Ovary development in cotton: (Gossypium hirsutum L.)

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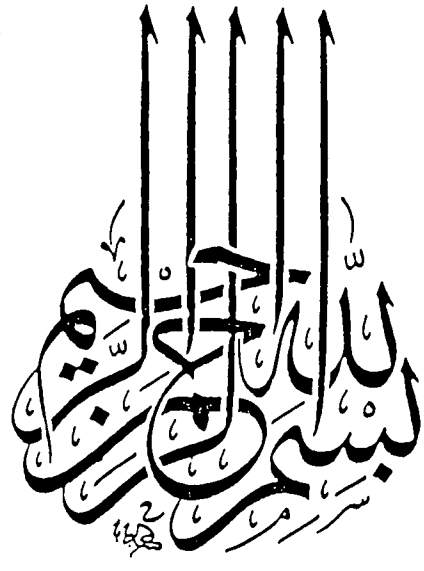

In the name of Allah (God),
Most Gracious, Most Merciful.

Ovary development in cotton
(*Gossypium hirsutum* L.)

by

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Declaration

The work in this thesis has not been submitted elsewhere for any other degree or qualification, unless otherwise referenced, it is the author's own work.

Dedicated to my
late father Noor Muhammad
and late mother Shah Khatoon

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Abstract

A histological study of developing cotton ovary has been carried out, examining differentiation in the major tissues in successive stages before and after fertilization. Histochemistry, immunocytochemistry and *in situ* hybridization have been used for localization of cell wall components and enzymes

The results show that the young ovary produces four regions of meristematic tissue, which enclose the locular areas and give rise to the ovules. The ovules form on the placentae as rounded masses of meristematic cells from which develop the outer and inner integuments and the nucellus. In early stages the outer integument develops more rapidly than the inner integument and nucellus. Cotton fibres develop from the outer epidermis of outer integument. The lint fibres appear on the surface of the ovule on the day of anthesis, and fuzz fibres appear from seven days after fertilization. The fuzz fibres cells are more vacuolate than lint fibres in their initial stages. At maturity the lint fibres become like twisted ribbon but fuzz fibres did not become twisted.

From the early zygote stage distinctive patterns of cell divisions were found in embryo and indicate that changes in group of cells undergoing mitosis are of fundamental importance in the development of form in the embryo.

Dehiscence of the boll in essential for the exposure of the cotton fibre crop. Results indicate that the lignification of endocarp of fruit wall and septum, the development of sclerenchyma fibre in the mesocarp, and the formation of thick-walled valves all play active roles in boll dehiscence.

Histochemical analysis has been used to examine patterns of cellular differentiation within the tissue of the developing fruit. Esterase activity is associated with cell expansion prior to, or in the absence of, subsequent lignification.

A group of monoclonal antibodies have been used for localization of arbinogalactans (AGPs), glycoproteins and pectins in the cell wall. Of those tested several showed uniform binding to most cell types, however JIM14 preferentially stained arbinogalactan (AGP) on the cell wall of the septum of just-fertilized ovary.

Cellulase has been implicated in dehiscence mechanisms. Using *in situ* hybridization, a high level of gene expression for cellulase mRNA is seen in inner epidermis of the outer integument, and the outer epidermis of the inner integument of the ovule before fertilization, and after fertilization.By 15 days after fertilization cellulase mRNA was associated with vascular bundles of fruit wall and fibres. The levels of cellulase mRNA had declined at fruit maturity.

Abbreviation

AGP	arbinogalactan
AF	after fertilization
β	beta
BF	before fertilization
BSA	bovine serum albumin
°C	degrees Celsius
cDNA	complementary DNA
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DNA	deoxyribunucleic acid
g	gram
mg	milligram
µg	microgram
FITC	fluorescein isothioeyanate
mm	millimeter
µm	micrometer
ml	milliliter
µl	microliter
M	molar
mM	millimolar
µM	micromolar
mRNA	messenger ribonucleic acid
PBS	phosphate buffered saline
rmp	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulphate
TBS	Tris buffered saline
TESPA	3-aminopropyl triethoxysilane
totuidene	toluidine
Tris	2-amino-2-(hydroxymethyl)- 1,3-propanediol
tRNA	transfer ribonucleic acid
v/v	volume per volume
UV	ultra violet
w/v	weight per volume

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Where used the bar is given in µms

INTRODUCTION

1.1 THE ORIGIN OF COTTON

The production of cloth has been one of the world's most important industries since prehistoric time. The processes in the modern factory remain basically the same as those used by earlier civilizations. Wool, silk, and flax were used for spinning and weaving, long before cotton became important. Cotton fibre is thought to have been used from about 3500BC, but its use was not recorded until about 500BC in the civilizations of Mesopotamia, Babylon and Egypt (Hutchinson *et al.*1947). Among early references of the twentieth century, Sir George Watt, wrote "The Wild and Cultivated Cotton Plant of The World" (Watt, 1907).

1.2 GEOGRAPHICAL DISTRIBUTION

Cotton is generally regarded as a tropical and sub tropical crop (DeJoode and Wendel 1992; Fryxell, 1979; Fryxell, *et al.* 1992) but at present about two-thirds of the world production comes from north of the latitude 30°, were the three major producers, USA, USSR, and China, are all located. Smaller cotton producing countries like Bulgaria, Russia, and Korea are located north of the 40°, however, at these latitudes the summers are too short for anything but varieties which mature very quickly. About 10 percent of the total crop comes from the southern hemisphere, where ripening occurs between May and July, while 25 percent comes from the northern tropics up to 30°, where it mostly ripens December to February. Outside of the tropical belt, the temperature rather than rainfall determines the cropping cycle. At north of 30°, crops can only be grown in the summer months and the crop ripens between September and November.

More than half of the world's production comes from three major producers, USA, USSR and China. Since 1947 India has produced more than twice as much cotton

crop as its nearest competitors in the old world, Pakistan, Turkey and Egypt, and also in the new world, Brazil, and Mexico. In Africa, Uganda, Tanzania, Zaire, Mozambique and Nigeria are all cotton producers. In the Middle East and Europe, the general pattern among the small producers has been an increase in production up to about 1970, with it remaining fairly steady since then. There has been some decline in Sudan, Iran and Spain, while Israel and Greece have continued to expand production. Considering, production of cotton among the smaller producers in Latin America, the fourfold increase in production in Paraguay in the last 10 years has been outstanding (Munro, 1987). See Table 1

1.3 CULTIVATED COTTON SPECIES

The cultivated cottons are found as four species of *Gossypium,* the diploid old world cottons. *G. arbreum* and *G. herbaceum*, and the tetraploid new world cottons. *G. hirsutum* and the *G. barbadense* (Wendel, *et al.* 1992).

G. arboreum. This species of cotton has 26 chromosomes (2X = 26), and is cultivated in Western India, the coastal areas of Tanzania, in Burma, Bengal, Assam, Indochina, Korea, Pakistan, and Japan. It grows as annual shrubs, and the cotton has good ginning outturn percentage but a coarsness of cotton fiber (poor fibre length and fibre fineness)

G. herbaceum. This annual shrub is found in Iran and surrounding countries like Balochistan, Afghanistan, Russian Turkistan, and Iraq. It spread southward into Pakistan, and North East China. It was spread widely by the Muslim Invasion in the twelfth century (Watt, 1907). It is also found as a perennial form in south-east Africa, Zimbabwe, Mozambique, Swaziland and the Transvaal Lawveld (Watt, 1907).

G. hirsutum. This upland cotton is an annual shrub and has spread from a comparatively small area in Mexico until it now produces well over half of the world's crop. Seed was taken from Mexico to the United States of America in about 1700AD, where forms were selected which were capable of fruiting irrespective of

TABLE. 1

PRODUCTION AND YIELD OF COTTON LINT BY COUNTRIES (5-years average, 1977/78 to 1981/82) (Munro, 1987)

Countries	Production in 1000 metric tons	Yield in Kgs/hec
North America		
Costa Rica	3	428
Cuba	1	268
Dominican Republic	1	207
El Salvador	60	776
Guatemala	132	1187
Haiti	1	268
Honduras	8	739
Jamaica	-	268
Mexico	338	934
Nicaragua	79	659
United States	2902	545
South America		
Argentina	155	290
Bolivia	10	424
Brazil	569	228
Colombia	110	506
Ecuador	12	673
Paraguay	88	289
Peru	91	641
Venezuela	14	407
Western Europe		
Greece	129	853
Italy	-	189
Spain	50	838
Yugoslavia	1	261
Africa		
Angola	1	170
Benin	7	277
Cameroon	26	260
Central Africa Republic	10	3
Egypt	370	903
Ethiopia	25	503
Kenya	9	74
Madagascar	11	584

Malawi	8	226
Mozambique	18	132
South Africa	53	504
Sudan	14	189
Zimbabwe	62	506
Socialist		
Albania	5	277
Bulgaria	9	404
China Peoples Republic	2421	505
Korea Democratic Republic.	1	134
USSR	2737	886
Vietnam	1	91
Asia		
Afghanistan	34	363
Bangladesh	3	294
Burma	17	82
Taiwan	-	537
India	1336	167
Iran	109	459
Israel	77	1358
Korea Republic	2	272
Pakistan	648	319
Sri Lanca	-	258
Syria	134	852
Thailand	47	387
Turkey	503	748
Yemen Arab Republic	9	359
Yemen Peoples Republic	4	281
Oceania		
Australia	83	1198
Indonesia	3	281

day length. They have been introduced successfully into all of the cotton producing countries of the world. The cotton has good qualities in terms of fibre length, fibre strength and fibre fineness.

G. barbadence. This perennial type occurs in Western South America from Colombia, where it was semi-cultivated. Some modern varieties are annual. Finelinted types were introduced into the West Indies in the eighteenth century. It is known for its fine fibre cotton all over the world, it is also called Egyptian cotton, although it is cultivated in Egypt, Mexico, Nigeria, South America, Central America, Africa and India.

1.4 PLANT BIOLOGY

The first comprehensive study of the taxonomy of cotton was *"The wild, and cultivated cotton plants of the world"* (Watt 1907). This is still a standard reference for nineteenth century literature. Current ideas on classification come from Zaitzev (1928); those were elaborated upon by Harland (1932 & 1940) and by Hutchinson and Ghose (1937). Cytological studies by Skovsted (1937) and Webber (1939) led to the concept of genomes and their nomenclature by Beasley (1942). In 1947 Hutchinson, Silow, and Stephen published the "Evolution of *Gossypium"*, reviewing the genus as a whole, and the related genera called *Gossypieae*. They discussed the evolution and current status of the genus with the aim of providing an adequate foundation for the proper planning of breeding work.

Fryxell (1979) published *"The Natural History of the Cotton Tribe"* which will probably supersede Hutchinson *et al.* (1947) as the taxonomic reference work on the *Gossypieae*. It does not attempt, however, to deal with classification within the cultivated species of cotton, which is dealt with in great detail by Hutchinson *et al.*(1947), Silow (1944) and Hutchinson (1950 & 1962).

1.4.1 MORPHOLOGY

The general plánt form of cotton plant is as annual shrubs or small trees, which are distributed in the tropical and subtropical regions of Africa, Asia, Australia, and America.

The whole plant is irregularly dotted with black oil glands, its branches are slightly angled, usually with *monopodial* (vegetative) branches and *sympodial* (fruiting) branches. On its fruiting branches bracteoles are usually foliar and persistent but rarely caducous. At anthesis they are inserted above the nectaries at the top of pedicel. The calyx is cup shaped with truncate 5-toothed or deeply 5-lobed leaves. The petals are 5-imbricate, often large and showy, and white, creamy or yellow in colour, usually with darken coloured spots on the claw. Each flower contains numerous stamens. The lower parts of the filaments are united into a tube which surrounds the ovary, while the upper parts are free and bear unilocular anthers. The stigma and style are clavate although rarely divided at the tip, and the ovary has 3-5 locules. The fruit forms as a dry, brittle, dehiscent capsule, with several seeds per locule. In some wild species the seeds are almost naked but in many varieties they are covered with 1 or 2 coats of unicellular hairs. These hairs are the fibre of the commercial cotton crop (Gore, 1935; Munro, 1987; Prentice, 1972).

1.4.2 CYTOLOGY

The first chromosome counts of cotton were reported by Nikolajewa in (1923) and Zaitzev (1927). New world cultivated cottons have 52 chromosomes. The old world Asiatic cottons were found to posses only half this number, with 26 chromosomes. It was postulated that the basic haploid number (x) for *Gossypium* was 13, the Asiatic cotton with 2x=26 are thus diploid, and the Upland cotton *G. hirsutum*, and Egyptian cotton *G. barbadense* with 4x=52 are tetraploids. A comprehensive survey of various

cotton species was under taken by Skovsted (1935) but within the genus no other chromosome numbers were discovered.

1.5 PLANT STRUCTURE

The cotton plant has a vertical stem carrying two kinds of branches, vegetative branches or monopodia, similar to the main stem in nature. The primary axis continues to grow from the same growing point, developing successive lateral branches. Fruiting branches or sympodia terminate at each node with flowers. The monopodial branches do not bear flowers directly, but produce fruiting branches which bear flowers. The first two nodes carrying the cotyledons are on opposite sides of the stem, at the same level. The main stem produces a series of nodes, each with a leaf subtending two or three dormant buds on the side of the stem, any of which may develop into branches. The flower bud first becomes visible as a small green cones, it is surrounded by three green bracts, or bracteoles, which are joined at their bases. The bracteoles are heart-shaped forming a pointed canopy over the bud, often referred to as the square. At the base of each bracteole it contains nectary glands. The ring of bracts is pushed open by the extending petals on the day the flower opens although the bracts remain attached to the developing fruit until the boll is about to ripen. The cotton boll is a dehisent schizocarp with 3-5 locules or lobes. The fruit usually ripens 50 to 70 days after fertilization, the time varying with the varieties, temperature, and humidity (Gipson, and Ray, 1970). The cotton seed measures about lOx6 mm and has a seed index of about 5-10 grams (wt. per 100 seeds). The seed has a hard seed coat covered by a slightly waxy cuticle. Beneath the epidermal, cells which produce the seed hairs, is a thin outer seed coat, but the two are fused together. The inner seed coat has a layer of palisade cells then a layer of protoplasmic or parenchymatous tissue, which is compressed between the palisade layer and swelling embryo as the seed matures (Balls, 1915). Although the endosperm fills the embryo sac by the third week after anthesis,

largely as undifferentiated protoplasm, the rapid growth of the embryo absorbs and digests the endosperm and fills the whole seed cavity.

The seed hairs are unicellular outgrowths of the epidermis of the seed. In the early stage of development they are thin walled, but cellulose is later deposited within the cuticle. In the ordinary seed hairs, or fuzz, the cellulose deposit builds up until at maturity the lumen is partially obliterated. Dry seed hair on a ripe seed is long and tapered; it is circular in cross-section and filled with cellulose, except for the small central cavity left by the drying up of the cell contents (Hutchinson, *et al.* 1947).

1.6 FERTILIZATION

The general pattern of reproduction in flowering plants was established during the nineteenth century. Pollen contains two vegetative nuclei, that are concerned with production of the tube, and the generative nucleus that gives two male gametes or sperm. Pollen germinates on the stigma, and its tube grows down through the style carrying the male gametes to the ovule. These are discharged from the pollen tube into the embryo sac, where one male gamete nucleus fuses with the egg nucleus to form the zygote (Johansen, 1950). A masterful summary of many details of the structure and development of the pollen, pollen tube, embryo sac and embryogensis, revealed through microscopic and histological techniques, was written by Maheswari, (1950)

1.7 OVARY DEVELOPMENT

According to Doak (1928) and Gore (1932) the pistil consists three to five carpels. The primordial points of the carpels arise from a dome-shaped growing points surrounded by the staminal column, and these ultimately become stigmatic, stylar, and other parts. At first they grow slowly as compared with the other floral parts; but the upward growth of the ovary

results in the projection of the young stylar structures into the staminal tube at an early age.

As the undiverged bases of the carpels increase in size, internal ridges are produced, which develop into the true septa of the ovary. These may be regarded as free inturned margins of the undiverged carpels, which grow centripetally and upwardly until the original cavity is divided in to as many locules or carpels. Continued growth of the reflexed margins of each carpel forms the placentae from which four to six ovules arise. In describing the formation of the placentae Gore (1932) stated that "from the two vertical edges of each septum the rounded placental ridges appear". The edges of these placental masses grow outward at an increasing angle toward the external carpel wall, finally becoming a flattened mass of meristematic tissues.

1.7.1 CAPSULE DEHISCENCE

Baranov and Maltzev (1937), Gubanob (1966), Joshi wadhwani and Johri (1967) and Morris (1964a, 1964b), have described boll dehiscence in general terms and Ashworth and Hine (1971) have reviewed the earlier literature. Joshi *et. al.* (1967) reported that the dehiscence zone consists of 4 to 5 layers of small parenchymatous cells and pericarp formed of thick-walled fibrous cells on both side of sutural area. Morris (1964b) indicated that dehiscence normally occur when the capsule is physiologically mature. The time and point of initial sutural dehiscence was reported by Baehr and Pinkard (1970); dehiscence begins at about 10 to 15 days after fertilization with the progressive schizogenous separation of the sutural area parenchyma located between the sclerenchyma fibres bundles. Splitting is produced from sutural area to the internal and external limits of the pericarp until 40-45 days of the fertilization, when the dehiscence is generally completed. The physical break down of the cuticle, covering the sutural area occurs over a period of several days, and the time at which the first external microbreak occurs depends on the weather, variety, and probably other factors.

According to Pickard and Baehr (1973) epidermal and subepidermal layers of pericarp, which are covered by thick cuticle, and the sutural area of dehiscence zone, consist of typical parenchyma cells with thin cell walls. The sutural area of the dehiscence zone forms a vertical column from epidermis to the seed chamber (locule) of the cotton boll.

1.7.2 OVULE DEVELOPMENT

Gore (1932) reported that the primodia of the ovule arise on the placentae as rounded masses of meristematic cells at about the time that the locules becomes closed. During the development of the nucellus, a basal fold appears growing upward to form the inner integument; and as the apex of the nucellus begins to curve, a second fold initiates the outer integument, which soon overgrows the inner one. Both continue their growth until they cover the nucellus, except for a small micropylar opening. Gore (1932) and Linthac and Jensen (1974) reported that at the time of flowering the inner integument of the ovule produced 10 to 12 cell layers; and outer integument layer produced 6 to 8 cell layers at the time of pollination. Joshi *et al.* (1967) reported that one or two days before of the anthesis the epidermis of outer integument produced stomata and guard cells at the chalazal end of the ovule. Stewart (1975) and Ryser (1992) reported that the epidermal layer of the ovule consists of three types cells; i.e. cotton fibre cells, guard cells of stomata and ordinary epidermal cells, fibre cells and guard cells usually occurring at the chalazal end of the ovule. Joshi *et al.* (1967) and Pollock and Jensen (1964) reported that the nucellus produced ovullar tissue, nucellar tissue and endosperm at later stage. During the late stage of the embryo development (at the time of maturity) the embryo absorbs most of the endosperm cell reserves and nearly crushes the nucellus, with remaining endosperm cells turned into storage reserve cells at senescence (Joshi, *et al.* 1967; Werker, 1980/1981).

According to Ryser *et al.* (1988) at the time of maturity (about 69 days after anthesis) the outer integument consists of mature fibre, the inner and outer epidermal layers and mesophyll cells; and inner integument consists of palisade cells, mesophyll, and the fringe layer.

1.7.3 EMBRYO DEVELOPMENT

Jensen and Fisher (1968) described how the two synergids are fully developed before anthesis. After pollination both synergids begin to degenerate, and pollen tube grows into the degenerating synergids through the filiform apparatus. Balls (1905), Gore (1932) and Jensen (1965a) reported that the pollen tube enters into the embryo sac and destroys one of the synergids and discharges the sperm into the embryo sac. Gore (1932), Jensen (1963, 1964, & 1965b) and Shulz and Jensen (1968) reported that the egg of cotton is a large and highly vacuolate cell and contains two polar nuclei. Pollock and Jensen (1964) reported that the fertilization in *Gossypium* usually take place after 20-30 hours of pollination, and that the zygote is formed approximately 14 hours after fertilization, although the zygote does not divide before 56-72 hours following of the pollination. According to Reeves and Beasly (1935) the first division of the zygote usually occurs during the second day after pollination and a short suspensor is formed at on early development stage. Balls (1905) however, disagreed with the idea that the embryo produces a suspensor during its early stage. Reeves and Beasly (1935) and Pollock and Jensen (1964) observed early heart-shape, late heart-shape, torpedo shape and mature embryo at its late development stages.

1.7.4 FIBRE DEVELOPMENT

Balls (1905), Hawkins and Serviss (1930), Gulati (1930), Beasly (1975), Stewart (1975), and Pandey (1987) observed the epidermal cells nearest the chalazal end of the ovule are the first to develop fibre and produce the longest fibres; those at the

micropylar end develops fibre later, or may fail to develop at all. Ryser (1992) distinguished between fibres and ordinary epidermal cells at the early stage. Aiyanger (1951), Joshi *et al.* (1967), and Peeters *et al.* (1987) reported that on the day of flowering, the individual epidermal cells that are to become fibres begin to swell slightly. At the end of the twelfth hour they have a bulbous appearance, and becomes more vacuolate. After twenty four hours, the tubular fibre cells are much more elongated and have usually attained their full diameter. During the second day there is a differentiation of the fibres, some becoming longer with pointed tips, while others are short and rounded. After three days lint fibres appear definitely tapered. Lang (1938) and Basra and Malik. (1984) reported that the short fibre called fuzz remains adhered to the seed coat after ginning. The longer fibre, collected by the ginning process is called lint fibre or commercial fibre. The lint fibre grows to approximately a quarter of the total length of the fibre within the first seven days and usually attains its full length by the end of the 15-25 days of growth. Beasly (1975) and Peeters *et al.* (1987) reported that the elongation of fibre ceases by 24-28 days after anthesis and by 45-70 days fibre completes its secondary thickening and becomes spiral and twisted. Ryser (1992) reported that the fibre develops its final elongation within 20 days of the anthesis, and secondary thickening completes within 48-69 days after anthesis. Joshi *et al.* (1967), Beasly (1977) and Ryser (1985) reported that the "lint fibre" and "fuzz fibre" originate as epidermal outgrowths of the ovule, and the fibre that initiates its elongation on the day of anthesis is destined to become "lint fibre", and those epidermal cells initiate its elongation after 4-12 days of the anthesis they develop only "fuzz" fibre. The period of elongation apparently depending upon several factors like environmental conditions and variety.

1.8 AIMS OF THE PROJECT

Cotton production in the tropical and subtropical areas of the world is a major factor in the economy of many countries, including Pakistan, India, Egypt and other small countries whose total production levels are smaller but still significant in national economic terms. The world production of cotton also plays a major role in the economy of the developed countries, including the United States of America.

The improvement of cotton production needs continuous efforts; this project will focus attention on the development of the ovary before and after fertilization. The primary aim will be to describe the cytology of the development of the endocarp and dehiscence zone and relate this to the /mechanisms of boll opening. Histochemical analyses will be used to identify key enzymes associated with differentiation of specific cell types and *in situ* hybridization will be used to identify associated patterns of differential gene expression.

A broad range of histological, cytochemical and molecular-histochemical techniques will be applied with the aim of identifying those techniques which may be potential aids to the classical techniques associated with plant breeding for crop improvement.

MATERIALS AND METHODS

2.1 PLANT MATERIAL

1.1 Cotton seed

Cotton seed was obtained with kind courtesy of Cotton Botanist Agriculture Research Institute Tando Jam Sindh, Pakistan.

2.1.2 Plant growth

Cotton plants (*Gossypium hirsutum* L.) were grown in I.C.I. compost in pots 28 × 24 cms under controlled temperature of 28°C. Daylight was supplemented by 14-16 hours with sodium (lamps 400 W). Plants were grown in the glass house at the Botanical Garden, University of Durham, England (UK), and were regularly watered as per requirement by sprinkler.

2.1.3 Types of ovaries

Two types of ovaries were collected for study

(1) Pre-Fertilized ovaries, whose maturity was graded by size (8, 10, 15, 18, 20 mms).

(2) Pre-and Post-Fertilized ovaries, whose maturity was graded by hours and days.

(a) 24 hours before fertitilization, just fertilization, 24 hours and 48 hours after fertilization.

(b) 7-days, 15-days, 21-days, 30-days, 45-days after fertilization.

2.1.4 Size of tissues samples

Prior to fixation and subsequent processing ovaries were cut into transverse sections with slices obtained from the middle area of each ovary

(1) 2.mm thick sections were used for processing to wax embedded tissue blocks

(2) 1.mm thick section were used for processing to L.R. White resin blocks.

2.2 FIXATION

Two fixatives have been used; (a) standard fixative for tissue, used in cytological studies, and (b) a milder fixation for tissue, used for immunological studies

2.2.1 Standard aldehyde fixative

Tissues were immediately placed in a freshly-prepared aldehyde fixative solution containing 2.5% (v/v) gluteraldehyde, 1.25% (w/v) formaldehyde and 0.05M phosphate buffer pH 7.0. Tissues were fixed for 24 hours at 20°C.

2.2.2 Immunological fixative

Tissues were placed immediately after cutting into a freshly-prepared solution containing 3% (w/v) paraformaldehyde 1.25% (v/v) gluteraldehyde and 0.05M phosphate buffer pH 7.0 Fixation was carried out for 24 hours at room temperature.

2.3 DEHYDRATION

Dehydration was carried out with graded aqueous ethanol series.

The time and series is given below.

Solutions	12.5%	25%	50%	75%	95%	100%	100%
Time minutes	90	90	90	90	90	90	90

Dehydration was carried out at room temperature on a 45° rotating platform at 2.rmp, and solutions were changed every 45 minutes

2.4 EMBEDDING

2.4.1 Wax embedding

Dehydrated tissues were placed in 50:50 Histoclear : ethanol overnight at room temperature on a 45° rotating platform. The samples were then placed in fresh

Histoclear for 16 hours, the solution was changed every 4 hours. The samples were then placed in 50:50 Paraplast wax : Histoclear overnight at 60°C in a oven. The 50:50 solution were changed to Paraplast wax for 48 hours at 60°C, and wax was changed twice a day. The tissues were embedded in Paraplast wax.

