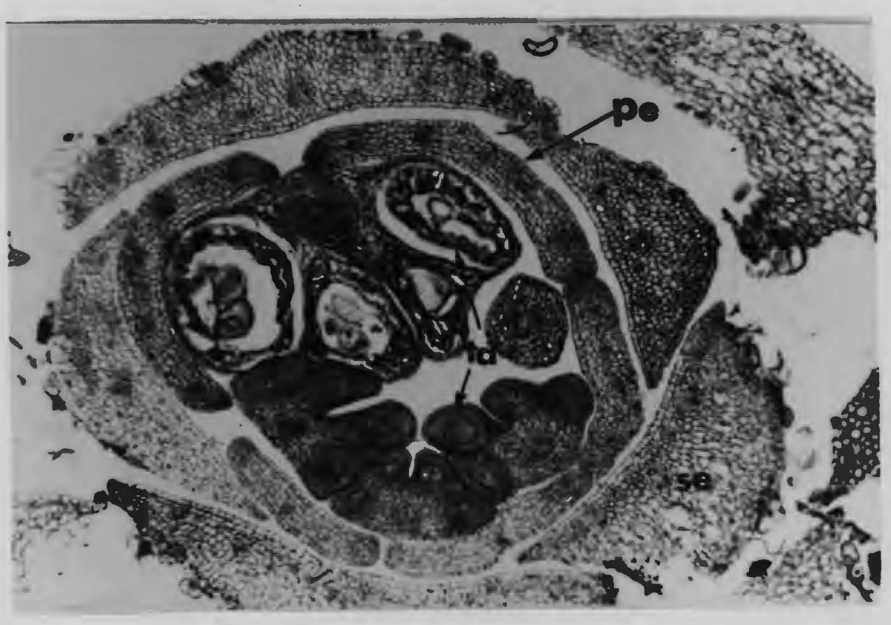
A. Transverse section of the apex of Pharbitis nil excised and cultured 7 days after the end of the dark period. After 2 weeks of in vitro growth, the apex shows 4 microspore mother cell groups in the stamens, and free petals (x 280).

B. Transverse section of the apex of Pharbitis nil, excised and cultured 7 days after the end of the dark period and examined 4 weeks after in vitro growth. The apex shows fusion of the petal margins. A few of the stamens have a well developed tapetal layer while in the others the inner layer had disintegrated (x 270).

PLATE XXIX



A



B

Medium longitudinal section of the apex of Pharbitis nil excised and cultured 8 days after the end of the dark period. After 4 weeks of in vitro growth, the apex shows initiation of the ovules and development of the carpels (x 210).

PLATE XXX