2.4.2 L. R. White

Dehydrated tissues were placed in mixture of equal volumes of resin and ethanol, and infiltrated overnight at room temperature on 45° rotating platform at 2.r.p.m. The 50%:50% mixture was replaced by 100% resin at room temperature until the tissues were judged to be fully infiltrated. The resin was changed twice a day. Tissues were embedded in resin which was polymerized at 65°C for 18 hours.

2.5 SLIDE PREPARATION

Twin frosted microscope slides (76/26 0.8-1.O mm) were washed in detergent, and then rinsed thoroughly with distilled water. Slides were then dipped for 15 seconds in a 2% solution of aminopropyl triethoxysilane (TESPA) in acetone and, rinsed for 60 seconds, twice, in acetone and rinsed for 60 seconds in distilled water. The slides were then air dried in a dust free environment in the oven.

2.6 SECTIONING

2.6.1 Wax

Wax-embedded tissues were cut at 10μm on a Leitz 1512 microtome. The sections were floated on distilled water at 50°C in a water bath and picked up on TESPA coated microscopic slides. Slides were allowed to dry overnight at 40°C on a hot plate.

2.6.2 L. R. White

L. R. White-embedded tissues were cut to 1µm on a Sorvall M T 2-B Ultramicrotome using glass knives. Sections were collected on TESPA coated slides and allowed to dry at 60°C on a hot plate for two minutes to ensure adhesion of the tissues to the microscope slides

Note. Serial sections were obtained for embryogensis.

2.6.3 Cryostat sectioning

The tissues were first treated in *iso*-Pentane (2- methyl butane) for 30 seconds to one minute, in liquid Nitrogen at -196°C. Then the tissues were transfer into liquid Nitrogen for 2-3 minutes or until properly frozen. The tissues were fixed to a specimen holder by cryomatrix liquid until the tissues were attached fixed and the cryomatrix liquid turned white. Then sections were cut at 10 µm thickness using a Bright OTF Cryostal microtome.

2.7 DEWAXING

Wax sections were placed in Histoclear for a minimum of two minutes, rinsed twice in 100% ethanol, washed thoroughly with tap water, and then left to air dry

2.8 HISTO- AND CYTOCHEMISTRY

2.8.1 ACRIDINE ORANGE

L. R. White and wax sections were stained with freshly filtered 0.01% acridine orange and incubated for 2-3 minutes at room temperature. Sections were then rinsed in distilled water, left to dry and mounted with D. P. X.

2.8.1.2 Calcofluor

Razor cut or cryostat section were incubated in fresh prepared 0.1% aqueous solution of Calcofluor for 10 minutes, then examined by fluorescence microscopy (Nikon TMD) using ultraviolet light with a UV filter.

2.8.2 General esterase

Solution preparation

Substrate solution: 5 mg Naphthol AS-D was dissolved in 0.5 ml dimethylformamide (DMF), 25 mls 0.2M Tris, pH 6.5, and 25 mls distilled water were added. 30 mg Fast Blue BB were than added, The mixture was shacked well and filtered into dark bottle.

Control solution: As above, without Naphthol AS-D acetate.

Frozen (10 μm) sections were washed in distilled water to remove the embedding medium. They were then rinsed in 0.2M Tris/NaCl pH 6.5 for 10 minutes, and incubated in substrate or control solution, in the dark, at 37°C until a blue colour developed in the test and was samples judged to be satisfactory. Section were rinsed in Tris buffer then mount in Citifluor. This method is based on that reported by Gahan (1981).

2.8.4 Toluidene blue

L. R. White and wax sections were stained with freshly prepared 0.1% toluidene blue in 1.0% Boric acid for 2-3 minutes, then rinsed in distilled water, air dried at room temperature and then mounted in D. P. X

2.9 IMMUNOCYTOCHEMISTRY

2.9.1 General immunocytochemical protocol L.R. White embedded (1μm) sections were rehydrated in 0.05% (w/v) BSA (bovine serum albumin) in TBS (Tris buffered saline) pH 8 0 for 30 minutes. Sections were incubated overnight at RT in primary antibodies using TBS buffered BSA as the diluent. Section were rinsed in buffered BSA for 30 minutes (fresh BSA was added every 10 minutes). Then sections were incubated in with the secondary antibodies, using buffered 2% BSA as the diluent, for 2 hours at RT, in the dark. Sections were rinsed in buffered BSA for 30 minutes (fresh BSA was added every 10 minutes).

2.9.2 Fluorescent detection

For fluorescent detection of bound primary antibodies appropriate secondary antibodies conjugated to FITC were used. Following the instruction of the commercial supplies the secondary conjugates were diluted in buffered 1% BSA and used for incubation, at RT, for 2 hours. Then sections were rinsed in BSA for 30 minutes (fresh BSA was added every 10 minutes) then mounted in Citifuor, and viewed under fluorescence light with blue filter.

The JIM monoclonal series (a gift from Dr. P. Knox and Professor K. Roberts, Norwich) were raised in rat, and secondary conjugate used goat anti-rat FITC. Polyclonal antibodies raised in rabbit were located using goat and rabbit-FITC.

2.10 *IN-SITU* HYBRIDIZATION

Wax embedded 10μm sections were dewaxed in Histoclear and rehydrated in 100%, 75% and 50% ethanol then DEPC treated water. DEPC water was prepared by 2%

(w/v) DEPC in distilled water, this was shaken vigorously, left overnight at room temperature and then autoclave at 121ºC, for 20 mins)

Hybridization buffer preparation

50% deionised formamide, 4X SSC, 1X Denhardts, 500 µg/ml sonicated denatured salmon sperm DNA, 250 µg/ml yeast tRNA, 10% dextran sulphate.

Probe preparation

The DIG (digoxigenin) probe was denatured by incubating for 5 minutes at 95°C and cooled in ice / NaCl bath. The denatured probe was added to the hybridization buffer (have given above) to final concentration of 1 µg/ml.

Sections were incubated over night in probe under parafilm cover slips in a humid chamber at 42°C. Cover slips were removed by dipping the slides in a solution of 4X SSC, 50% formamide at 42°C. Sections were rinsed twice in buffer solution containing of 2X SSC, 0.1% SDS at RT for 15 minutes each then twice at 42°C for 15 minutes each. Sections were dehydrated in a graded ethanol series buffered containing 300mM ammonium acetate then air dried.

Sections were incubated in 2% sheep serum in buffer (100mM Tris pH 7.5, 150mM NaCl) for 30 minutes at RT. Then sections incubated in 1:500 dilution of alkaline phosphatase-conjugated, polyclonal sheep anti-digoxygenin antibody in buffer of 100mM Tris pH 7.5, 150mM NaCl for 2 hours at RT then rinsed in buffer (100mM Tris, 150mM NaCl) for 1 hour (fresh buffer was added every 15 mins).

Sections were incubated in darkness at RT in freshly prepared substrate solution containing 0.5mM naphthol AS-MX phosphate, 2.0mM fast red TR in Tris buffer pH 8.0. Sections were monitored until colour had developed sufficiently. the sections were rinsed in distilled water, air dried and mounted in D.P.X.

2.11 PHOTOGRAPHY

Light microscopy of sections was done using a Nikon Diaphot or Optiphot photo microscope equipped with conventional bright field and fluorescence illumination. Monochrome photographs were taken on Technical Panchromatic 2415 emulsions, and colour photograph on Fuji colour (RH 100 or 400), Agfa 50 or Konica 200 at different magnifications.

RESULTS

3.1 STRUCTURE AND MORPHOLOGY

Light microscopy studies of the histological development of cotton ovary have been undertaken at sequential stages of development. Particular attention was paid to the carpels, and to seed development (i.e. ovule, seed coat and embryo), septa position, dehiscence mechanism and fibre development processes. Histochemical and cytochemical studies were also carried out to identify key enzymes associated with differentiation of specific cell types, and *in situ* hybridisation was used to identify associated patterns of differential gene expression. Tissue sections were cut from either L. R. White resin-embedded material, sectioned at 1μm, or wax-embedded material sectioned at 10μm.

3.1.1 GROSS MORPHOLOGY

The plant height was recorded at 1 meter (average of 5 plants) at maturity. The plants produced an average of 3 monopodial and 18 sympodial branches (average of 5 plants). The first square bud was observed 35 days after planting. The buds appeared as extra-axillary, axillary, terminal and solitary, on the fruiting branches (Fig. 1a).

Initially bracts were entire but later they became serrated with 9-10 teeth, and formed a pointed canopy on the developing bud. The bracts had rough and hairy outer surfaces and smooth inner surfaces. The canopy of bracts opened early on the day of flowering, and remained attached until boll ripening (Fig. 2, d2).

The gamosepalous calyx was found to be persistent, consisting of five unequal lobes of mostly undiverged sepals; it formed a shallow cup around the base of the petals. After the petals and staminal column fall of, the calyx covers the base of young boll loosely (Fig. 2.b1), but as the boll develops it adheres tightly at the base. The calyx remains attached to the base of the boll until boll opening (Fig. 2. b3).

The first flower opened fully 52 days after planting (Fig. 1b), and exposed the creamy-white corolla, anthers with staminal column, and the stigma. The tubular corolla consisted of five obcordate petals with lobes which were alternated with the calyx, and each glandular petal overlapped the next in series in a convoluted manner.

Figure 1

Floral development in ovary before and after fertilization.
(a) Square bud 24 hours before fertilization (real size).
(b) Flower on the day of fertilization (real size).
(c) Flower 24 hours after fertilization (" ").
(d) Flower 48 hours after fertilization (" ").

a, anther; br, bracts; co, corolla; st, staminal column; stig, stigma.

Figure 1

Figure 2

Developing stages of ovary before and after fertilization.

(a) Pre-fertilization ovaries (square buds).
1 (8 mm), 2 (15 mm) and 3 (20 mm).
(b) Fertilized ovaries (along with calyx)
1 (3 days), 2 (7 days) and (21 days).
(c) Fertilized ovaries (along with bracts)
1 (7 days), 2 (15 days) and 3 (21 days)
(d) Mature stages of the ovary (before and after dehiscence)
1 (before opening), 2 (at the time of opening) and 3 (fully opened)

br, bracts; c, calyx; d, dehiscence area; f, fibre.

Figure 2

The outer base of the corolla was covered by the calyx, and inside it was joined with the staminal column. 24 hours after anthesis the colour of corolla turned from creamy white to light pink (Fig. 1c), after 48 hours it turned from light pink to dark pink or red (Fig. 1d) and after 72 hours it withered and fell off along with staminal column and stigma.

The staminal column produced 8-10 vertical rows of stamens with double-loculated anthers. The young anther developed on the small filament, initially capitate and finally becoming reniform. Numerous stamens formed the staminal column which completely surrounded the pistil (ovary) except for a short portion of style and stigma (Fig. 1b). The bilocular anthers dehisced along a single line running over the crest of the anthers, liberating large, spinose pollen grains. The staminal column fell off along with corolla and stigma 3 days after anthesis (Fig. 2. b1).

The young boll was exposed after the staminal column dropped off (Fig. 2.b1). It developed quickly during the first three weeks and was observed as green, thick and leathery structure with an oval shape (Fig. 2. d1). As bolls reached maturity the colour changed from green to yellow, and they started to dehisce, from the chalazal end, 64 days after anthesis (Fig. 2. d2). This exposed the seed cotton (commercial fibre) (Fig. 2 d3). The ovary was observed to be formed with three to five locules (Fig. 3. a2, b2, and c2).

3.1.2 FLORAL DEVELOPMENT

Histological studies of the development of cotton flower have been carried out. The youngest stage examined was taken when the ovary was about 8 mm old (along with floral part) and covered by the staminal column, stamens, corolla and calyx (Fig. 4a).

A gamosepalous calyx forms a ring surrounding the rest of the flower; it consists of about 7 layers of parenchymatous cells which possess different size and shape. The outer epidermis was found as a single row of mostly isodiametric cells, while the inner epidermis of calyx consists of a row of different type of cells and is covered by thick cuticle. The outer epidermis of calyx produced multicellular hairs (Fig. 4b). The vascular bundles, containing xylem, and also resin ducts were observed within the calyx wall (Fig. 4a).

The corolla consists of five obcordate petals with overlapping lobes (Fig. 4a). The longer half of the petal carried stellate hairs on both sides of corolla wall (Fig. 4b),

Figure 3

Ovaries with different number of carpels (locules).
Transverse sections were stained with toluidene blue and photographed under bright field illumination.

(a1) Three loculary ovary.
(b1) Four loculary ovary.
(c1) Five loculary ovary.

(a2) Three loculary ovary
(b2) Four loculary ovary
(c2) Five loculary ovary

d, dehiscence area; l, locules or locular area; ov, ovules; s septum.

In all Figures the bar in given in μm

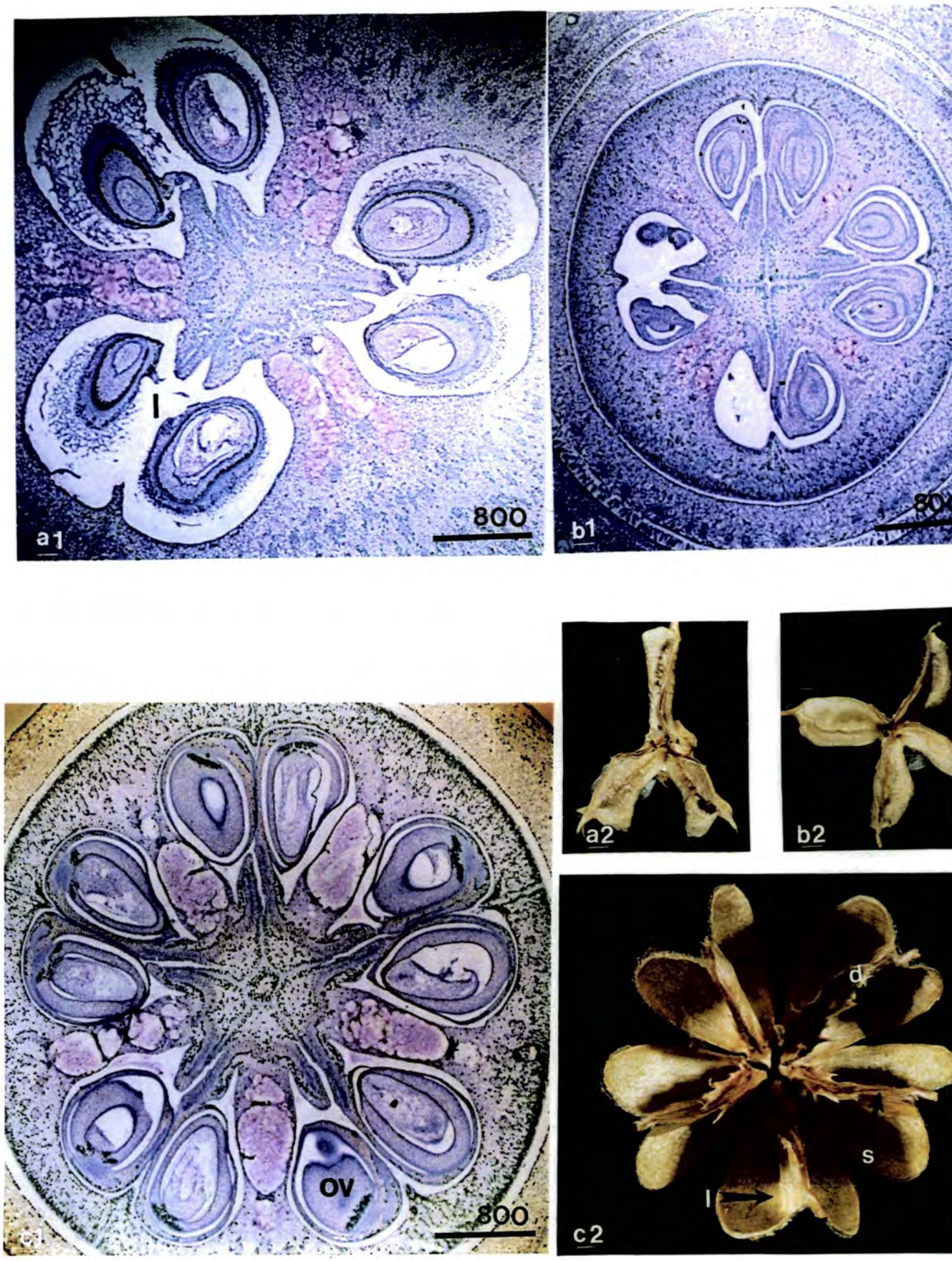


Figure 3

Figure 4

Histological development of floral parts.
Transverse sections were stained with toluidene blue and photographed under bright field illumination.

(a) Calyx, corolla, staminal column and stamens
(b) Calyx and corolla.
(c) Stamens.

a, anther; c, calyx; co, corolla; fl, filament; mh, mullticellular hairs; p, pollen grains; s, stamens; st, staminal column; sh, stellate hairs; r, resin ducts; vb, vascular bundles.

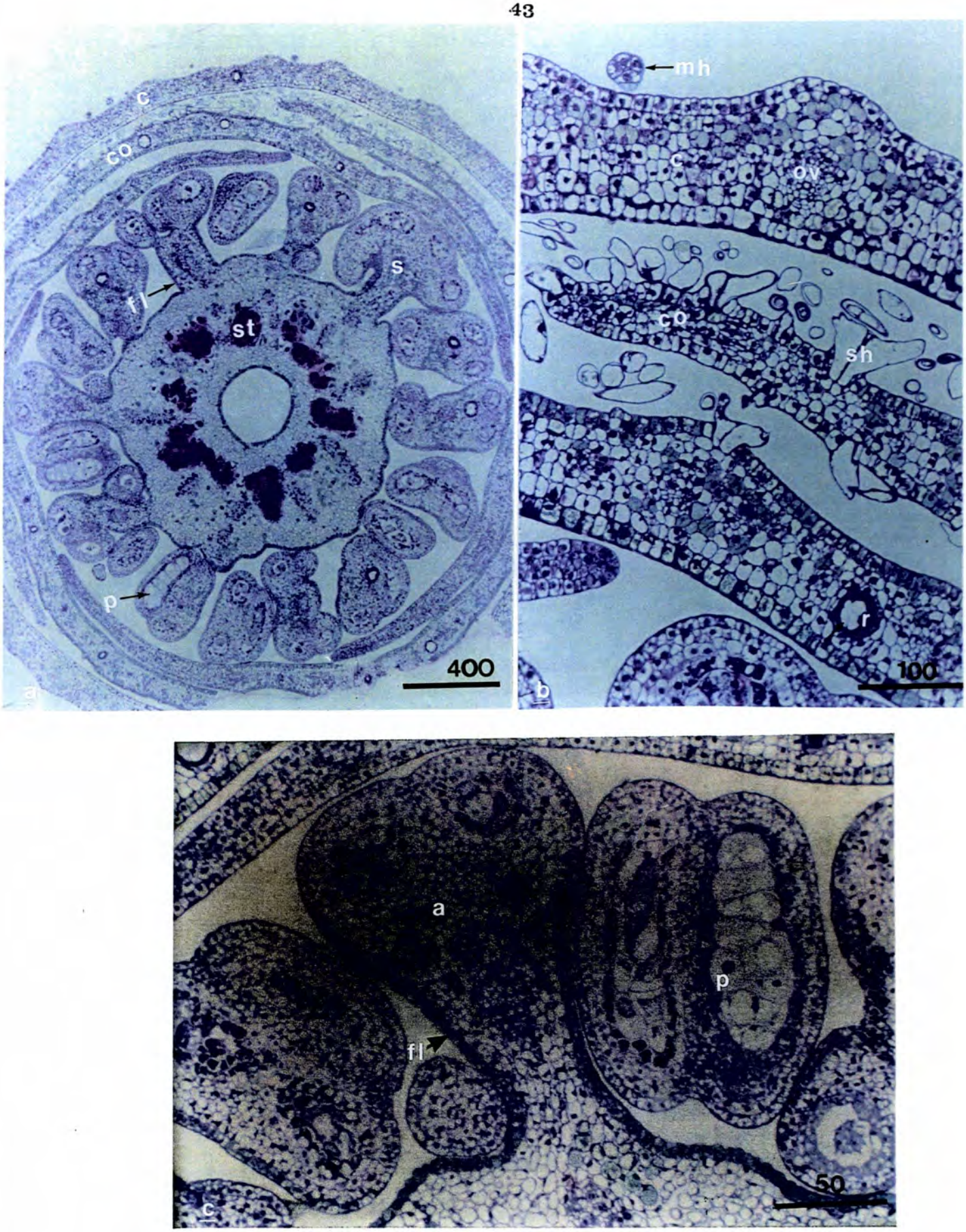


Figure 4

while the shorter half remained glabrous (Fig. 4a). The petal wall contains 6-7 layers at its middle area, and 3-4 layers at both ends. The outer and inner epidermis consist of a single row of isodiametric cells with interspersed gland cells. The petal wall also contains vascular bundles and resin ducts (Fig. 4b).

The staminal column in the young ovary produced numerous stamens. The stamens have bilocular anthers attached to the staminal column by a filament; they contained several small, developing pollen grains (Fig. 4c).

3.1.3 EARLY OVARY DEVELOPMENT

The ovary of cotton was observed with three different numbers of locules (Fig. 3. a1, b1, c1) most commonly with 4 locules but occasionally with 3 or 5 locules. The primary development of the ovary is shown in Figure 5a; the ovary has produced four regions of meristematic tissue.

In the next stage (Fig. 5b) the ovary formed locular ridges which initially progressed and produced the locular spaces. As the carpel increased in size the internal ridges were produced, which developed into the true septa of the ovary. The free internal margins of the carpel grow centripetally and upward, and then the original cavity is divided into four locules (Fig. 5c). Continuous growth of the reflexed margins of each carpel formed the placentae. Each placenta developed two ovules which were attached to the placenta by a funicle, a tubular structure. The funicle carried the ovular vascular supply to the ovule. The carpel margins remained unclosed in this stage (Fig. 5c).

Continuous growth of the carpel margins towards the centre of the ovary allowed the margin of carpels to fuse with each other at centre of the ovary, although the endocarp cells are apparent in a zigzag pattern (Fig. 6a). The ovules completed development of their three zones i.e. outer integument, inner integument and nucellus (Fig. 6a).

During the last stage of ovary development the locules were separated by well-developed septa. This septa contain vascular bundles and pink-coloured mucilage glands. The carpel margins fused completely at the centre of the ovary (Fig. 6b).

Figure 5

Histological development of ovary (primordial stages).
Transverse sections were stained with toluidene blue and photographed under bright field illumination.

(a) 8 mm ovary showing the four regions of meristematic tissues
(b) 8 mm ovary (late stage) initial locular area.
(c) 10 mm ovary.

cm, carpel margin; ed, endocarp; f, funicle; l, locular area; mer, meristematic tissues; mr, middle region of the ovary; ov, ovule; sep, septum wall; vb, vascular bundles.

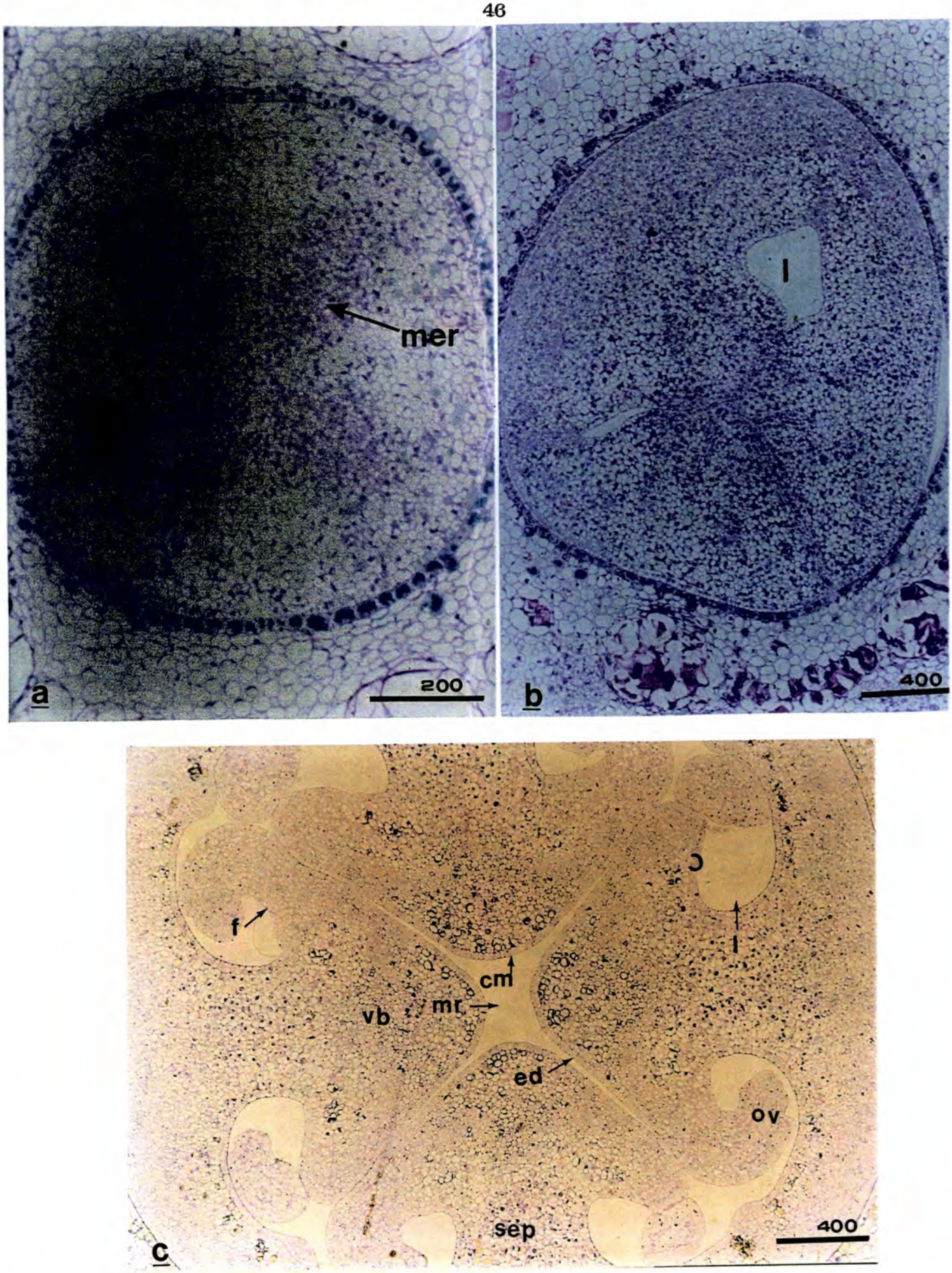


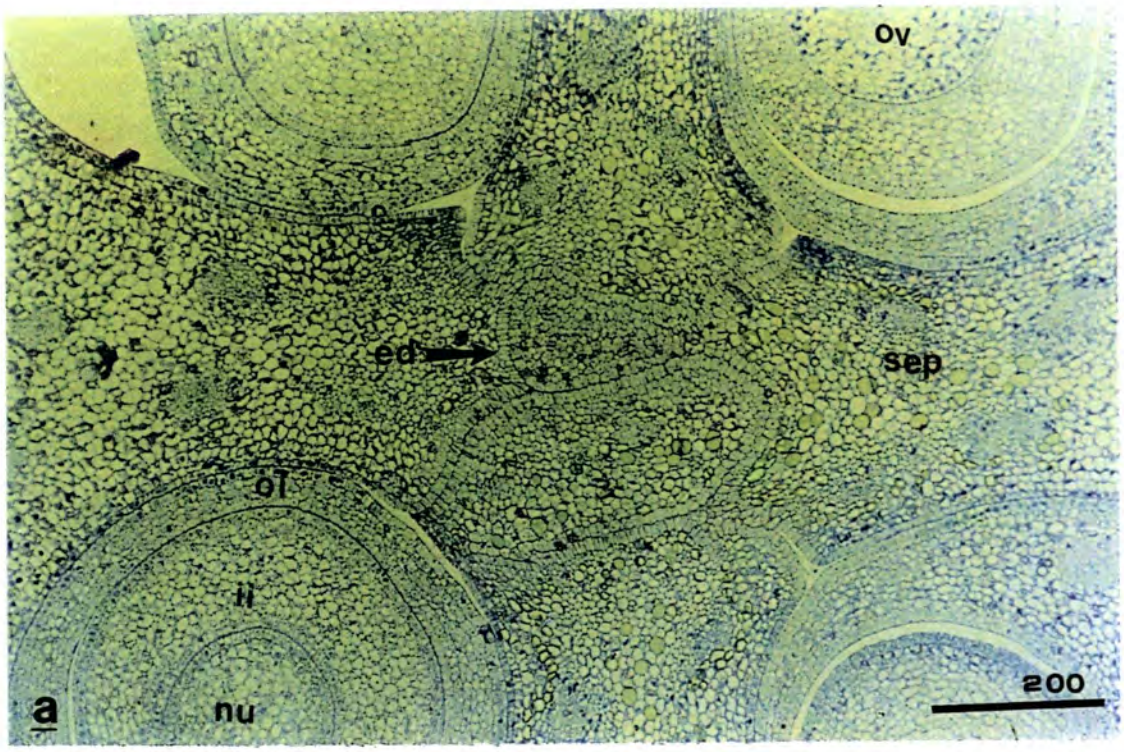
Figure 5

Figure 6

Ovary development stages (15 mm and 18 mm).
Transverse sections were stained with toluidene blue and photographed under bright field illumination.

(a) 15 mm ovary.
(b) 18 mm ovary.

ed, endocarp; l, locular area; ii, inner integument; mr, middle region of the ovary; nu, nucellus; oi, outer integument; ov, ovules; p, placenta; sep, septum wall; vb, vascular bundles; mu, mucilage gland.



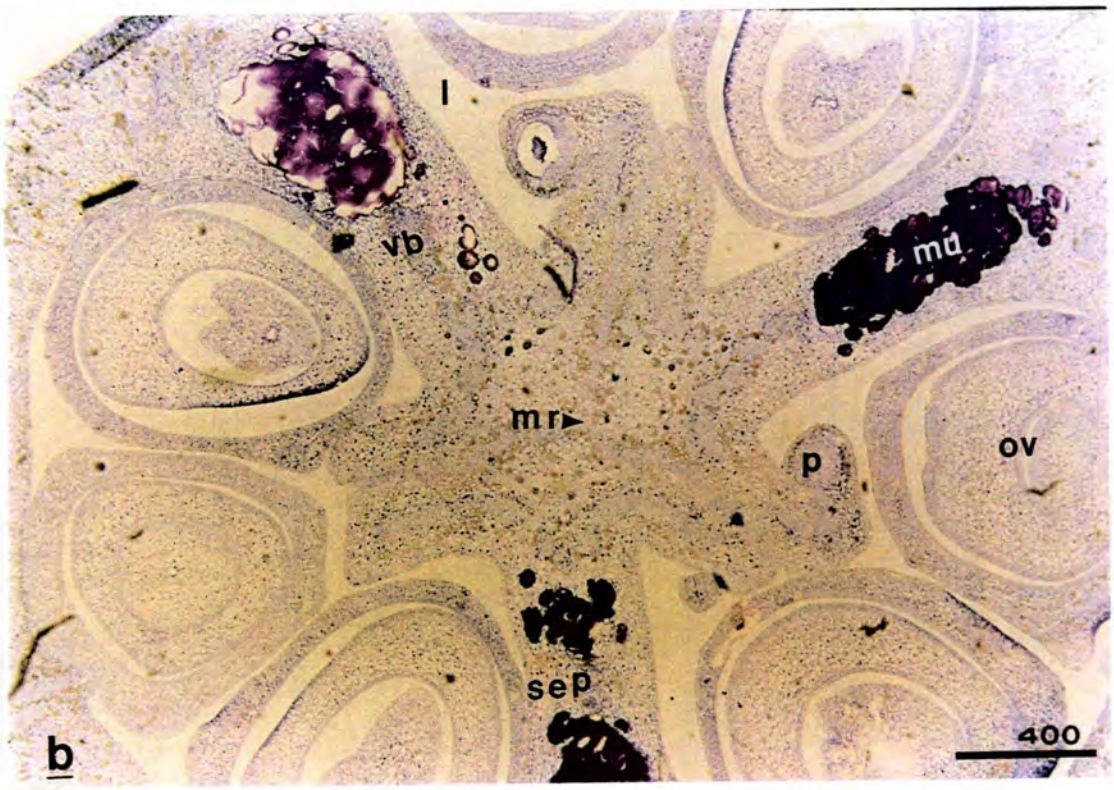


Figure 6

3.2 HISTOLOGY AND CYTOLOGY OF SEED DEVELOPMENT

3.2.1 OVULE DEVELOPMENT (PRE-FERTILIZATION)

The primordia of the ovules arose on the placenta as rounded masses of meristematic cells at about the time that the locules become closed. During the development of integument, and as the apex of nucellus begins to curve, a second fold was formed on the outer integument (Fig. 7a). The outer integument soon overgrew the inner integument, both continued their growth and made hook-like structures surrounding the nucellus (Fig. 7b).

With continuing growth of the ovules, the outer integument of the ovule covered the micropylar end of the developing nucellus. The inner integument lagged behind and did not fill the space enclosed between the outer integument and the tip of the nucellus (Fig. 7c).

Further curving development of the inner integuments lobes results in the inner integument covering the space between the nucellus and outer integument, and the ovule becomes anatropous (Fig. 8a).

When the ovary reached about 18 mm in size, the outer integument contained 6-8 layers and the inner integument 10-13 layers (Fig. 8b).

By twenty four hours before anthesis the epidermis of the outer integument had produced guard cells and stomata, and both fibre cells and ordinary epidermal cells were present. Within the nucellus the embryo sac developed (Fig. 8c).

3.2.2 SEED COAT DEVELOPMENT (POST-FERTILIZATION)

At fertilization the outer integument of seed coat consists of three layers: the outer and inner epidermal layers consist of a single row of small cells slightly different in size, and the mesophyll 3-4 layers of small cells of different sizes. Cell division was observed in mesophyll cells at this stage. The inner integument also shown to consist of three layers: the outer epidermal layer is a single row of small cells, slightly different in size and shape, the mesophyll contains 15-16 layers of small cells of different sizes and shapes, and cell division was observed in many cells; the inner most epidermis is a single row of small cells (Fig. 9a).

Figure 7

Seed development (early stages of the ovule).
Transverse sections were stained with toluidene blue and photographed under bright field illumination.

(a) 10 mm ovary.
(b) 12 mm ovary.
(c) 12 mm ovary.

f, funicle; ii, inner integument; l, locular area; mi, micropyle end; nu, nucellus; oi, outer integument; pl, placenta; vb, vascular bundles.

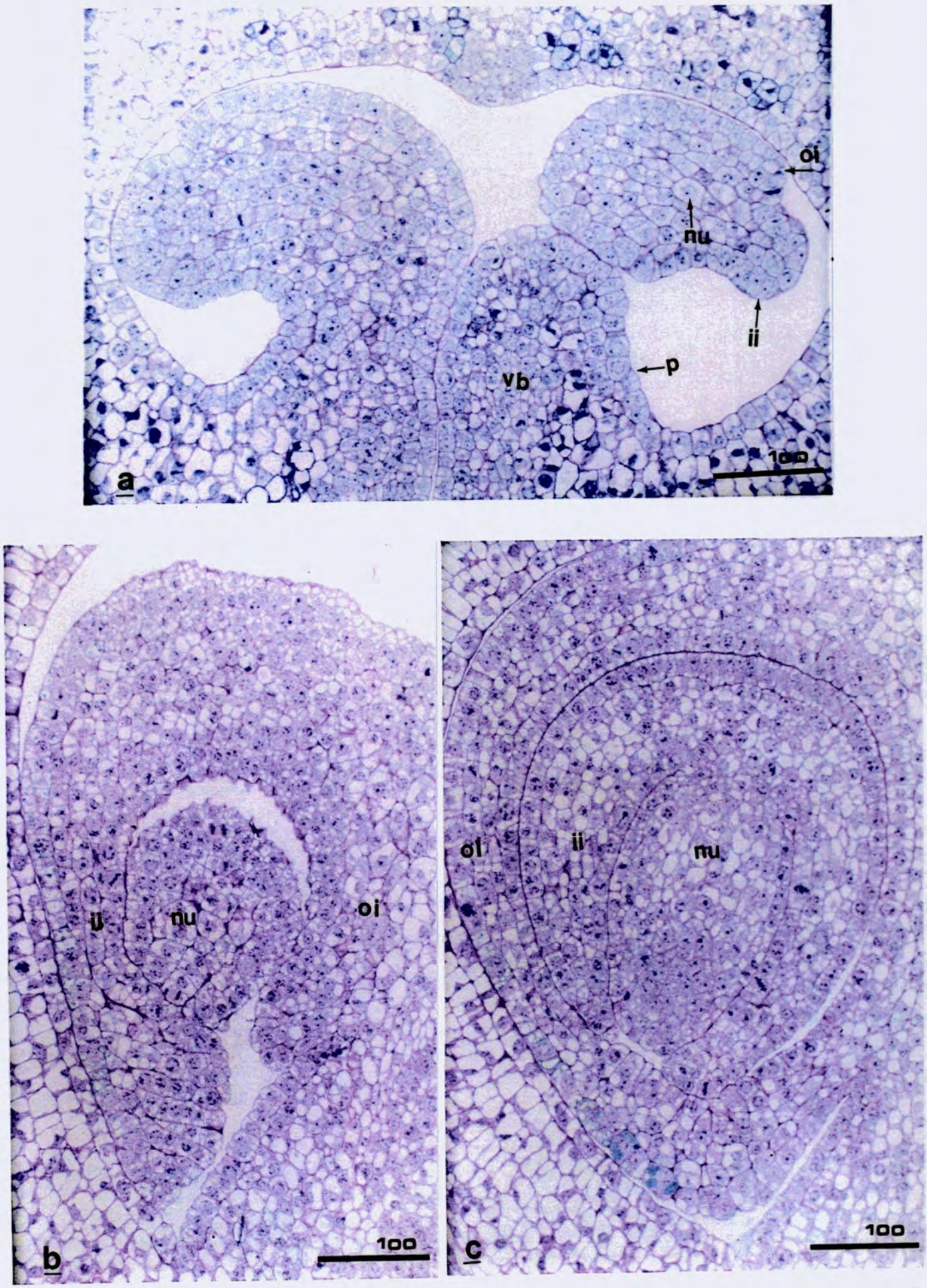


Figure 7

Figure 8

Seed development (late stages of the ovule).
Transverse sections were stained with toluidene blue and photographed under bright field illumination.

(a) 12 mm ovary.
(b) 15 mm ovary.
(c) 24 hours before fertilization.

emb, embryo; ii, inner integument; nu, nucellus; oi, outer integument; single dart, guard cells; double dart, fibre cells

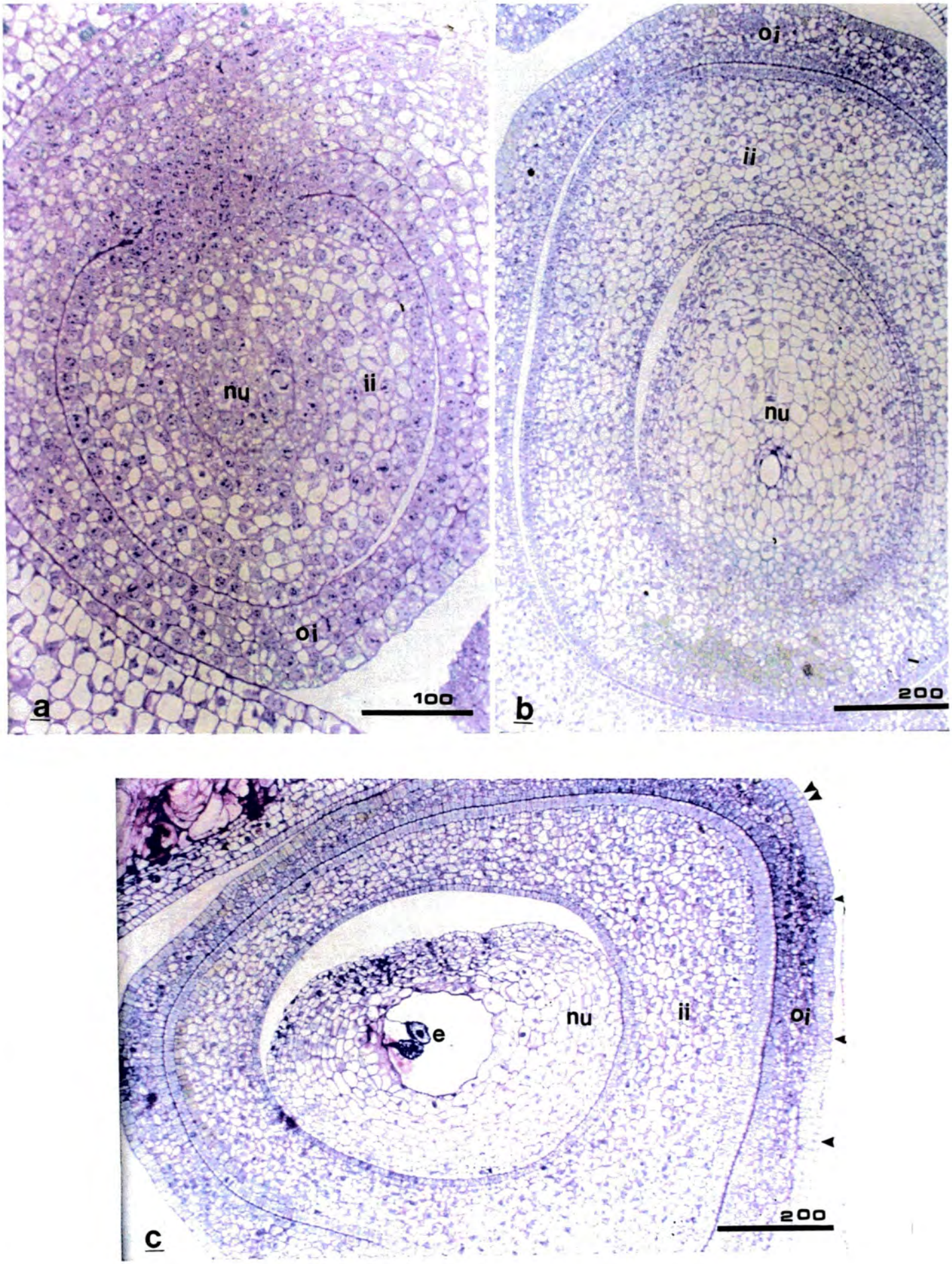


Figure 8

Figure 9

Seed coat development (early stages).
Transverse sections were stained with toluidene blue and photographed under bright field illumination.

(a) 48 hours after fertilization.
(b) 7 days after fertilization.
(c) 15 days after fertilization.
(d) 21 days after fertilization.

e, outer and inner epidermis layer; f. fibre; ii, inner integument of the ovule; fl, fringe layer; m, mesophyll; nu, nucellus; oi, outer integument layer of the ovule; arrow, cell division.

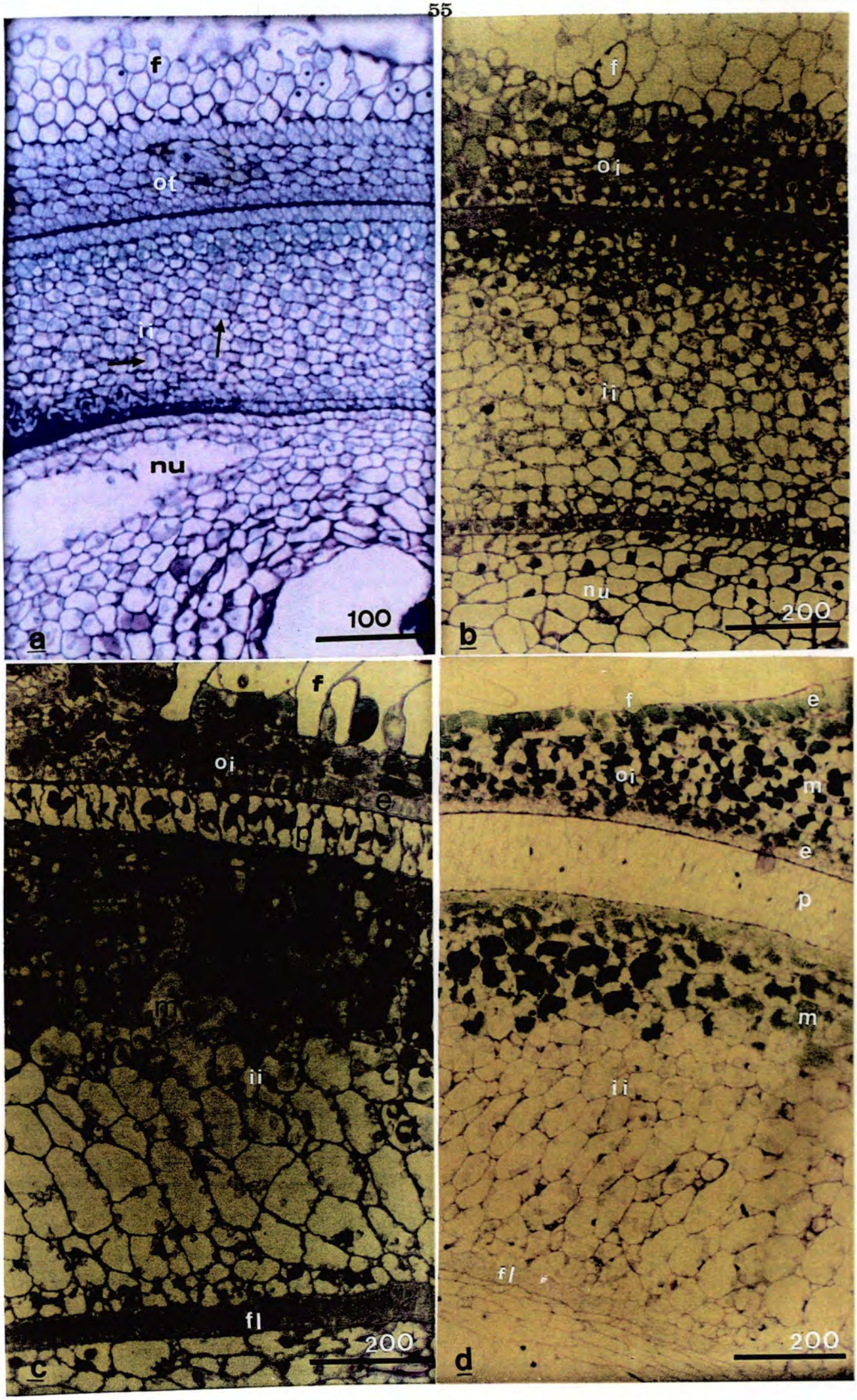

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Figure 9

Seven days after pollination, the outer integument of the seed coat consisted of 6-7 layers. The outer epidermis is shown to cotain a single row of irregular cells of different shapes and sizes and cell division was apparent in some cells; nuclei contained more than one nucleolus. The mesophyll of the outer integument has 4-5 layers containing different cell types, including some cells in mesophyll stained light green with toluidene blue. The inner epidermis of the outer integument has one row of isodiametric cells. The inner integument of the seed coat consisted of about 14-16 layers, and outer epidermis consisted of single row of irregular cells. The mesophyll layer consists of 12-14 layers of cells different sizes and shapes; the upper three rows of mesophyll stained light green. The innermost layer (fringe layer) consisted of one layer of isodiametric cells (Fig. 9b).

By 15 days after pollination the epidermal cells of the outer integument appeared larger in size, and cells stained green with toluidene blue, but fibre cells were not stained (Fig. 9c). The mesophyll cells had also enlarged since the earlier stages. The epidermal layer of the outer integument consisted of one row of isodiametric cells, and the inner integument of the seed coat of 16 layers. A palisade layer had developed, containing two rows of cells. The mesophyll has 14 layers cells of different sizes and shapes; the green staining of the upper 6 rows may be due to tannin. The cells near the fringe layer were found to be large and contained starch grains near the boundary of cells wall. The vertical cell wall of fringe cells stained darkly (Fig. 9c).

No significant developmental changes occurred in the seed coat between 15 days and 21 days after pollination, except in the palisade layer. The palisade layer doubled in size between the two stages. The palisade consisted of 4-5 rows of palisade cells (Fig. 9d).

The final development of seed coat had occurred by 45 days after pollination. The outer epidermis consisted of large cells with irregular shape; these cells stained light green with toluidene blue. The mesophyll consisted of four rows with different types of cells; some mesophyll cells stained dark green in colour. The inner epidermis of outer integument layer consisted of single row of irregular cells with different sizes and shapes. The palisade layer consisted of 3-4 layers of larger cells (Fig. 10a) The upper 6-7 layers of mesophyll cells contained tannin and starch grains; the tannin formed ring-like structures and stained dark green. Starch grains, found mostly near the cell wall, stained light pink in colour (Fig. 10b) The fringe cells are restricted to the lateral walls and interconnected ridges oriented longitudinally (Fig .10c).

Figure 10

Seed coat development (late stage).
Transverse sections of seed coat of 45 days after fertilization were stained with toluidene blue and photographed under bright field illumination.

(a) Outer integument and inner integument (only palisade layer) of the ovule.
(b) Mesophyll layer of the inner integument of the ovule.
(c) Mesophyll and fringe layer of the inner integument and endosperm and cotyledon of the ovule.

c, cotyledon; e, outer and inner epidermis layer; end, endosperm; fl, fringe layer; ii, inner integument; m, mesophyll; outer integument; palisade layer; single dart, tannin fill cells; double dart, starch grains.

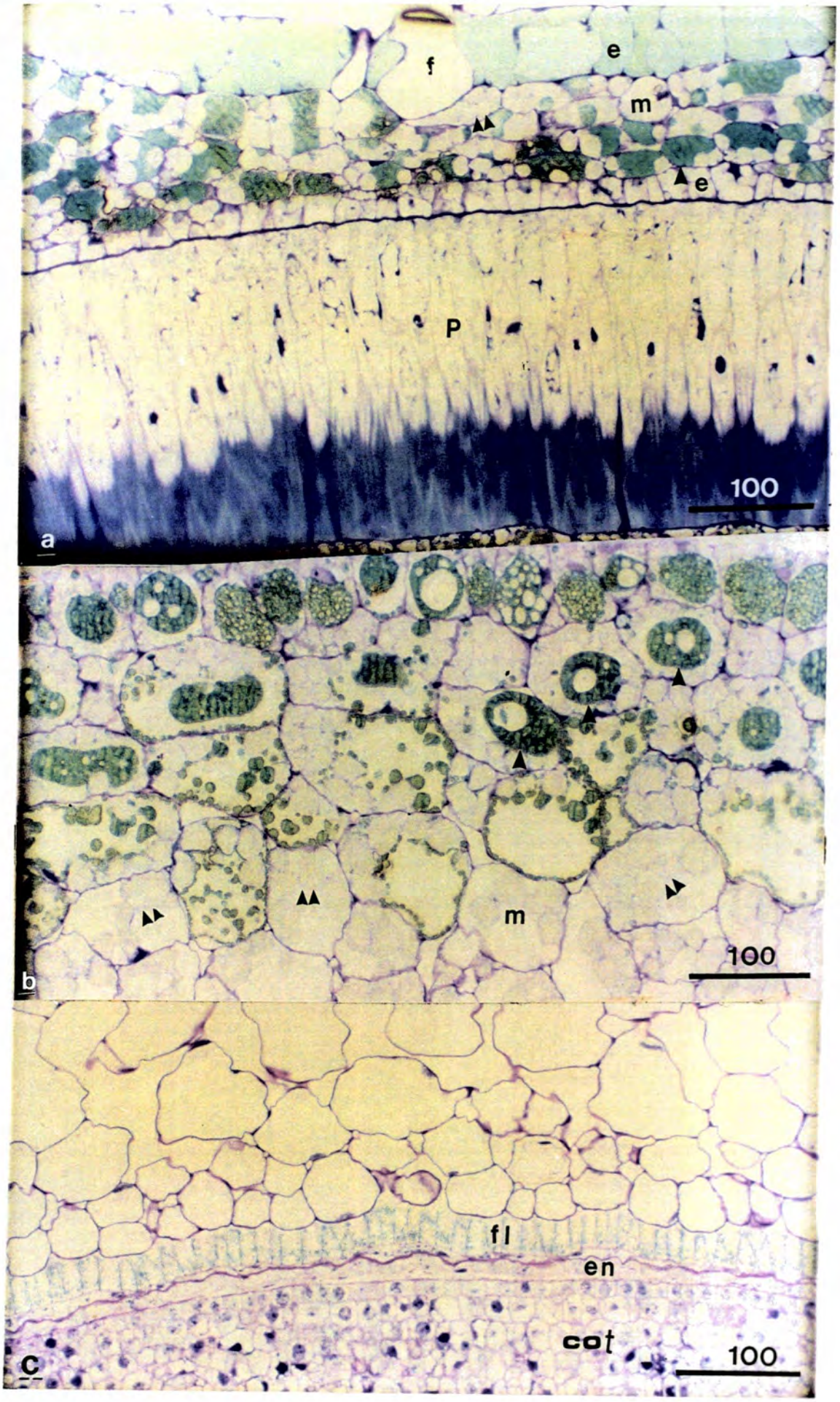


Figure 10

3.2.3 FIBRE DEVELOPMENT

On the day of pollination the outer epidermis of the outer integument of the ovule developed unicellular fibre hairs. They appeared on the surface of the ovule as balloon- shaped enlargements. Such cells were vacuolated and contained a large nucleus (Fig. 11a).

The low-magnification micrograph of the ovule at the time of anthesis showed that the fibres were distributed all over the surface of the seed, and fibre cells could easily be distinguished from the other cells like guard cell and ordinary epidermal cells (Fig. 11b).

Forty-eight hours after anthesis the fibres became elongated with tapered tips . The nuclei migrated towards the outer ends of the fibres (Fig. 12a).

Seven days after anthesis the fibre hairs (the lint fibres) lengthened to approximately half their final length, and developed a substantial primary cell wall. The thinner-walled fuzz fibres were initiated at this stage (Fig. 12b)

The cotton fibre reached its final length 30 days after anthesis, and fibres developed grooves and pits in their walls toward the attached end; due to this the fibres were making contact with neighbouring cells (Fig 12c). The primary wall developed a secondary layer due to additional depositing of cellulose (Fig. 13a, 13b). The cell wall of the mature cotton fibre consisted many layers, and fibre formed its secondary wall (Fig. 13c).

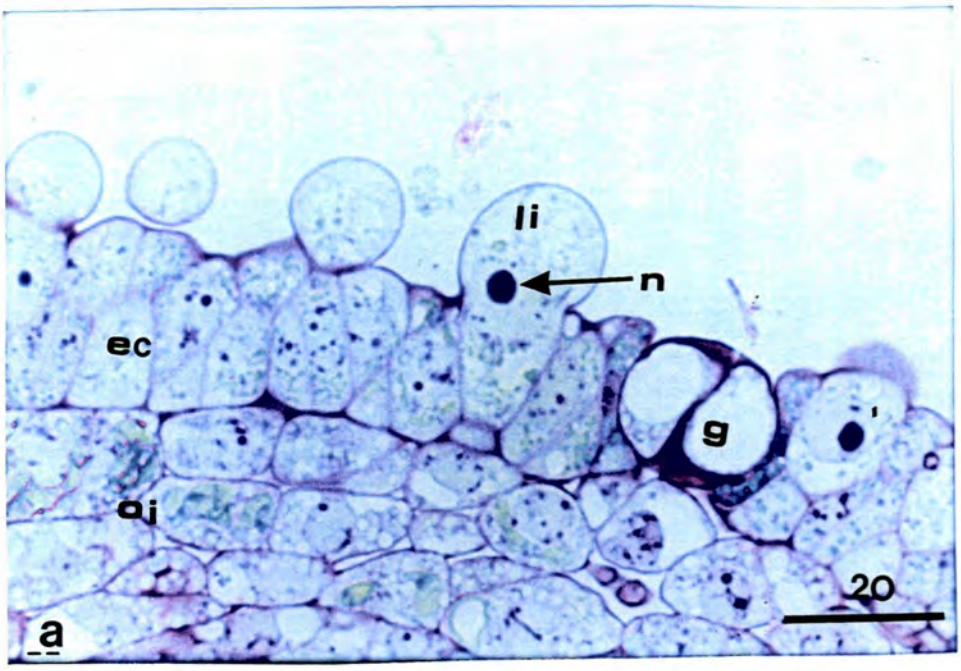
Lint fibre and **fuzz fibre.** Lint fibres were initiated and appeared on the surface of outer epidermis layer of the ovule on the day of anthesis (Fig. 11a) but fuzz fibres were initiated later, 7-10 days after anthesis (Fig. 12b). Lint fibres had spiral convolutions, which repeatedly changed their direction along their length (Fig 12b) but fuzz fibres did not showed any convolution and they grew straight (Fig. 12c). As compared to fuzz fibre (Fig. 14c) the cell wall of lint fibre is thinner due to less deposition of cellulose (Fig. 14b). Lint fibre is the longer fibre collected by ginning process and used for commercial purpose (Fig. 14,a1); at maturity it had become like twisted ribbon (Fig. 14d). The fuzz fibre that remained adhered to the seed coat after ginning (Fig. 14,a2) is very short and it was not twisted at maturity (Fig. 14e).

Figure 11

Fibre development (Just fertilized ovary).
Transverse sections were stained with toluidene blue and photographed under bright illumination.

(a) First day of pollination.
(b) Longitudinal section same as above stage.

ec, ordinary epidermal cells; g, guard cells; li, lint fibre; n, nucleus; vb, vascular bundle; oi, outer integument; ii, inner integument.



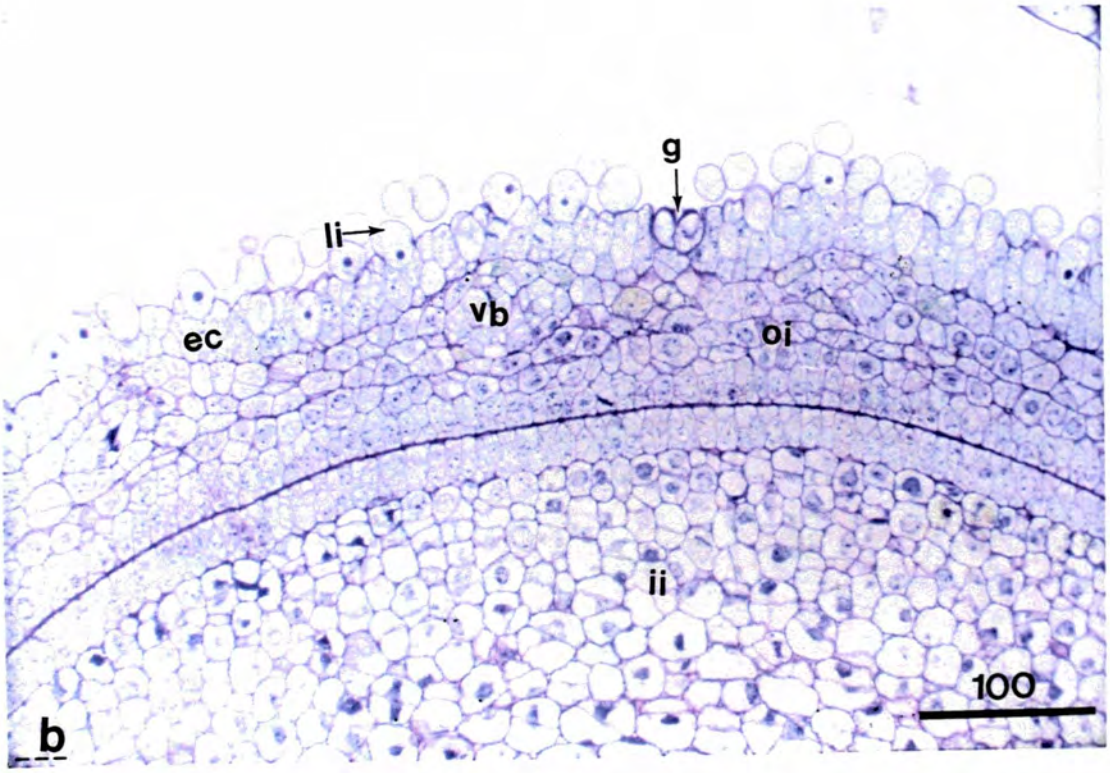


Figure 11

Figure 12

Fibre development stages (48 hours, 7 days and 30 days AF). Transverse sections were stained with toluidene blue and photographed under bright field illumination.

(a) 48 hours after fertilization.
(b) 7 days after fertilization.
(c) 30 days after fertilization.

ec, epidermal cells; cell, cellulose; fi, initiation of fuzz fibre cells; li, lint fibre cells; pw, primary cell wall; n, nucleus; va, vacuole; dart, pits.

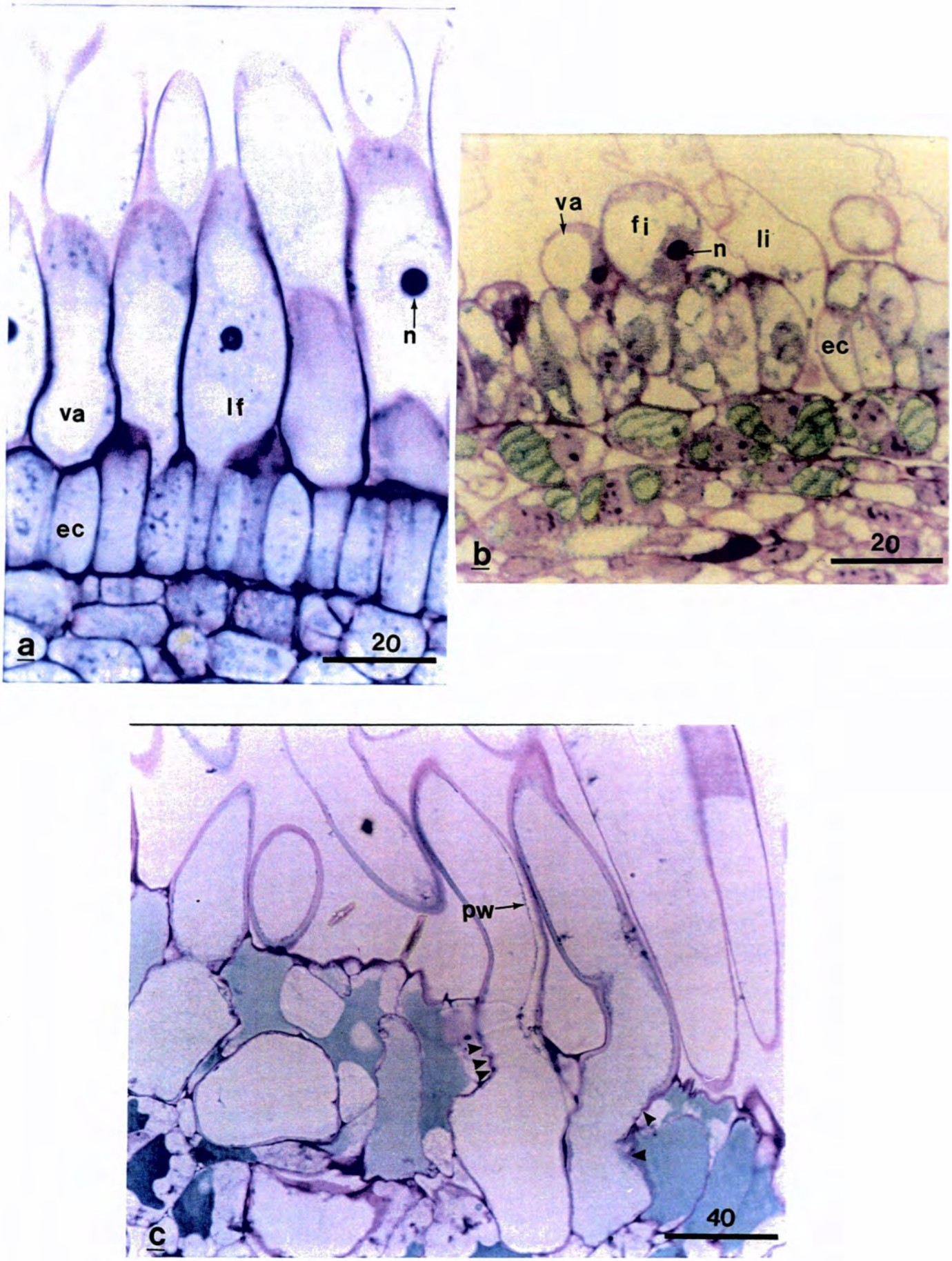


Figure 12

Figure 13

Fibre development stages (30 days, 45 days and mature fibre)
Transverse of section a was stained with calcofluor and photographed under fluorescence illumination; b and c were stained with toluidene blue and photographed under bright field illumination.

(a) 30 days after fertilization.
(b) 45 days after fertilization.
(c) Mature fibre.

ec, epidermal cells; f, fibre; vb, vascular bundle; cell, cellulose deposition; dart, secondary growth of the cell wall.

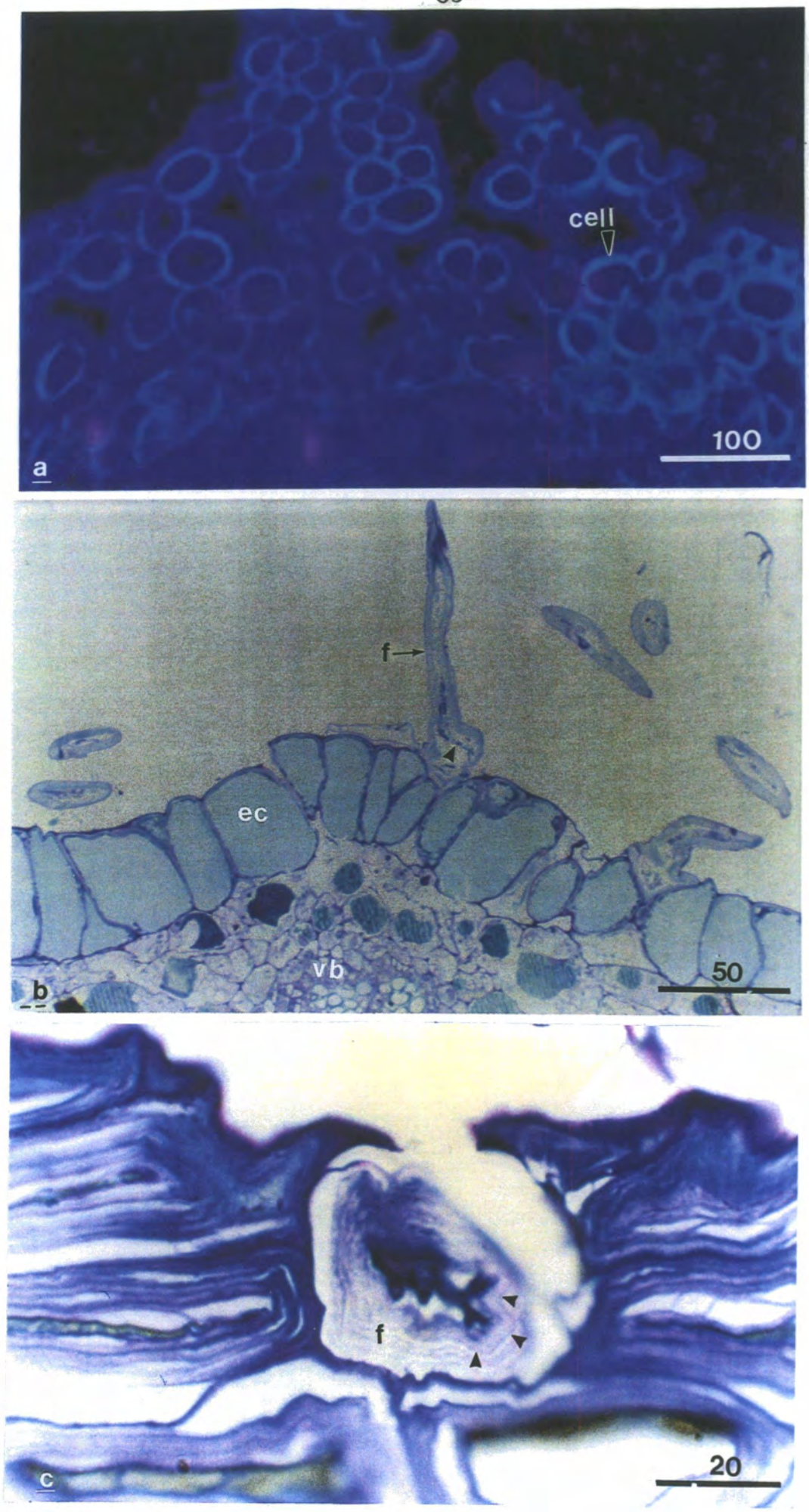


Figure 13

Figure 14

Fibre development (fuzz fibre and lint fibre)

(a) 1. Lint fibre, 2. seed with fuzz, 3. seed without fuzz
(b Lint fibre stained with calcofluor.
(c) Fuzz fibre stained with calcofluor.
(d) Lint fibre.
(e) Fuzz fibre.

fu, fuzz fibre; li, lint fibre; s, seed; single dart, fuzz fibre shows cellulose depositions; double dart, lint fibre shows cellulose deposition.

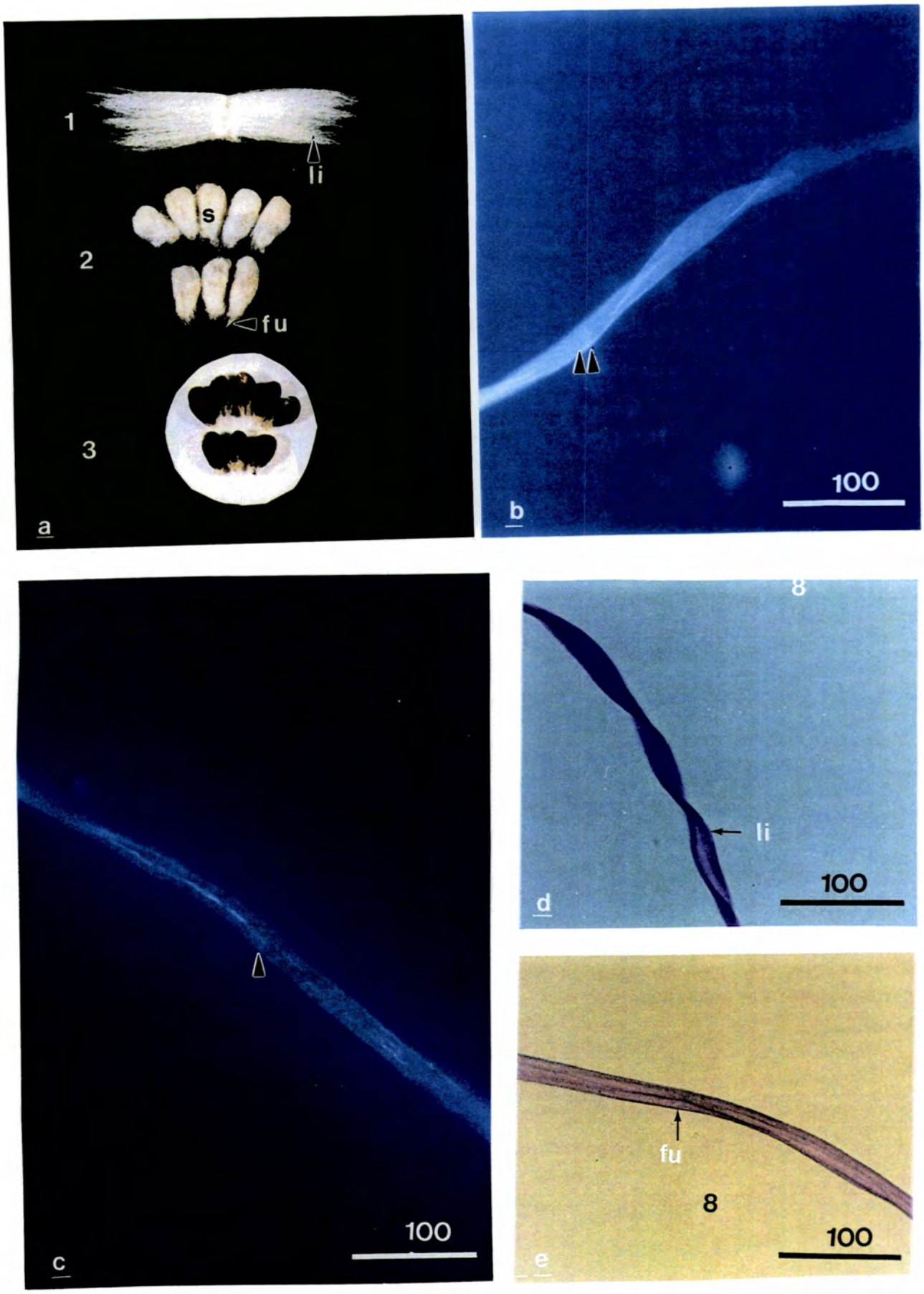


Figure 14

3.2.4 EMBRYO DEVELOPMENT

The two synergids were fully developed 24 hours before pollination; they lie adjacent to each other within the nucellus, and contained many vacuoles (Fig. 15a).

Twenty-four hours after pollination the pollen tube entered the micropyle of the ovule, and reached the embryo sac. The alignment of exostome and endostome determined whether there is a straight or a zigzag course for the pollen tube (Fig. 15b). The tube grew for a short distance between the outer and inner integuments and entered the nucellus of the ovule.

By 48 hours the pollination the pollen tube had grown into the degenerating synergid through a filiform apparatus and discharged the sperm into the synergid by release of a small pore called the discharging plug. After discharging the sperm into the degenerating synergid, the synergid collapsed to half its size (Fig. 15c)

The zygote was divided by a transverse wall and formed two cells, to basal cell (cb) and the terminal cell (ca). The terminal cell divided again transversely forming ci, and divided oblique-vertically forming cii; and formed four-cell embryo (Fig 15d). The terminal cell (cii) occupied one quarter of the embryo and the basal cell (cb) occupied a second quarter of the embryo, and ci and ca occupied the remaining part of embryo.

The embryo increased its growth by subsequent division of cii, ci and ca cells and attained its globular stage. The embryo cells contained large nuclei that possessed several nucleoli. The surface of the embryo appeared uneven (Fig. 16a).

Fifteen days after pollination the embryo reached its full globular stage, and was still attached to the suspensor at its basal portion. Within the cell vacuoles are being initiated, and the nuclei often contain more than one nucleolus (Fig. 16b).

Twenty one days after pollination the embryo reach its heart-shape stage. The embryo was flattened on the top and had formed cotyledonary primordia (Fig. 17a).Some embryos cells now contain a predominant central vacuole.

By 30 days after pollination two cotyledons are seen to have developed from the embryo axis (Fig. 17b) . Between the cotyledons a small meristematic region is present, which will later develop into the shoot apex.

Figure 15

Embryo development (early stages).
Transverse section (a) and longitudinal sections (b, c & d) were stained with toluidene blue and photographed under bright field (a, b, & c) and Nomarski (d).

(a) 24 hours before fertilization.
(b) 24 hours after fertilization.
(c) 48 hours after fertilization.
(d) 7 days after fertilization.

dsy, degenerating synergid; dp, discharging plug; emb, embryo; fa, filiform apparatus; ii, inner integument; oi, outer integument; pt, pollen tube; sy, synergids; vt, vascular tissues; ca, cb, ci and cii, embryo's cell (four cell embryos).

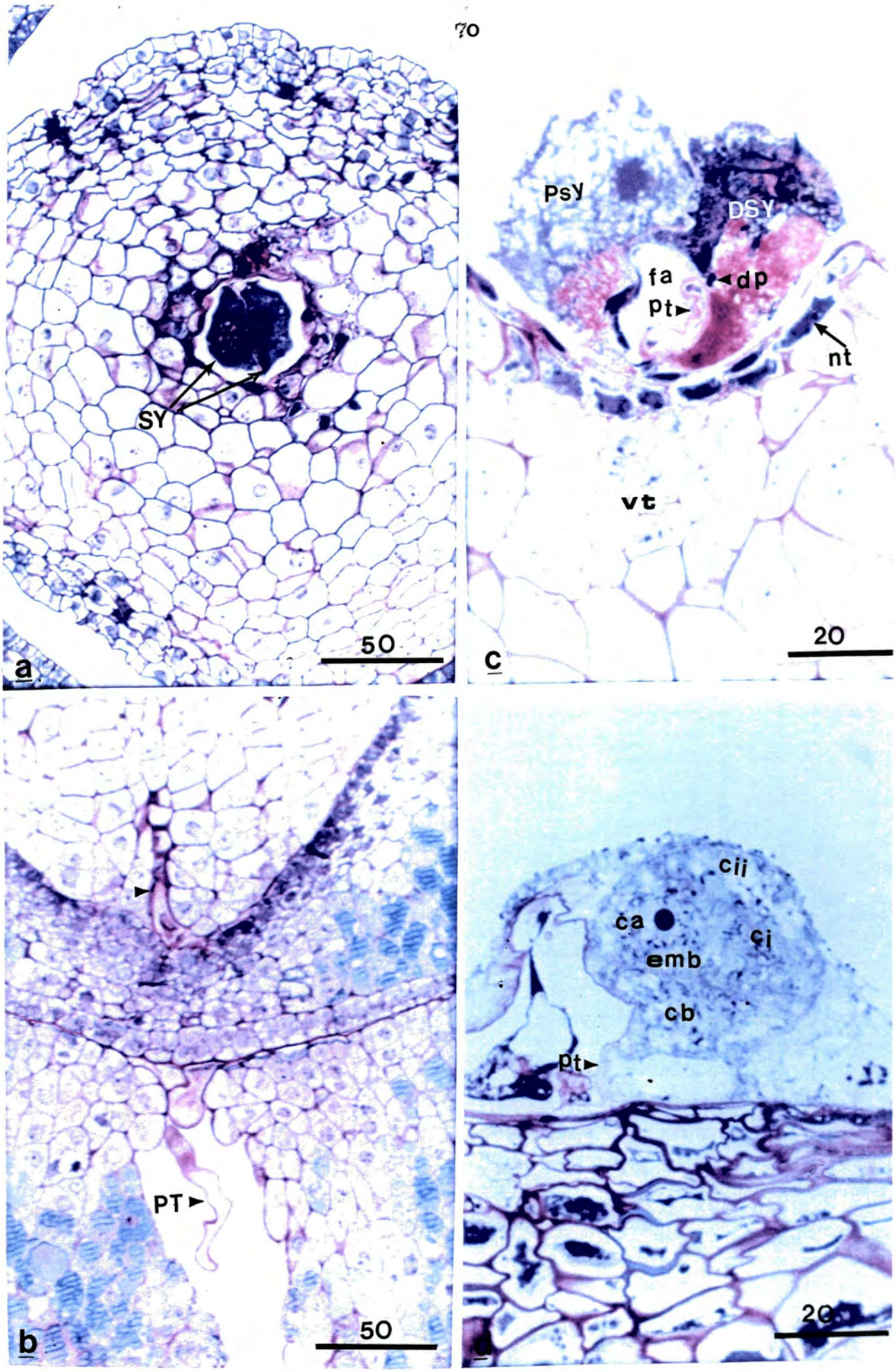


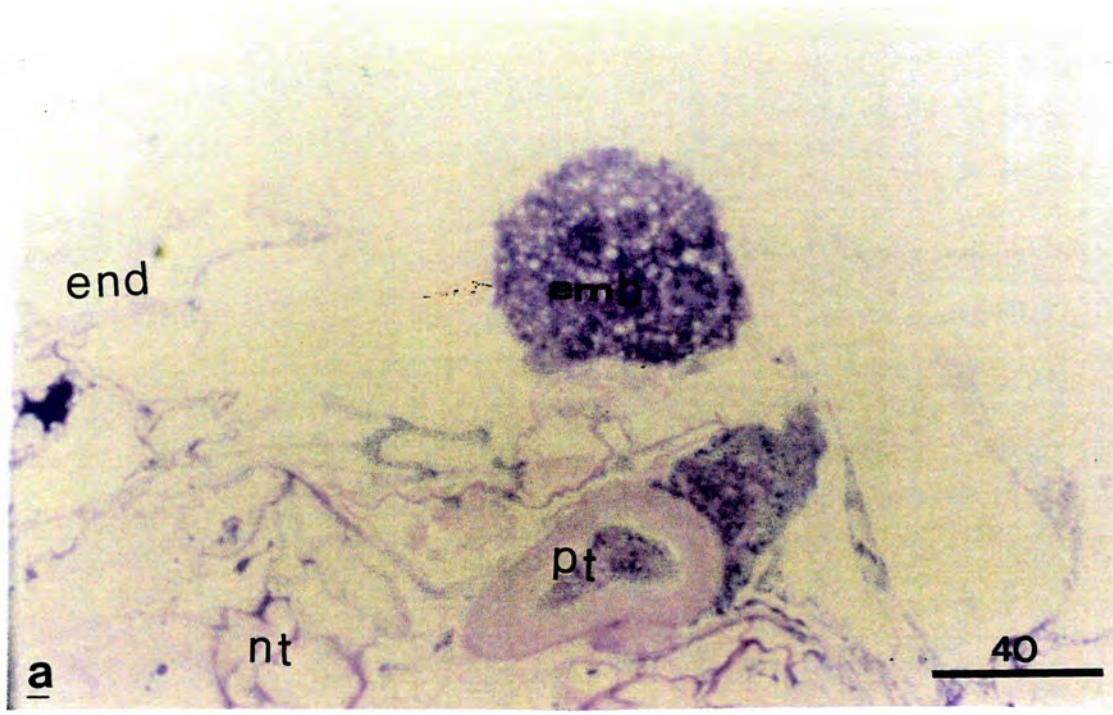
Figure 15

Figure 16

Embryo development (globular stages).
Transverse sections were stained with toluidene blue and photographed under bright field illumination.

(a) Early globular stage of the embryo.
(b) Full globular stage of the embryo.

emb, embryo; en, endosperm; nt, nucellus tissues; pl, pollen tube; sup, suspensor; vt, vascular tissues.



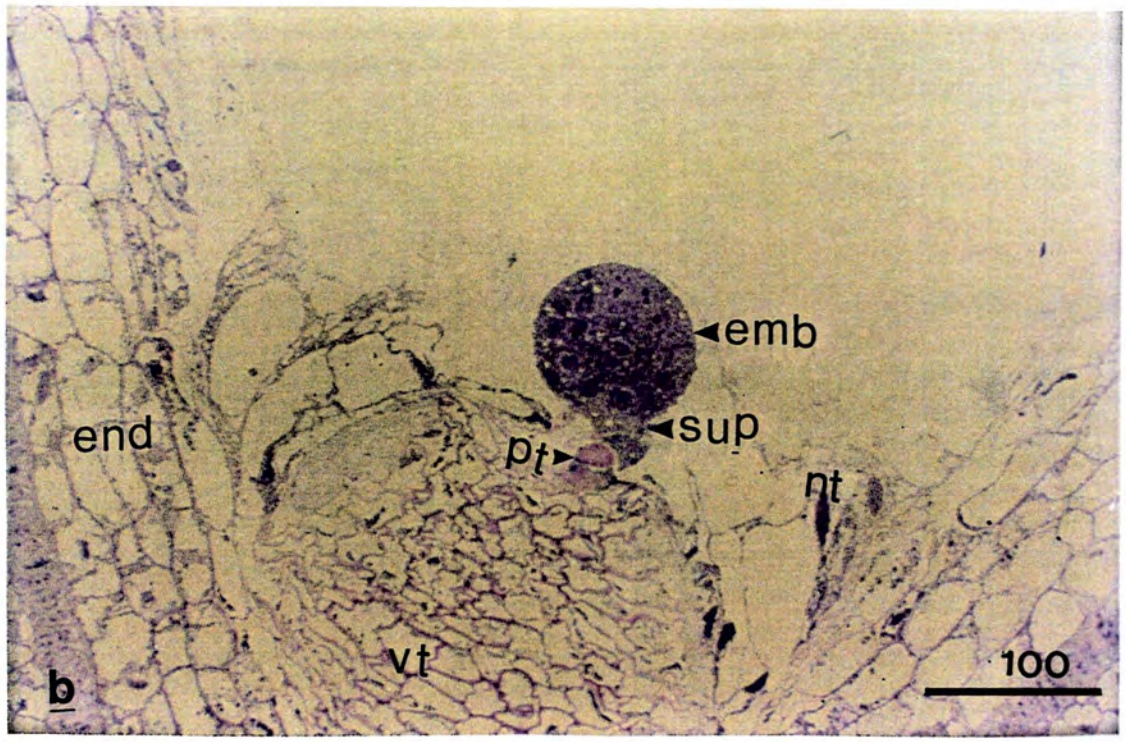


Figure 16

Figure 17

Embryo development stages (heart and torpedo-shaped, and mature embryo).
Transverse sections were stained with toluidene blue and photographed under bright field illumination.
(a) Heart-shaped embryo.
(b) Torpedo-shaped embryo.
(c) Mature embryo.

cot, cotyledons; emb, embryo; end, endosperm; nt, nucellus tissues; pc, pro cambium; rd, resin duct.

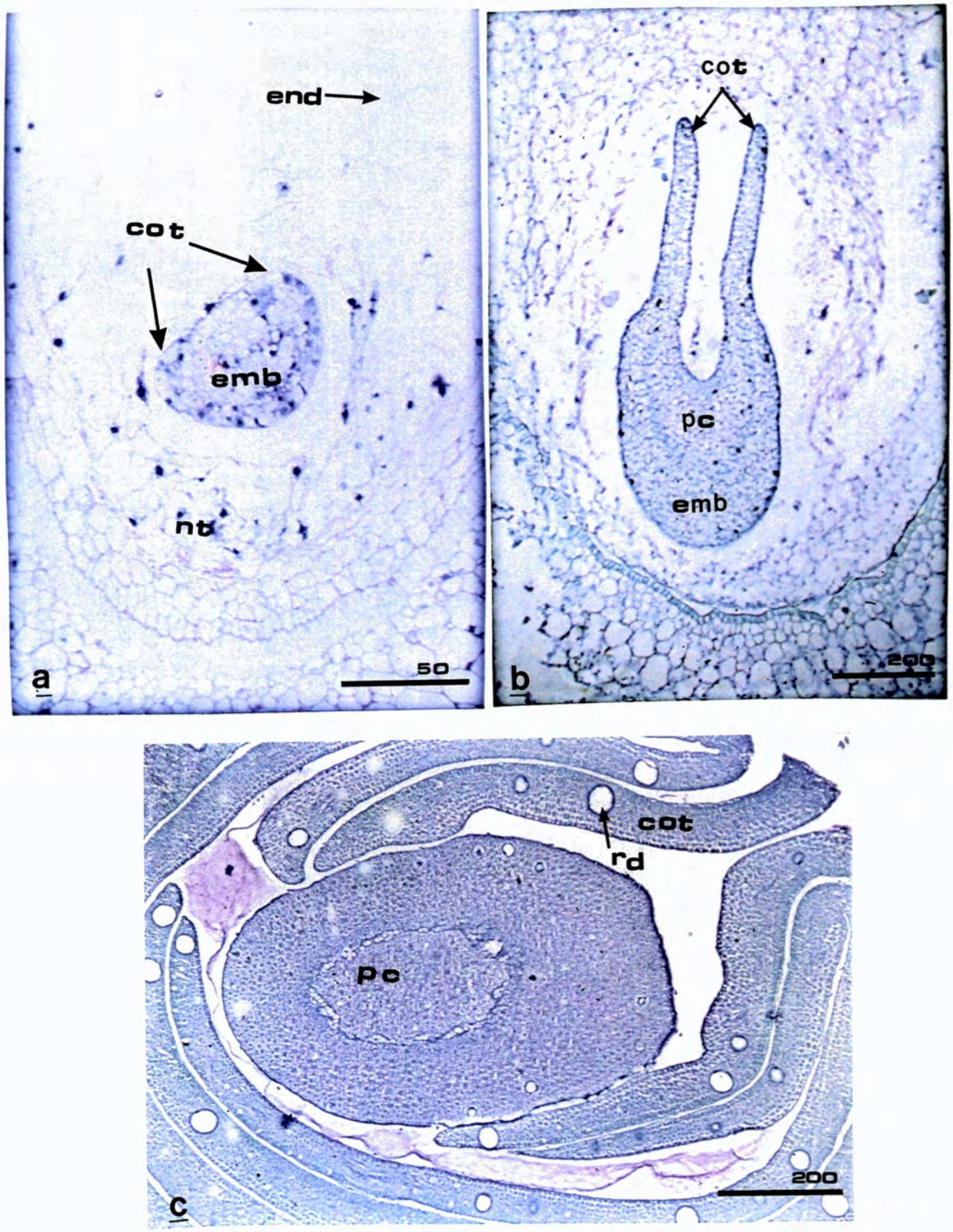


Figure 17

Forty-five days after pollination the cotyledons are further developed, they are flattened and the margins of cotyledons curved inward and then they coil around each other. The cotyledons contained several layers of mesophyll, which are not clearly distinguished into palisade and spongy layers at this stage, vascular elements and resin ducts (Fig. 17c).

3.3 HISTOLOGY AND CYTOLOGY OF FRUIT WALL

A study of the fruit wall (pericarp) in cotton was carried out to examine the development of the major tissues and the patterns of cellular differentiation in the exocarp (the outer layer), mesocarp and endocarp (inner layer) at different stages.

3.3.1 EXOCARP DEVELOPMENT

At the initial stage examined (10 mm ovary; before flowering) the exocarp consisted of a single layer of isodiametric cells with thin cell walls, and was covered with a thin cuticle. The arrangement of cells was compact and cell division occurred in many cells. The nuclei were large and often contained more than one nucleoli (Fig. 18a).

Later (15 mm ovary B.F.) the exocarp still consisted of single layer of isodiametric cells with thin cell walls, and was covered with thin cuticle; however these cells now contained many vacuoles (Fig. 18b).

With increasing size of ovary, to 18 mm and 20 mm (before fertilization) the exocarp remained as a single layer of cells; these show increasingly irregular forms of different sizes and shapes that retained thin cell walls, nuclei with more than one nucleolus, and the many vacuoles (Fig. 18c & 18d.). The exocarp was covered with a thin cuticle.

At 7-days after fertilization the exocarp consisted of a single layer of irregular thin-walled cells of different sizes and shapes. The layer was, however, now covered with thick-walled cuticle which formed an irregular and pointed border on the outer surface of ovary (Fig. 19a).

By 15-and 21-days after fertilization the exocarp still contains single layer of irregular cells. Further enlargement of the ovary by 21-days (AF), was linked with expansion of the exocarp cells (Fig. 19b and 19c). The layers were covered with a thick cuticle.

Figure 18

Exocarp development stages (before fertilization).
Transverse sections were stained with toluidene blue and photographed under bright illumination.

(a) 10 mm ovary before fertilization.
(b) 15 mm ovary before fertilization.
(c) 18 mm ovary before fertilization.
(d) 20 mm ovary before fertilization.

ex, exocarp; me, mesocarp; cu, cuticle; va, vacuole; n, nucleus.

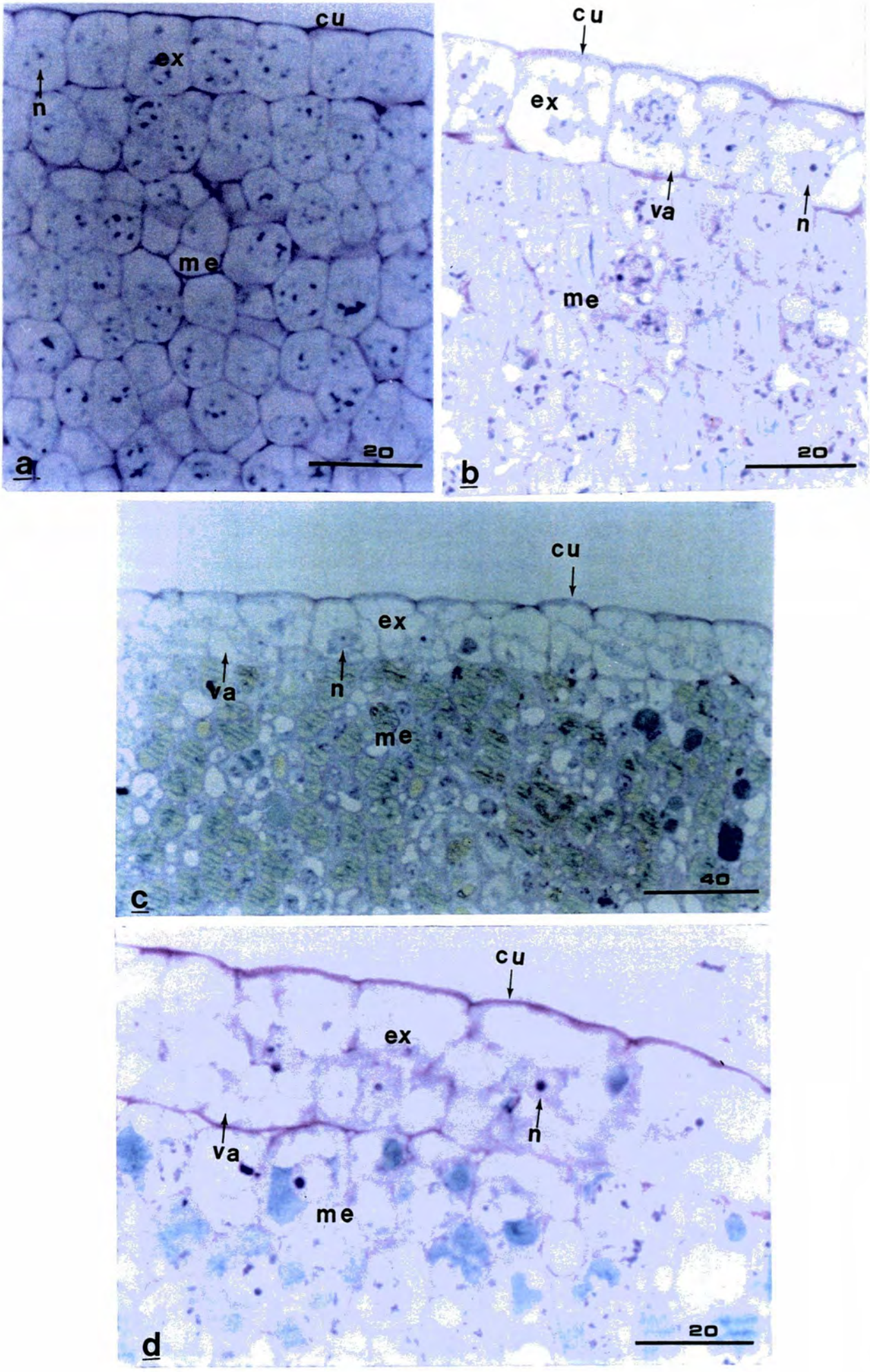


Figure 18

Figure 19

Exocarp development stages (after fertilization).
Transverse sections were stained with toluidene blue and photographed under bright field illumination.

(a) 7 days after fertilization.
(b) 15 days after fertilization.
(c) 21 days after fertilization.
(d) 45 days after fertilization.

ex, exocarp; me, mesocarp; cu, cuticle; and tann, tannin-filled parenchyma cells

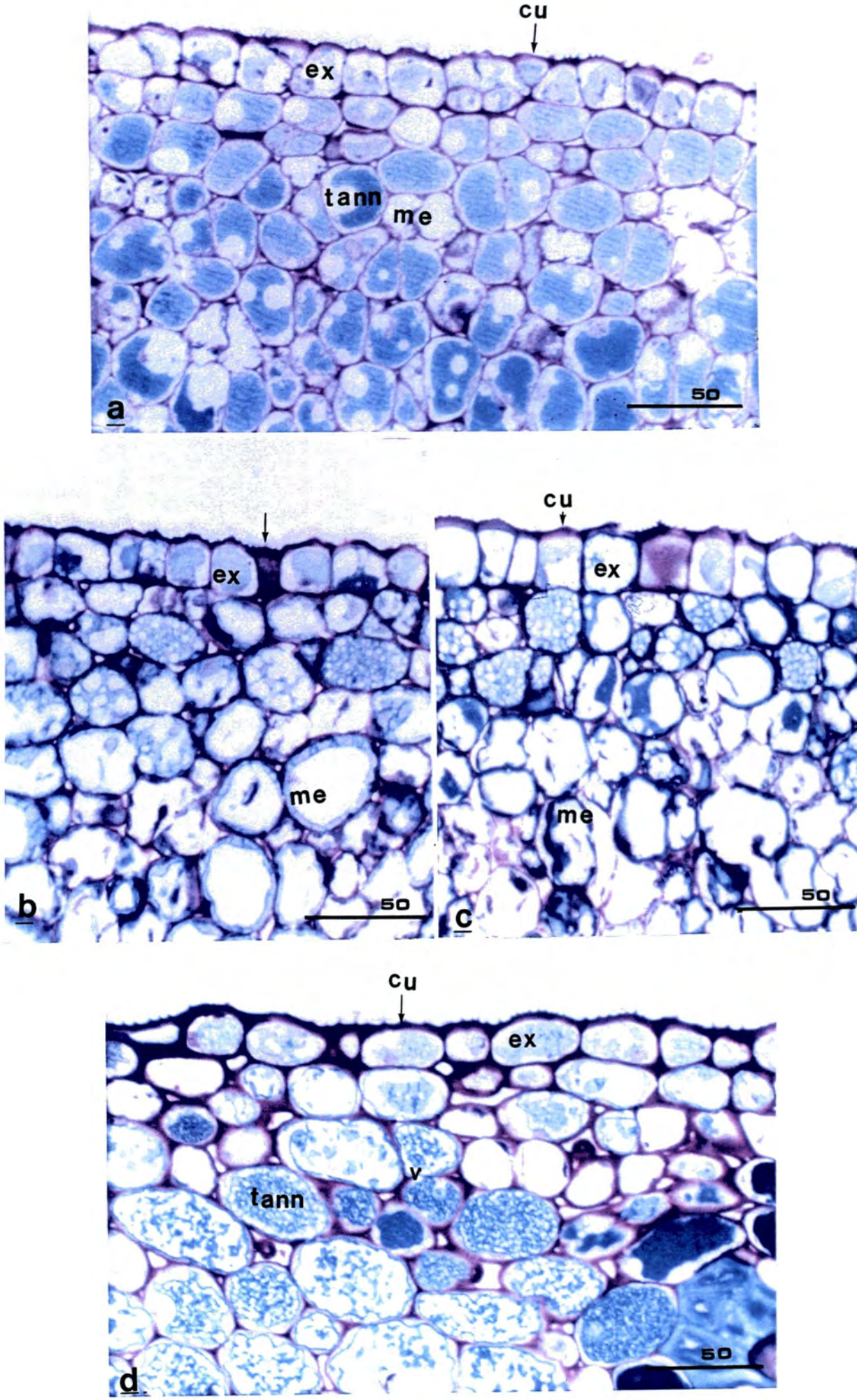


Figure 19

However by 45-days after fertilization the exocarp, which is covered by a thick cuticle, is no longer distinguishable as a single layer, instead it appears as 3-4 layers of large, irregular cells, with thick cell wall; most walls are thickened although some radial walls are not (Fig. 19d).

3.3.2 MESOCARP DEVELOPMENT

The mesocarp of 10 mm ovary (B.F.) consists of 11-12 layers of irregularly-shaped, thin walled parenchymatous cells of different sizes and shapes. Strands of provascular tissue have developed, and cell division within these is evident (Fig. 20a). The larger 15 mm ovary (B.F.) contained 21-22 layers of irregular, parenchymatous mesophyll cells. The nucleus of cells stained darkly with toluidene blue, and had more than one nucleoli. The cells of the pro vascular bundles possessed thin cell walls (Fig. 20b).

By the time that the ovary had enlarged to 18 mm the mesophyll contained 34-36 layers of irregular, thin-walled cells. The outer portion of mesocarp contained tannin-filled parenchyma cells, but in the inner portion, near the endocarp, the cells did not contain tannins. Within the primary vascular bundles differentiation of lignified xylem had occurred (Fig. 20c & 20d).

At 7-days after fertilization the tannin-filled parenchyma cells in the mesocarp stained green and chloroplast contained cells stained light blue with toluidene blue staining (Fig. 21a). The vascular bundle had some thick-walled xylem elements (Fig. 22a)

By 15-days after fertilization the tannin-filled parenchyma cells stained light green. However, those parechyma cells which were near the endocarp layer expanded and developed intercellular spaces (Fig. 21b). The vascular bundle had a few thick-walled xylem cells, and they had lignified, as shown by the acridine orange staining (Fig. 23a).

Thirty days after fertilization the mesocarp contained two type of parenchyma cells, (a) tannin-filled parenchyma, stained light green in colour, and (b) "colourless" parenchyma, which had larger intercellular air-spaces, near the endocarp (Fig. 21c). The vascular bundle contained phloem, and thick-walled xylem and phloem fibre cells (Fig. 22b) shown by acridine orange staining to be heavily lignified (Fig. 23b). The lignification occurred in the xylem after additional deposition of the cellulose, which is showed in Figure 23c, after staining with calcofluor and examined with fluorescence light and UV filter.

Figure 20

Mesocarp development stages (before fertilization).
The transverse sections were stained with toluidene blue for a, b, & c and photographed under bright field illumination. d was stained with acridine orange and photographed under fluorescence illumination using a blue filter, to show extent of lignification (yellow).

(a) 10 mm ovary before fertilization.
(b) 15 mm ovary before fertilizatin.
(c) 18 mm ovary before fertilization.
(d) 18 mm ovary before fertilization.

en, endocarp; ex, exocarp; me, mesocarp; par, parenchyma; tann, tannin-filled parenchyma cell vb, vascular bundle and dart, lignified cell (stained by acridine orange).

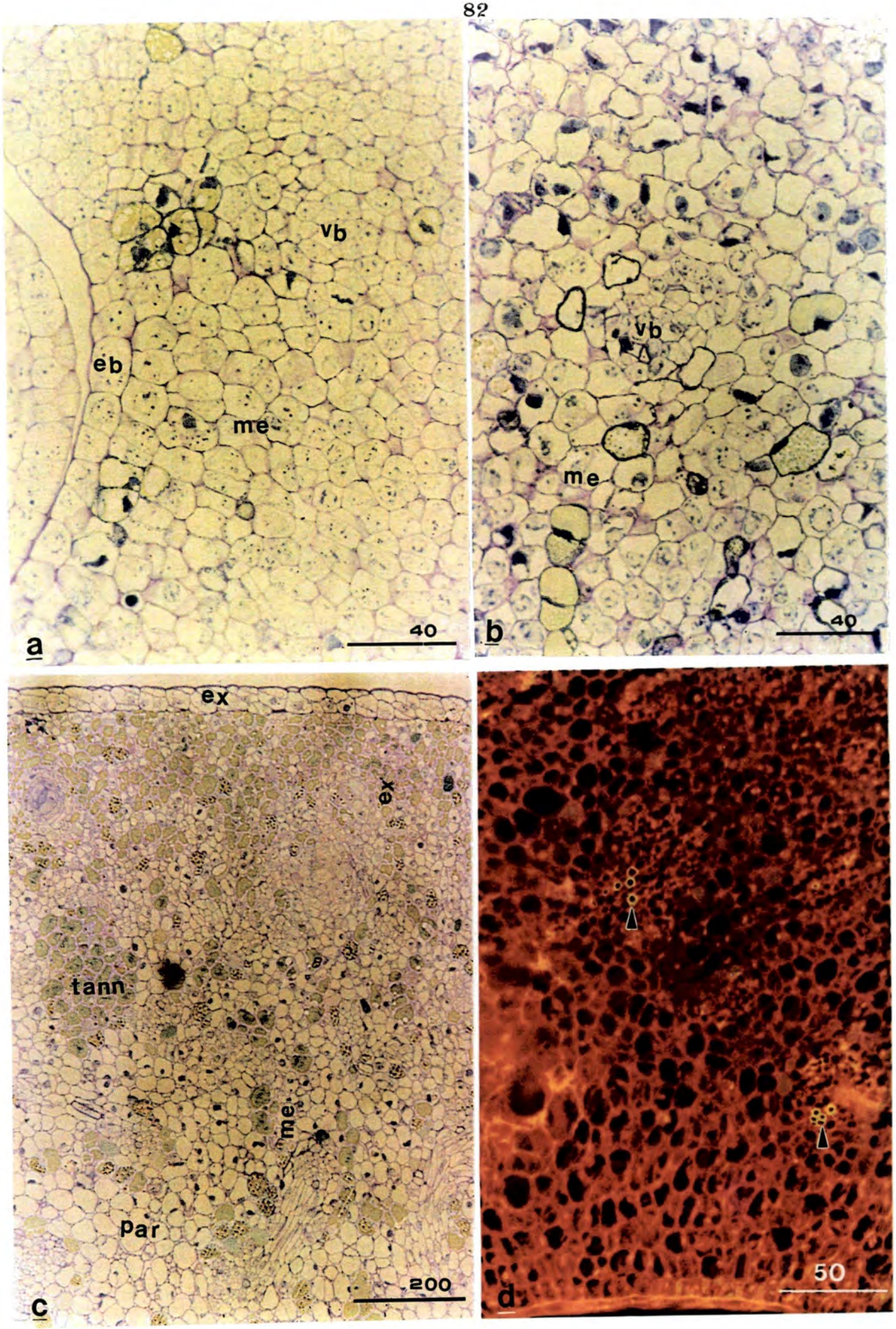


Figure 20

Figure 21

Mesocarp development stages (after fertilization).
transverse sections were stained with toluidene blue and photographed under bright field illumination.

(a) 7 days after fertilization.
(b) 15 days after fertilization.
(c) 30 days after fertilization.
(d) 45 days after fertilization.

cu, cuticle; en, endocarp; ex, exocarp; ch, chloroplast-containing parenchyma; me, mesocarp; in, intercellular space; r, resin duct; par, parenchyma; ph, phloem fibre; sc, sclerenchyma fibre; tann, tannin-filled parenchyma; vb, vascular bundle; xy, xylem.

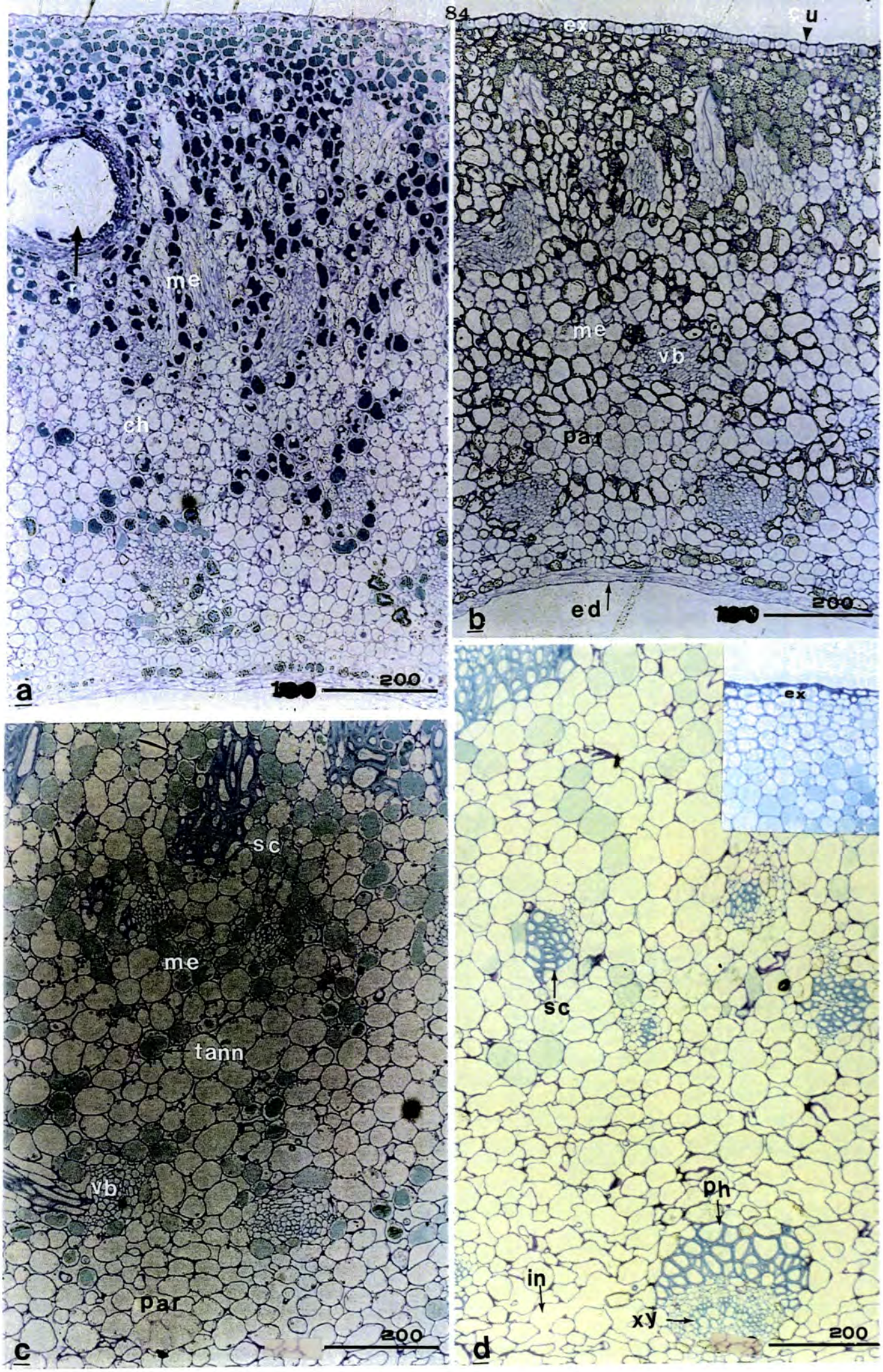

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Figure 21

Figure 22

Vascular bundles development in mesocarp at late stages (AF). Transverse sections were stained with toluidene blue and photographed under bright field illumination.

(a) 7 days after fertilization.
(b) 30 days after fertilization.
(c) 45 days after fertilization.

in, intercellular space; par, parenchyma; tann, tannin-filled parenchyma; dart, chl, chloroplast and arrow, lignified cell in the vascular bundle.

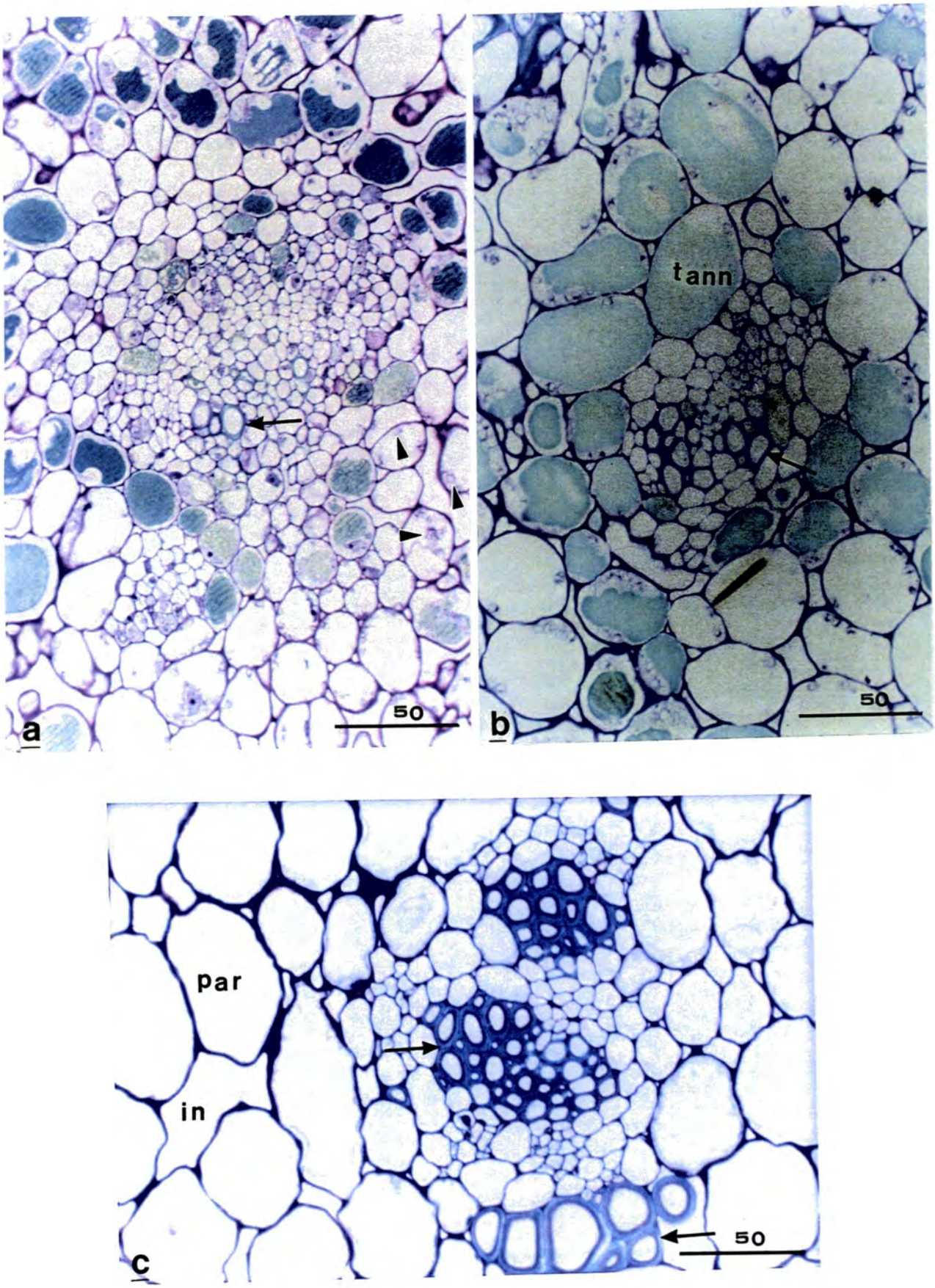


Figure 22

Figure 23

Vascular bundle development in mesocarp (showing lignified cells). Transverse sections a, b & d were stained with acridine orange and photographed under fluorescence illumination using blue filter, and c was stained with Colcofluor and photographed under fluorescence illumination using UV filter.

(a) 7 days after fertilization.
(b) 30 days after fertilization.
(c) 30 days after fertilization.
(d) 45 days after fertilization.

cell, cellulose deposition; ph, phloem fibre; vb, vascular bundle: a. b & d show lignification as bright yellow, after staining with acridine orange, and c shows deposition of the cellulose as blue bright by after staining with calcofuor; xy, xylem; dart, lignified cells

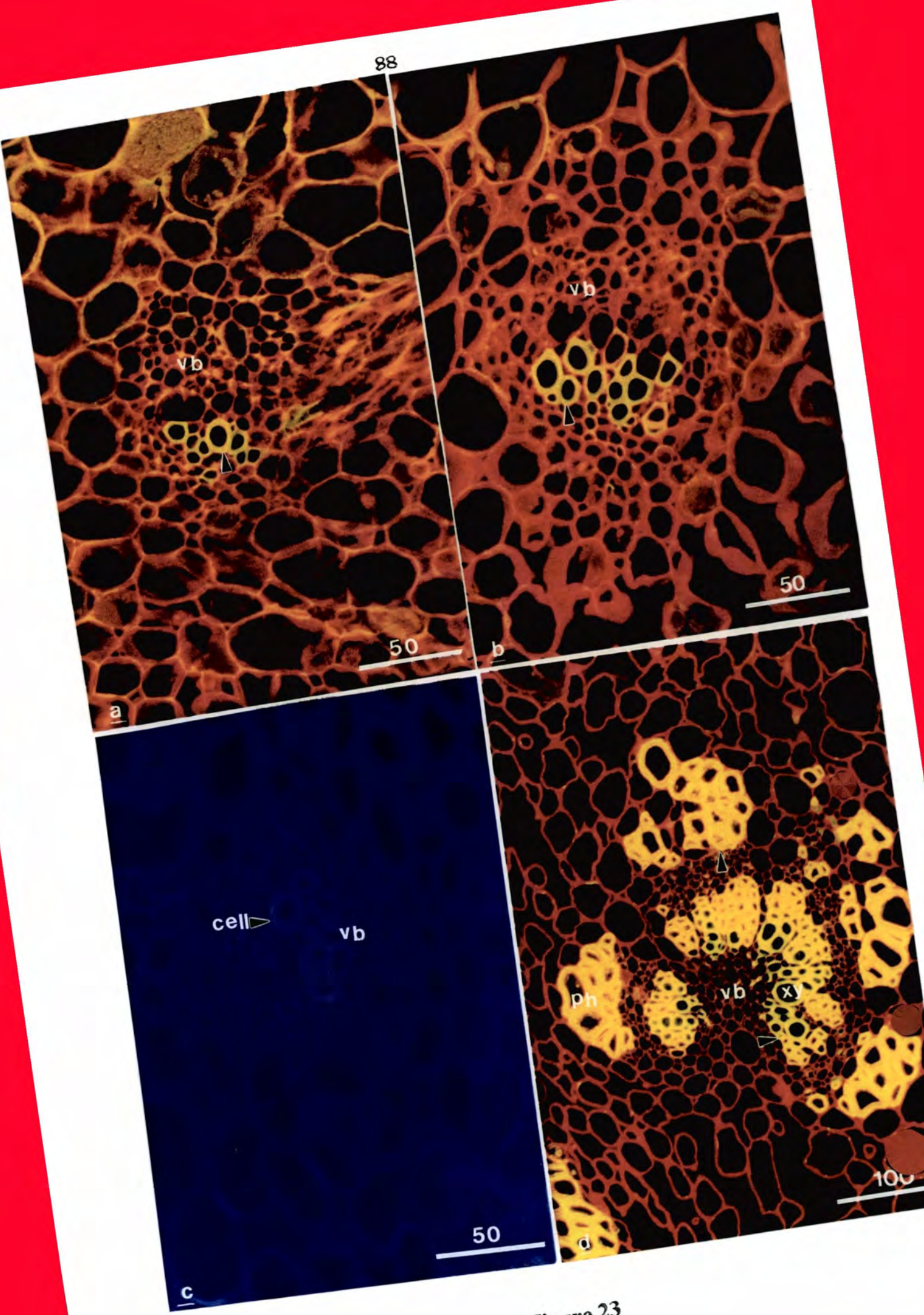


Figure 23

By 45 days after fertilization the mesocarp develops very thick-walled sclerenchyma fibre, and large parenchyma cell of irregular shapes. The parenchyma had large intercellular spaces adjacent to the endocarp (Fig. 21d). The vascular bundle had very thick-walled sclerenchyma fibre and xylem cells (Fig. 22c), and acridine orange staining resulted in substantial fluorescence under UV light indicating that the walls were now heavily lignified (Fig. 23d).

3.3.3 ENDOCARP DEVELOPMENT

At the earliest stage examined (10 mm ovary; B.F.) the endocarp consisted of a single layer of isodiametric cell with thin cell walls. The non-vacuolate cells contained a prominent nucleus with more than one nucleolus. A thin cuticle is seen on the outer wall (Fig. 24a).

The endocarp of the 15 mm ovary (before fertilization) showed a layer of slightly irregularly arranged isodiametric cells. The cells were thin-walled and contained prominent nuclei often showing multiple nucleoli. Vacuolation had started to occur (Fig. 24b).

Following further enlargement of the developing ovary (18 mm, B.F.) the endocarp had formed more than two layers of radially-elongated cells. The highly-vacuolate cells are seen to be thin-walled and with prominent central nuclei (Fig. 24c).

Further periclinal divisions result in the endocarp of the 20 mm ovary (before fertilization) containing four layers of elongate cells (Fig. 24d). The cells remain thin-walled, although the inner surface of the fruit-wall is accumulating a slightly thicker cuticle.

At 7 days after fertilization the endocarp still contains four layers of cells, elongated in longitudinal plane of the fruit, indicating that cell division within the layer ceased just prior to fertilization. The cells remain thin-walled (Fig. 24e).

By 21 days after fertilization the endocarp still contained four layers of thin-walled cells which had elongated in the longitudinal plane (Fig. 24f). Within the carpel wall the adjacent mesocarp is seen to containing an increasing amount of intercellular air-spaces around the expanding parenchyma cells.

Figure 24

Endocarp development stages (before and after fertilization).
Transverse sections (a and d), and longitudinal sections (c, d, e & f) were stained with toluidene blue and photographed under bright field illumination.

(a) 10 mm ovary before fertilization.
(b) 15 mm ovary before fertilization.
(c) 18 mm ovary before fertilization.
(d) 20 mm ovary before fertilization.
(e) 7 days after fertilization.
(f) 21 days after fertilization.

en, endocarp; cu, cuticle; int, intercellular space; me, mesocarp; n, nucleus; vb, vascular bundle and dart, cell division.

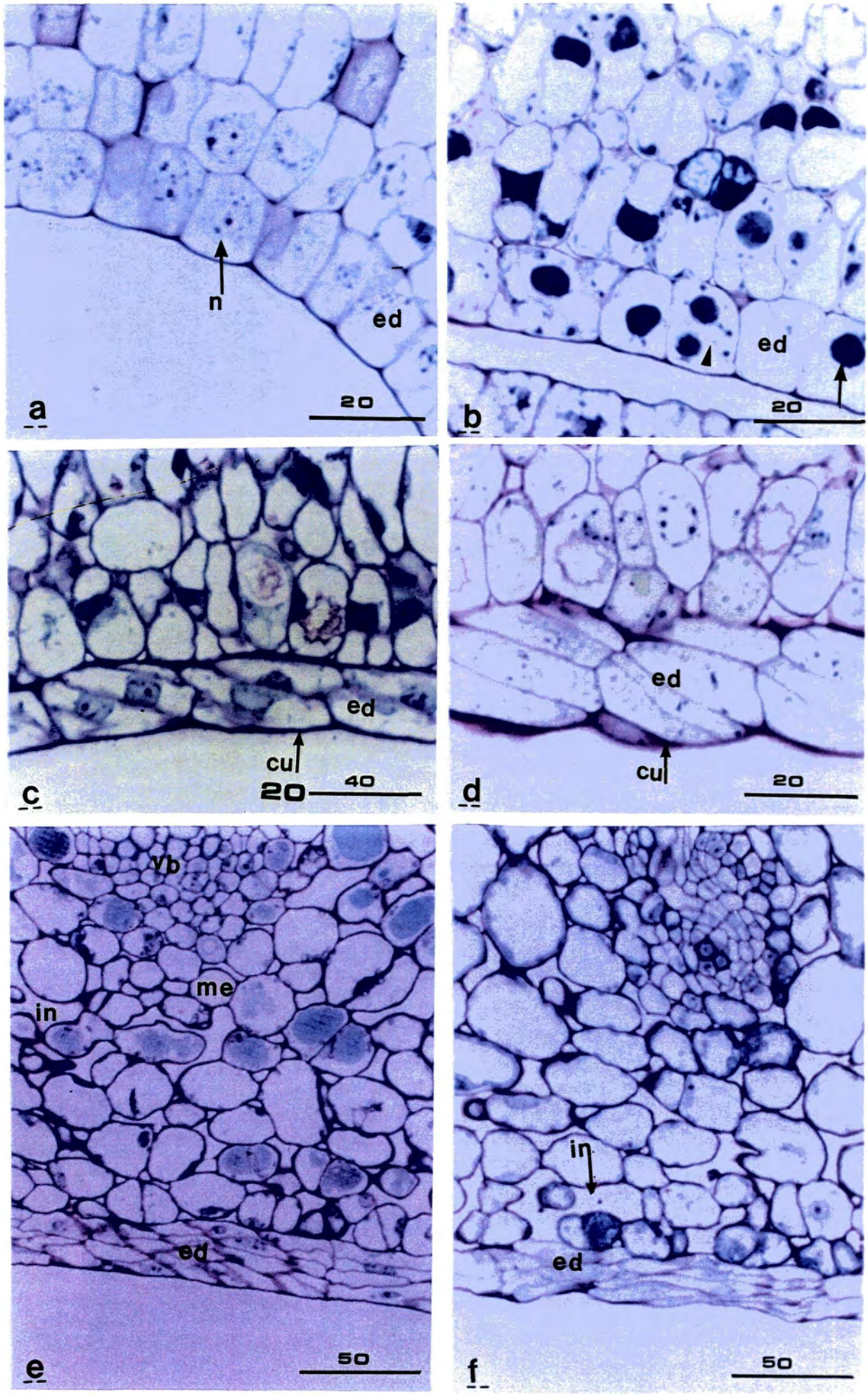


Figure 24

At 30 days after fertilization the endocarp had developed thick-walled cells (Fig. 25a). Staining with acridine orange indicated that few of the thick-walled cells had lignified (Fig. 25c). However by 45 days after fertilization not only had all of the endocarp cells developed thick walls (Fig. 25b) but acridine orange staining resulted in substantial fluorescence under UV light, indicating that the walls were now heavily lignified (Fig. 25d).

3.3.4 SEPTA DEVELOPMENT

Pre-fertilization

Septa were initiated at the first stage (10 mm) examined before fertilization, when the locular areas were closed by the developing ovary. The septa are bordered on both sides by a single layer of isodiametric cells of the endocarp. The septum wall contained 14-15 layers of slightly-irregular parenchymatous cells (Fig. 4c).

At a later stage (15 mm, B.F.) the septum had developed more cells layers, and contained 17-18 layers of irregular parenchymatous cells with different shapes and sizes, although still covered by a single-layered endocarp (Fig. 5a).

By 18 mm (B.F.) the septum had developed pink staining coloured mucilage glands, and primary vascular bundles (Fig. 5b).

Post-fertilization

At 7 days after fertilization the septum is covered by the multilayered endocarp which consisted elongated cells with thin cell walls. Within the septum developing vascular bundles, containing some lignified tissue, and also pink-coloured mucilage glands are seen (Fig. 26a).

Two weeks after fertilization the septum started to split from the base of the carpel to its apex, with the split developing through the central aerenchyma of the septum, and around the septal vascular elements. The septum wall was bordered by the multilayered endocarp which consisted elongated cells (Fig. 26b). The vascular elements within the septum become increasingly lignified, compare Fig. 26c and Fig. 26b although the bundles are far less lignified than those in the mesocarp (compare with Fig. 23c).

Figure 25

Endocarp development at the late stages (after fertilization). Longitudinal (a) and and transverse section (b) were stained with toluidene blue and photographed under bright field illumination, and longitudinal (c) and transverse section (d) were stained with acridine orange and photographed under fluorescence illumination using blue filter.

(a) 30 days after fertilization.
(b) 45 days after fertilization.
(c) 30 days after fertilization.
(d) 45 days after fertilization.

en, endocarp; dart, lignified cells; in, intercellular space; ph, phloem fibre; sc, sclerenchyma fibre, vb, vascular bundle; xy, xylem.

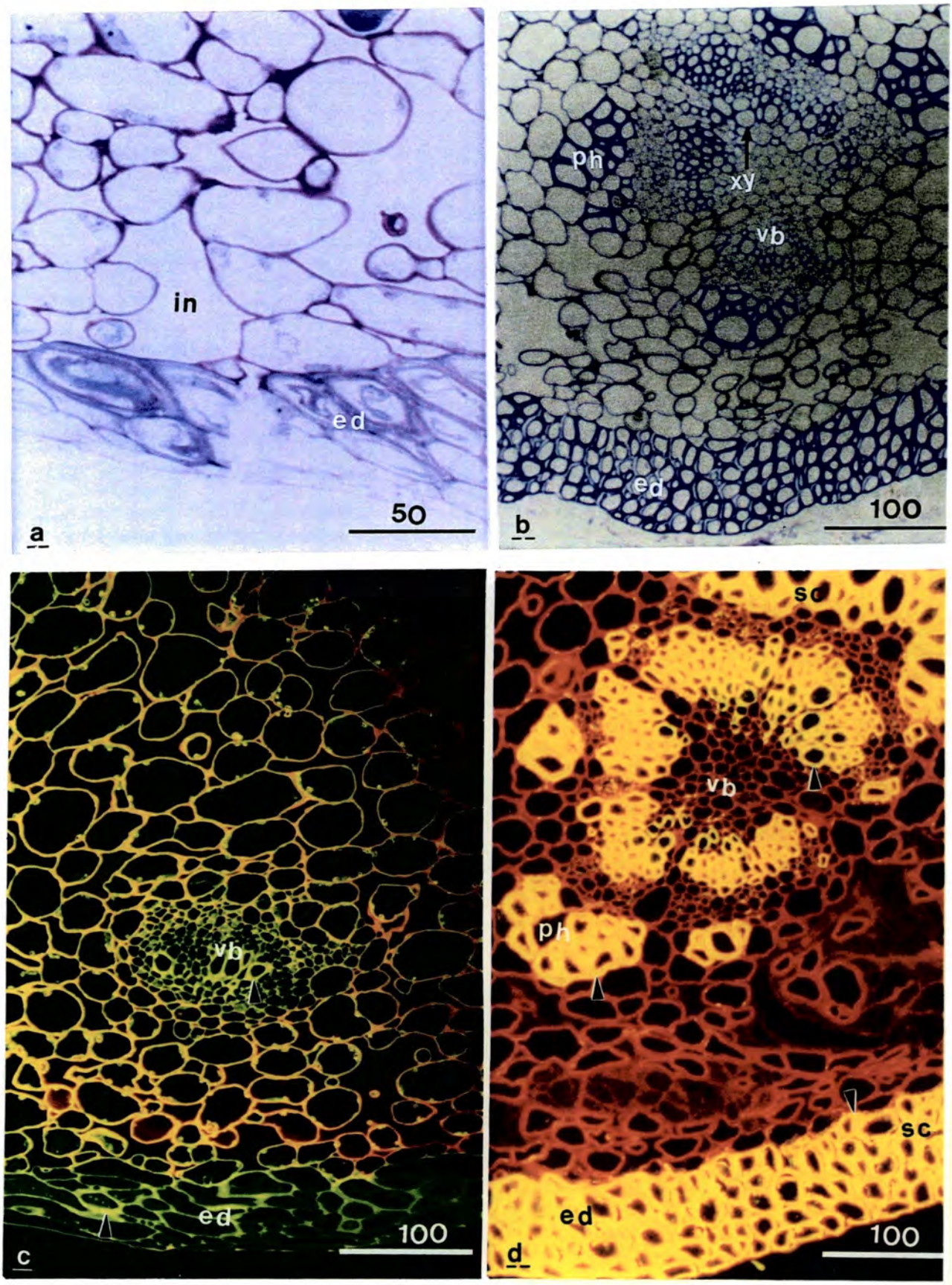


Figure 25

Figure 26

Septa development at different stages (after fertilization).
Transverse sections (a, b and c) were stained with toluidene blue and photographed under bright field illumination, and (d & e) were stained with acridine orange and photographed under fluorescence illumination using blue filter.

(a) 7 days after fertilization.
(b) 15 days after fertilization.
(c) 15 days after fertilization.
(d) 21 days after fertilization.
(e) 21 days after fertilization.

d, dehiscence zone; mu, mucilage glands; par, parenchyma; vb, vascular bundle; dart, lignified cell.

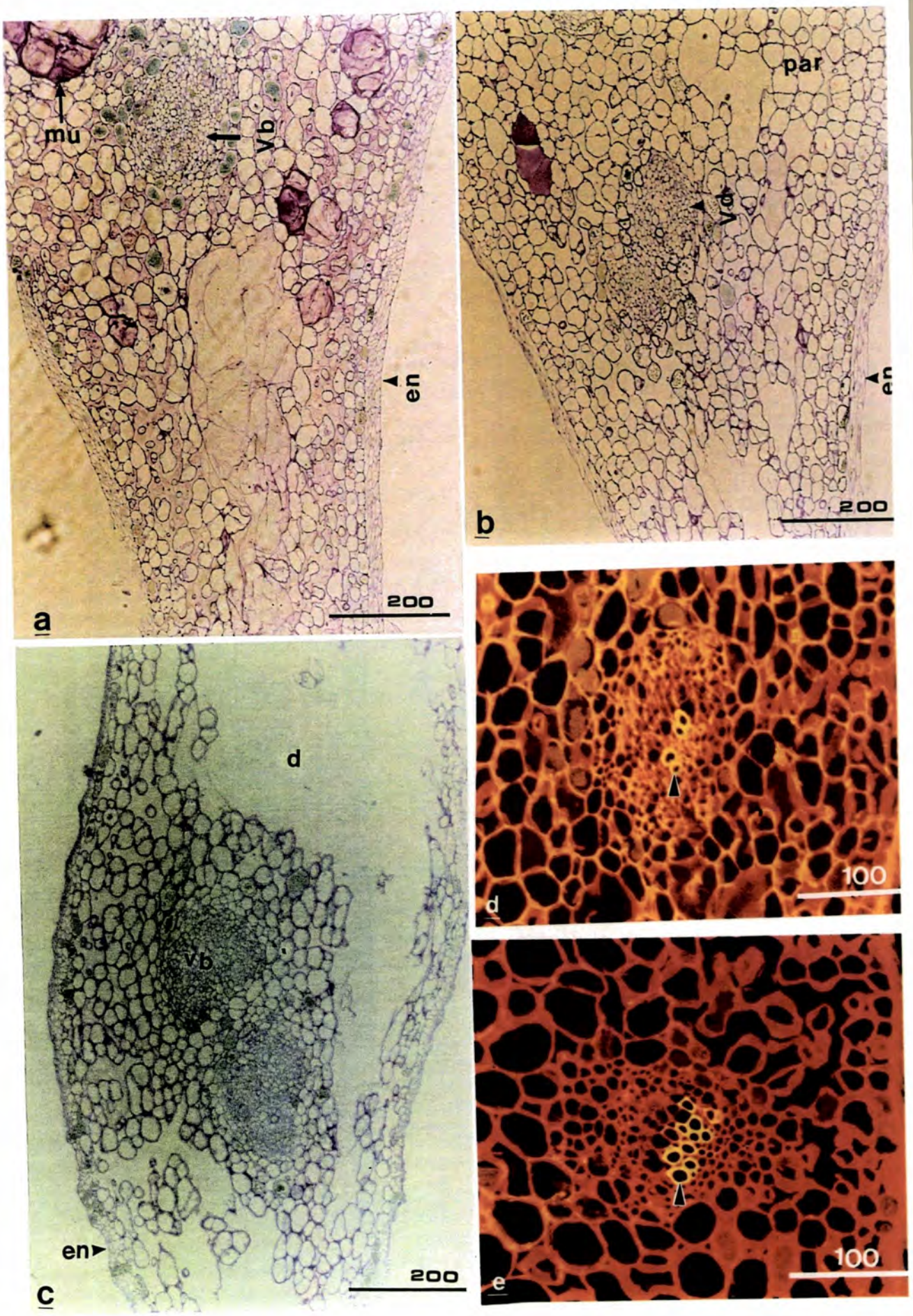


Figure 26

By 3 weeks after fertilization the septum has divided into two branches by a suture between the parenchyma cells. The outer sides of both branches were bordered by the mulltilayered endocarp consisted elongated cells with thin cell walls. The vascular bundle contained thick walled lignified cells (Fig. 26d, e).

At 4 weeks after fertilization the septum is fully divided into two parallel branches from the base to the top of the carpel. The multilayered endocarp shows accumulation of a substantial secondary wall (fig. 27a), and the vascular bundles contain many highly lignified cells (Fig. 27b).

An increase in lignification of the endocarp and vascular elements of the septum is apparent between 30 and 45 days after fertilization (Fig. 27b, d) by which time the fruit is nearly mature.

3.3.5 DEHISCENCE ZONE DEVELOPMENT

Pre-fertilization

The dehiscence zones (sutural areas) in the cotton boll are initiated at early stage of development of the ovary (10 mm B.F.), at which time 3-4 layers of isodiametric cells are seen as radial strips running through the mesocarp from the exocarp to the inner surface of the fruit wall. This made a vertical wall into the locular area of ovary against the mesocarp layers (Fig. 28a).

With the further development of the ovary to 15 mm size a vertical column of 4-5 layers of parenchyma cells has formed as a dehiscence zone. The dehiscence zone contains slightly smaller cells than the other adjacent mesocarp cells (Fig. 28b). With further development of the ovary (18 mm, B.F.) the dehiscence zone becomes more clear in comparison to the previous stages (Fig. 28c).

Post-fertilization

After fertililzation there is no further division of the cells of the dehiscence zones which have formed in the ovary wall. However, substantial and complex changes occur in the adjacent cells of the mesocarp. The dehiscence zone started to dehisce internally from 15 days after fertilization by spitting between parenchyma cells at the outer side of the zone, forming a intercellular space beneath the endocarp (Fig. 28d).

Figure 27

Septa development at late stages (AF).
Transverse sections (a and b) were stained with toluidene blue and photographed under bright field illumination, and (c and d) were stained with acridine orange and photographed under fluorescence illumination using blue filter.

(a) 30 days after fertilization.
(b) 45 days after fertilization.
(c) 30 days after fertilization.
(d) 45 days after fertilization.

ac, aerenchyma; en, endocarp; in, intercellular space; ph, phloem; xy, xylem; dart, lignified cell.

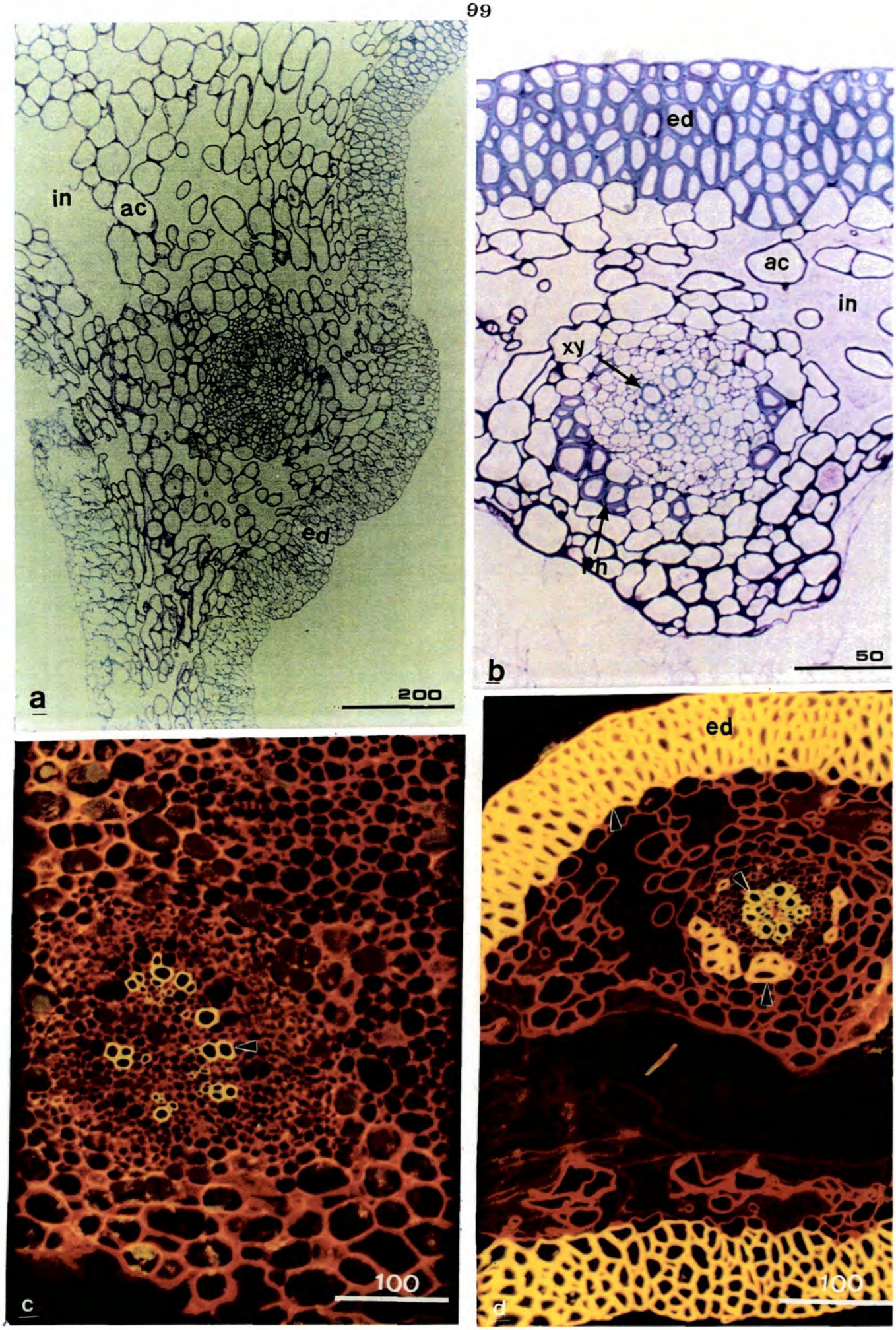


Figure 27

Figure 28

Dehiscence zone development at different stages (before and after fertilization).
Transverse sections were stained with toluidene blue and photographed under bright field illumination.

(a) 10 mm ovary before fertilization.
(b) 15 mm ovary before fertilization.
(c) 18 mm ovary before fertilization.
(d) 15 days after fertilization.
(e) 30 days after fertilization.

d, dehiscence zone; en, endocarp; ex, exocarp; in, inter cellular space; l, locular area; me, mesocarp; par, parenchyma; sc, sclerenchyma; vt, vascular tissue.

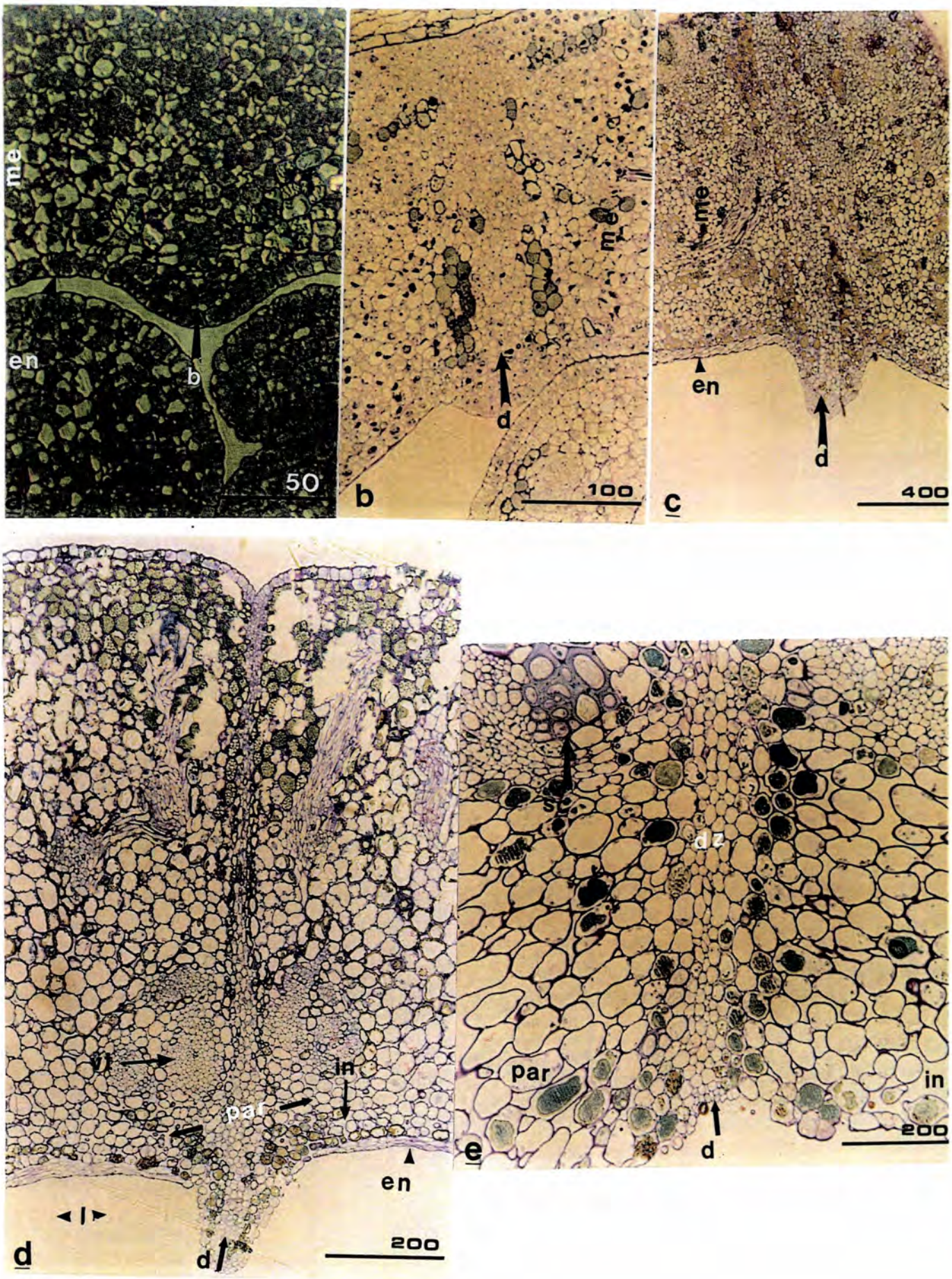


Figure 28

To the inside, the development of a layered split separates the endocarp from the adjacent regions of the dehiscence zones (Fig. 28e)

On either side of the dehiscence zones mesocarp cells develop highly-thickened, and subsequently-lignified walls (Fig. 29a, b). At 45 days after fertilization the dehiscence zone consisted of 4-5 layers of small parenchyma cells with thin cell walls. The outer portion of zone contained typical small parenchyma cells and their arrangement was very compact. They were covered in a half circle by a valve which contained lignified cells with thick cell walls (Fig. 30a). The outer end of dehiscence zone was covered with a thick cuticle, exocarp and subexocarp layers, and both sides were bordered by tannin-filled parenchyma (Fig. 29a). The inner portion of the dehiscence zone was bordered by the highly lignified sclerenchyma fibres (Fig. 30b) with very thick cell walls. The sutural parenchyma formed very large intercellular space and the sutural area dehisced by the splitting of sutural parenchyma cells (Fig. 29b). Later, at time of boll opening, the carpels were fully separated from each other (Fig. 3c).

3.4 HISTOCHEMISTRY OF THE OVARY

3.4.1 ESTERASE

Pre-fertilization

Esterase activity in the cotton ovary showed as blue precipitation on cryostat sections after staining with Fast blue BB, when naphthol was used as a substrate reagent. The control sections did not show blue deposition throughout any stages. Pre-and post-fertilized tissues at different stages of ovary development were used for esterase enzyme localisation.

At the earliest stage of the ovary examined (15 mm) highest esterase activity was found in the vascular bundles of the sepal wall, in the vascular tissues in the staminal column, and in the nucellus of the ovule (Fig. 31a, 31c and 31d). The inner and outer integument (excluding the outer epidermal layers of outer integument), the mesocarp of staminal column, placenta, septum, endocarp and mesocarp of sepal wall, all showed low esterase activity. No esterase activity was found associated with the inner few layers and outer epidermal layers of the sepal wall (Fig. 31a), the inner and outer border of the staminal column (Fig. 31c), or the exocarp of the fruit wall (Fig. 31d).

Esterase activity was evident in the vascular bundles of the sepal wall, vascular tissues of the staminal column and the nucellus of the ovule of the 18 mm ovary (Fig. 32a, 32b,32e) apparently at a slightly higher level, but activity was apparently less in the

Figure 29

Dehiscence zone development at stage of maturity.
Transverse sections were stained with toluidene blue and photographed under bright field illumination.

(a) 45 days after fertilization.
(b) 45 days after fertilization.

d, dehiscence zone; ex, exocarp; in, intercellular space, sc, sclerenchyma fibre; val, valve.

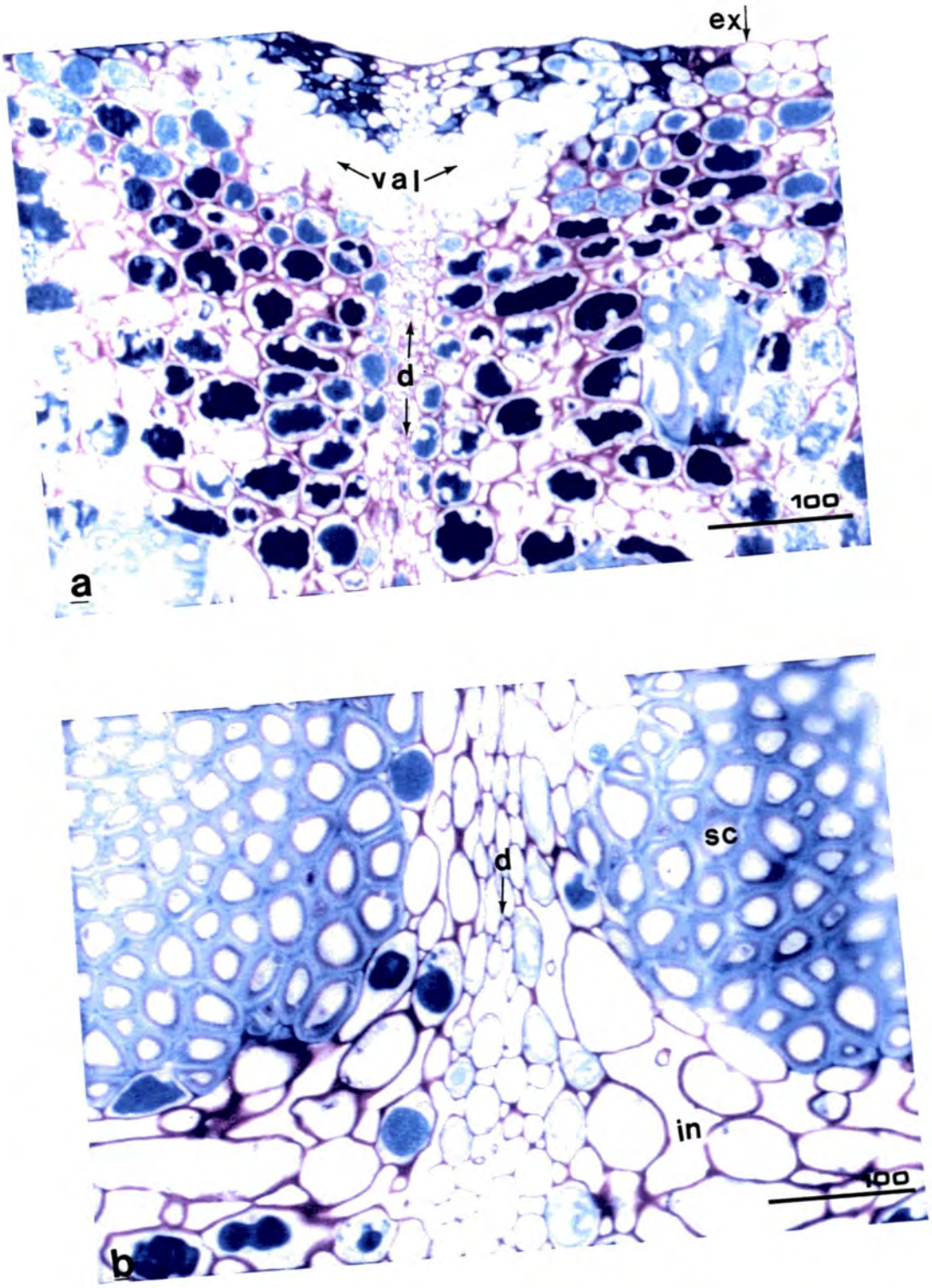


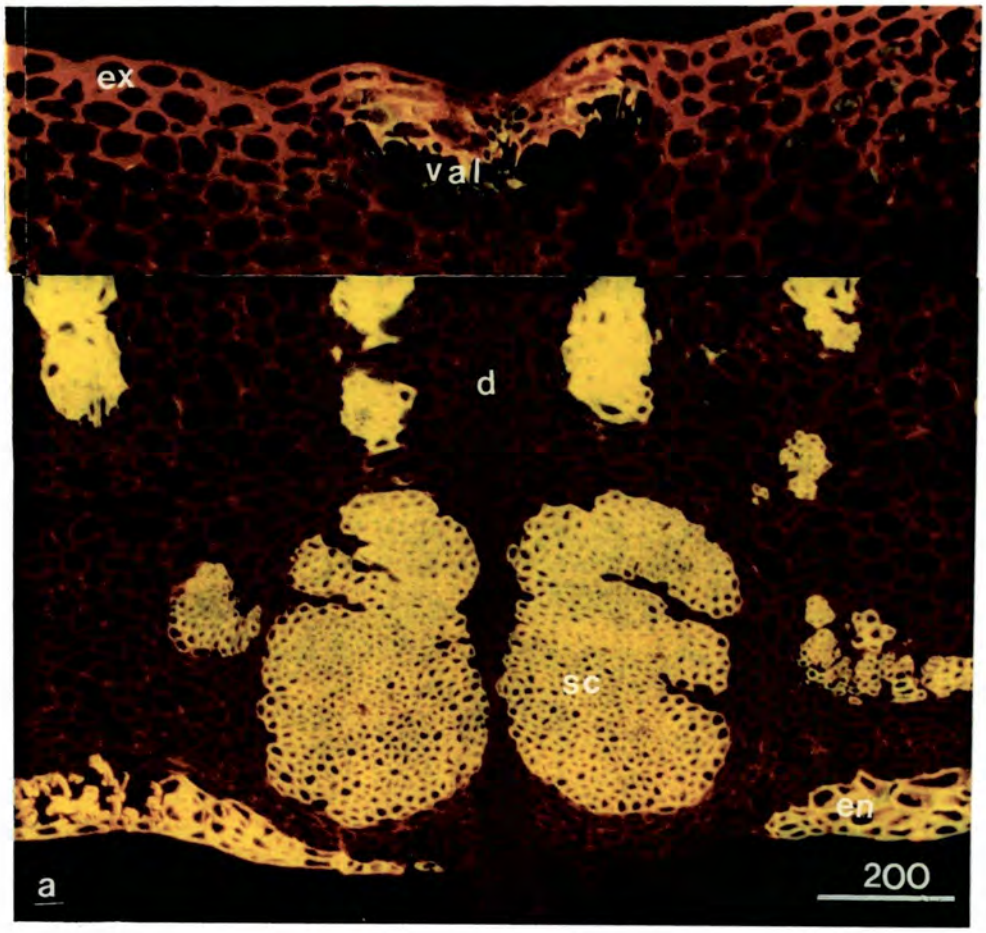
Figure 29

Figure 30

Dehiscence zone development at stage of maturity (showing the lignification of sclerenchyma fibres).
Transverse sections were stained with acridine orange and photographed under fluorescence illumination using blue filter.

(a) 45 days after fertilization.
(b) 45 days after fertilization.

d, dehiscence zone; en, endocarp; ex, exocarp; l, locular area; sc, sclerenchyma fibre; val, valve.



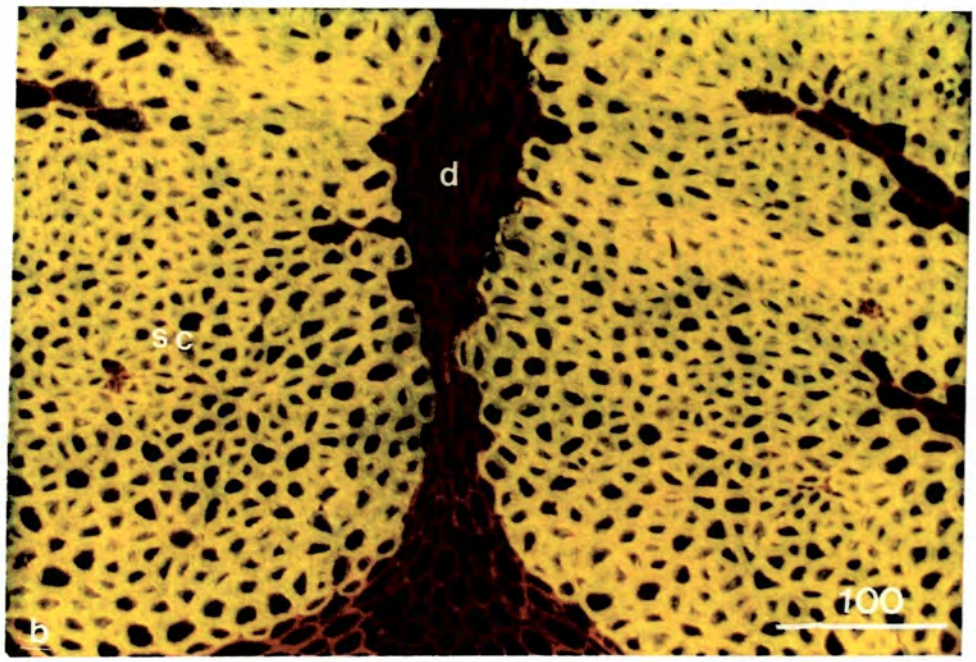


Figure 30

Figure 31

Localization of esterase on the floral organs of 15 mm ovary (AF). Esterase activity is shown as blue deposits.
Transverse sections were stained with naphthol and Fast blue BB and photographed under bright field illumination.

(a) Sepal wall.
(b) Sepal wall. (control)
(c) Staminal column.
(d) Ovary.

e, inner and outer epidermis; en, endocarp; ii, inner integument; oi, outer integument; me, mesocarp; nu, nucellus; s, septum; vb, vascular bundle; vt, vascular tissue.

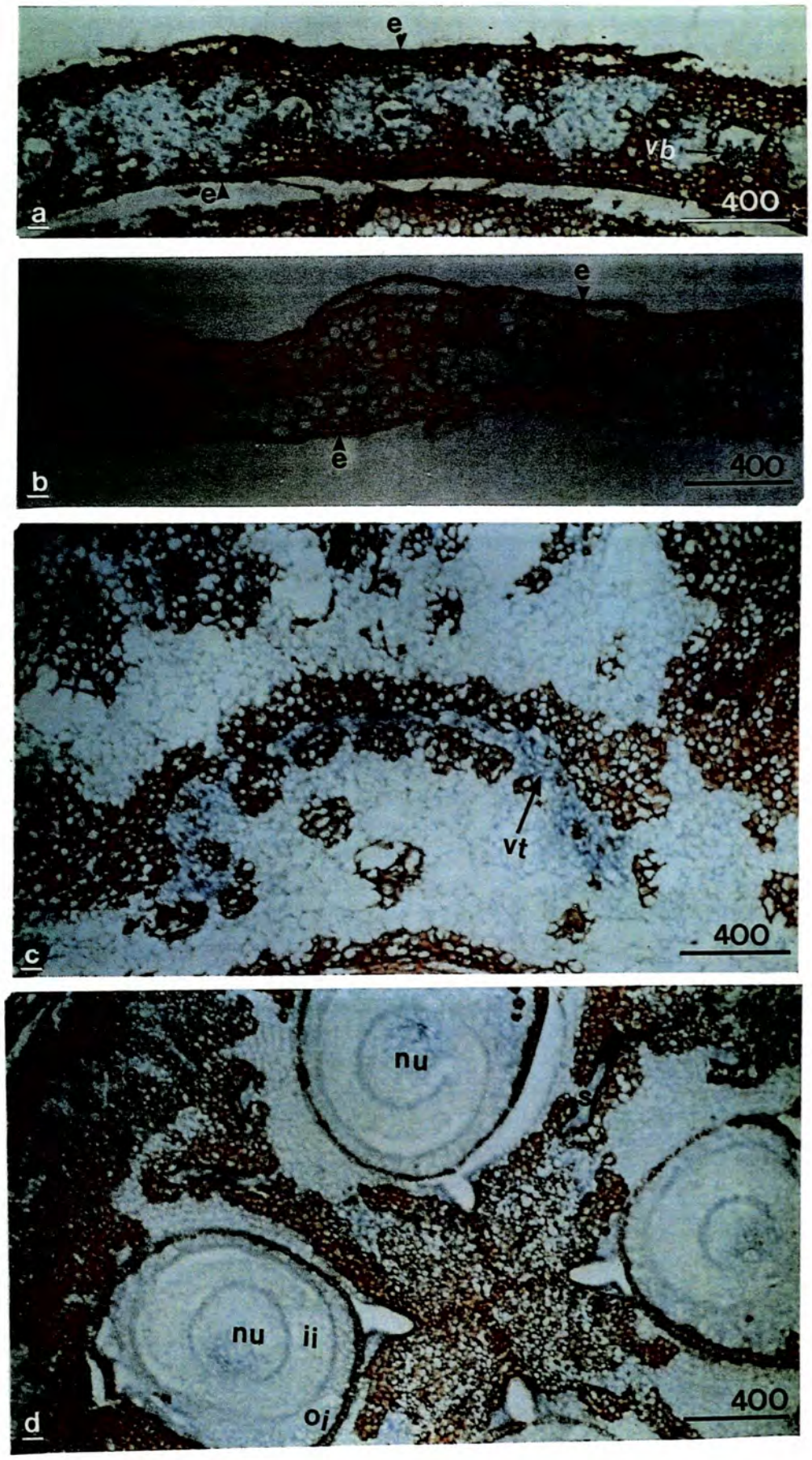


Figure 31

Figure 32

Localization of esterase on the floral organs of 18 mm ovary (AF). Esterase activity is shown as blue deposits.
Transverse sections were stained with naphthol and Fast blue BB and photographed under bright field illumination.

(a) Sepal wall.
(b) Staminal column.
(c) Staminal column. (control)
(d) Ovary. (control)
(e) ovary.

e, inner and outer epidermis; en, endocarp; ii, inner integument; me, mesocarp; nu, nucellus; oi, outer integument; s, septum.

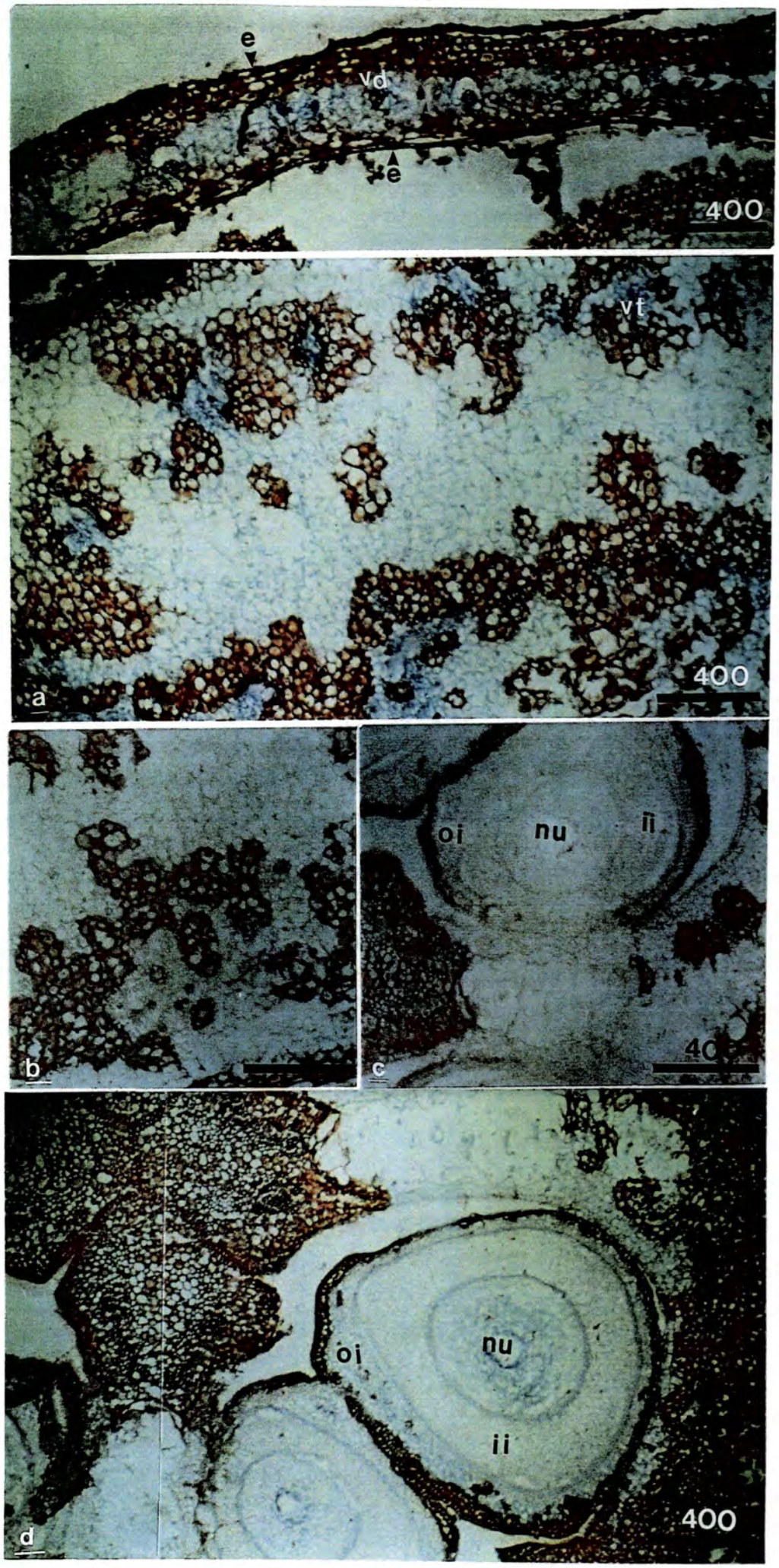


Figure 32

septum, placenta, mesocarp of the sepal wall, and endocarp (Fig. 32a, 32d); the control treatment did not show any esterase activity (Fig. 31b, 32c, 32d).

Post-fertilization

The highest levels of esterase activity recorded within the early developing seed were found associated with the nucellus of the ovule and the tip of placenta, and the lowest activity was observed on the inner integument of the ovule and carpel wall (Fig. 33a). Esterase activity was absent from the outer epidermis layer of the ovule, septum, and exocarp of the fruit wall.

At 7 days after fertilization esterase activity was greater in the tissues of the ovary and highest esterase was detected on developing fibres, in the nucellus of the ovule (Fig. 33b and 34a), vascular bundles of the carpel wall (Fig. 35a) and associated with the septum (Fig. 35c). Low activity was noted in the inner integument of the ovule. Esterase was absent from the outer epidermis of inner integument, the outer epidermis of outer integument, exocarp, and mesocarp excluding the vascular bundles, and the endocarp. The controls did not show esterase activity (Fig. 34b, 35b and 35d).

No significant difference was observed in esterase activity in fibres and septum wall of 7 day and 15 day-old ovaries. Esterase in the inner integument of the ovule was apparently more active than in the previous stage (Fig. 33c). The esterase activity declined on the vascular bundle of carpel wall and the nucellus of ovule. The controls showed no esterase activity (Fig. 34c).

The esterase activity reached its lowest level in all parts of the ovary, except the fibres, 21 days after fertilization, when only the fibres showed esterase activity (Fig. 33d). At a later stage, 30 days after fertilization, no significant esterase activity was observed in any parts of ovary.

3.4.2 CELLULOSE

Cellulose was detected as bright white or bright blue staining on fresh tissues of ovaries, after staining with Calcofluor and examination under fluorescence light (using a UV filter).

Figure 33

Localization of esterase on the tissues of ovule of just fertilized, 7 day, 15 day and 21 day (AF) fruits.
Esterase activity is shown as blue deposits.
Transverse sections were stained with Fast blue BB and photographed under bright field illumination.

(a) Just-fertilized ovary.
(b) 7 days after fertilization.
(c) 15 days after fertilization.
(d) 21 days after fertilization.

ii, inner integument; f, fibre; oi, outer integument; nu, nucellus.

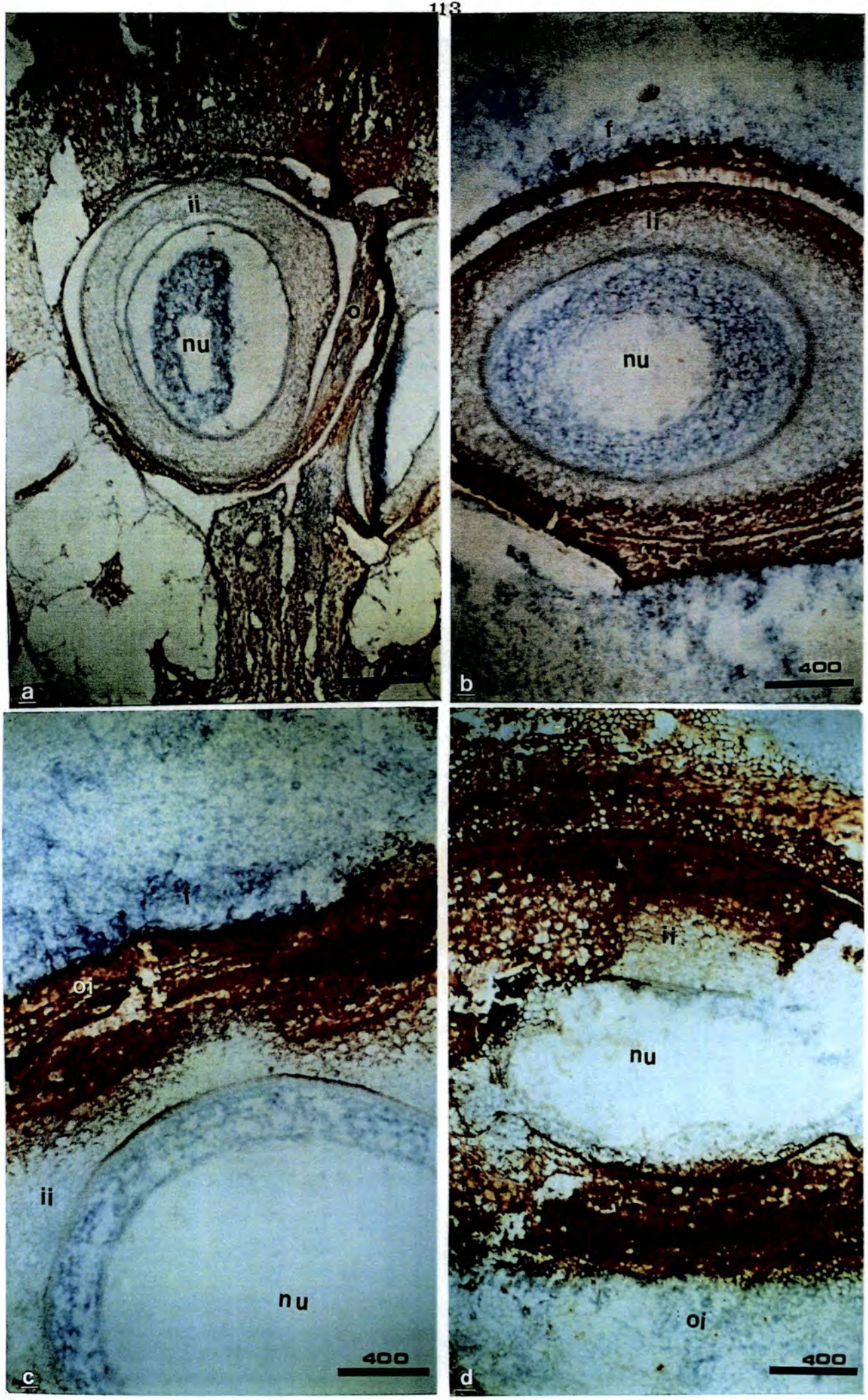


Figure 33

Figure 34

Localization of esterase on the seed coat fibre at 7 days and 15 days (AF)
Esterase activity is shown as blue deposits.
Transverse sections were stained with naphthol and Fast blue BB and photographed under bright field illumination.

(a) 7 days ovule & fibre.
(b) 7 days ovule & fibre. (control)
(c) 15 days ovule & fibre. (")

ii, inner integument; f, fibre; oi, outer integument; nu, nucellus.

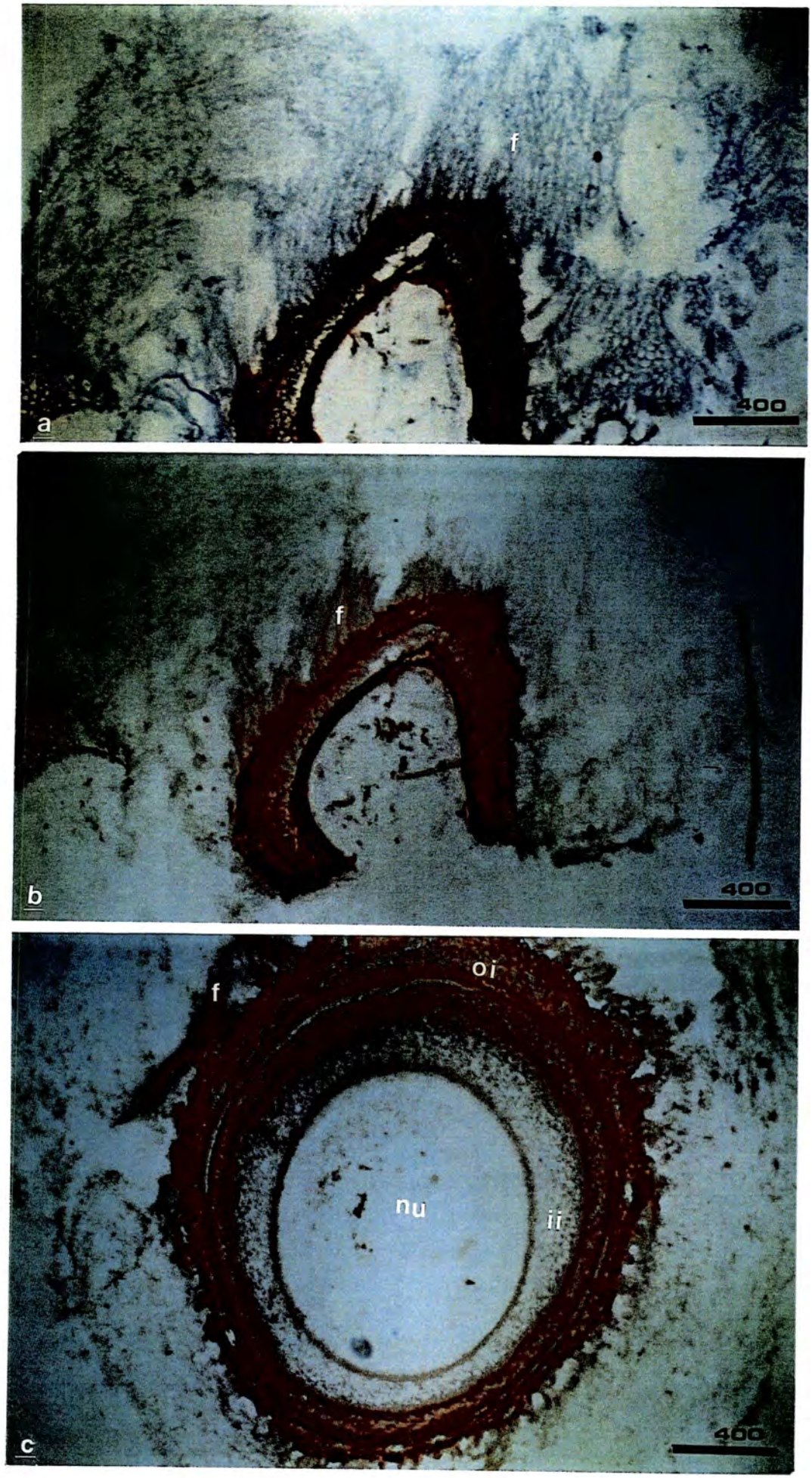


Figure 34

Cellulose was observed in the vascular bundle of the carpel wall and septum of the just-fertilized ovary. The vascular bundle showed their primary growth by the deposition of cellulose as show in Fig. 36a and 36b. Cellulose deposition increased gradually with the development of the ovary, as detected in sections of ovaries at 7 days (Fig. 36c) and 21 days after fertilization (Fig. 36d). The vascular bundles showed secondary cell wall growth by the deposition of cellulose at late stage of the ovary development (Fig. 23b, c) and then became highly lignified. Cellulose staining was not strong in the endocarp, mesocarp (except vascular bundle), septum wall (except vascular bundle), and sclerenchyma fibres at late stages of the ovary (Fig. 36c, d, e).

The ovules within the just-fertilized ovary showed Calcofluor staining of the guard cell walls, the vascular bundles of the outer integument, and the outer border of inner epidermis of the inner integument. There was, however, very low staining observed in the nucellus and the cell of the outer epidermis layer of the outer integument (Fig. 37a).

The fibres developing on the seeds showed very low staining for cellulose at 3 days after fertilization indicating very limited wall deposition at this stage, although the guard cells and the vascular bundles showed high levels of staining at this stage (Fig. 37b). Fibres developed their primary cell wall by depositing cellulose; this is seen by the increasing extent of Calcofluor staining during fibre maturation (Fig. 37c). At maturity the fibres showed highest cellulose deposition (Fig. 14b).

3.5 IMMUNOCYTOCHEMISTRY OF THE OVARY

A group of monoclonal antibodies were used for localization of specific antigenic targets in cell walls of fertilized ovaries. Immunolocalization was carried out using sections of resin-embedded material. The fluorescent label FITC conjugated to the secondary antibody was used as the marker, and specific localization was detected as bright green fluorescence which was absent from the controls, when sections were examined by epifluorescent illumination with a green filter block.

3.5.1 ARABINOGALACTAN (AGP) ANTIBODIES

A group of six monoclonal antibodies i.e. JIM4, JIM5, JIM13, JIM14, JIM15 and MAC207 were used to localized arbinogalactans (AGPs) on the tissue sections of the just-fertilized ovary. The JIM4, JIM5, JIM13, and MAC207 did not show any

Figure 35

Localization of esterase on the tissues of septum and carpel wall of 7 day ovary (AF).
Esterase activity is shown as blue deposits.
Transverse sections were stained with naphthol and Fast blue BB and photographed under bright field illumination.

(a) Carpel wall.
(b) Carpel wall. (control)
(c) Septum wall.
(d) Septum wall. (control)

en, endocarp; me, mesocarp; s, septum; vb, vascular bundle; dart, showing the esterase activity.

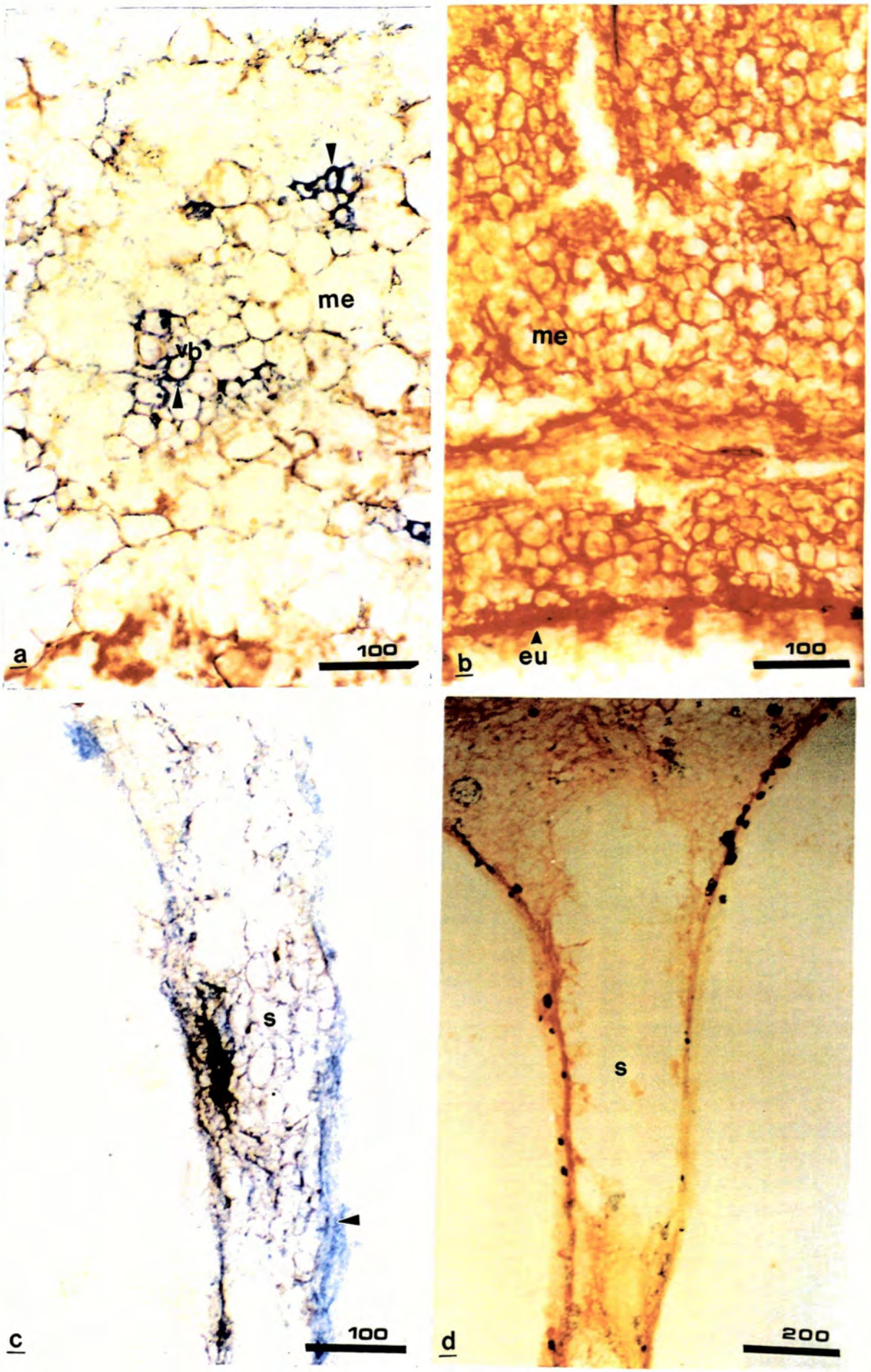


Figure 35

Figure 36

Localization of cellulose on the tissues of septum and carpel wall of just-fertilized, 15 day and 21 day ovaries (AF).
Cellulose is shown as bright blue or bright white.
Transverse sections were stained with Calcofluor and photographed under epifluorescent illumination with UV filter.

(a) Septum wall of just-fertilized ovary.
(b) Carpel wall of just-fertilized ovary.
(c) Carpel wall of 15 day ovary.
(d) Carpel wall of 21 day ovary.
(e) Carpel wall of 45 day ovary.

dehiscence zone; e, epidermis; en, endocarp; ii, inner integument; oi, outer integument; me, mesocarp; s, septum; sc, sclerenchyma; dart, showing cellulose deposition.

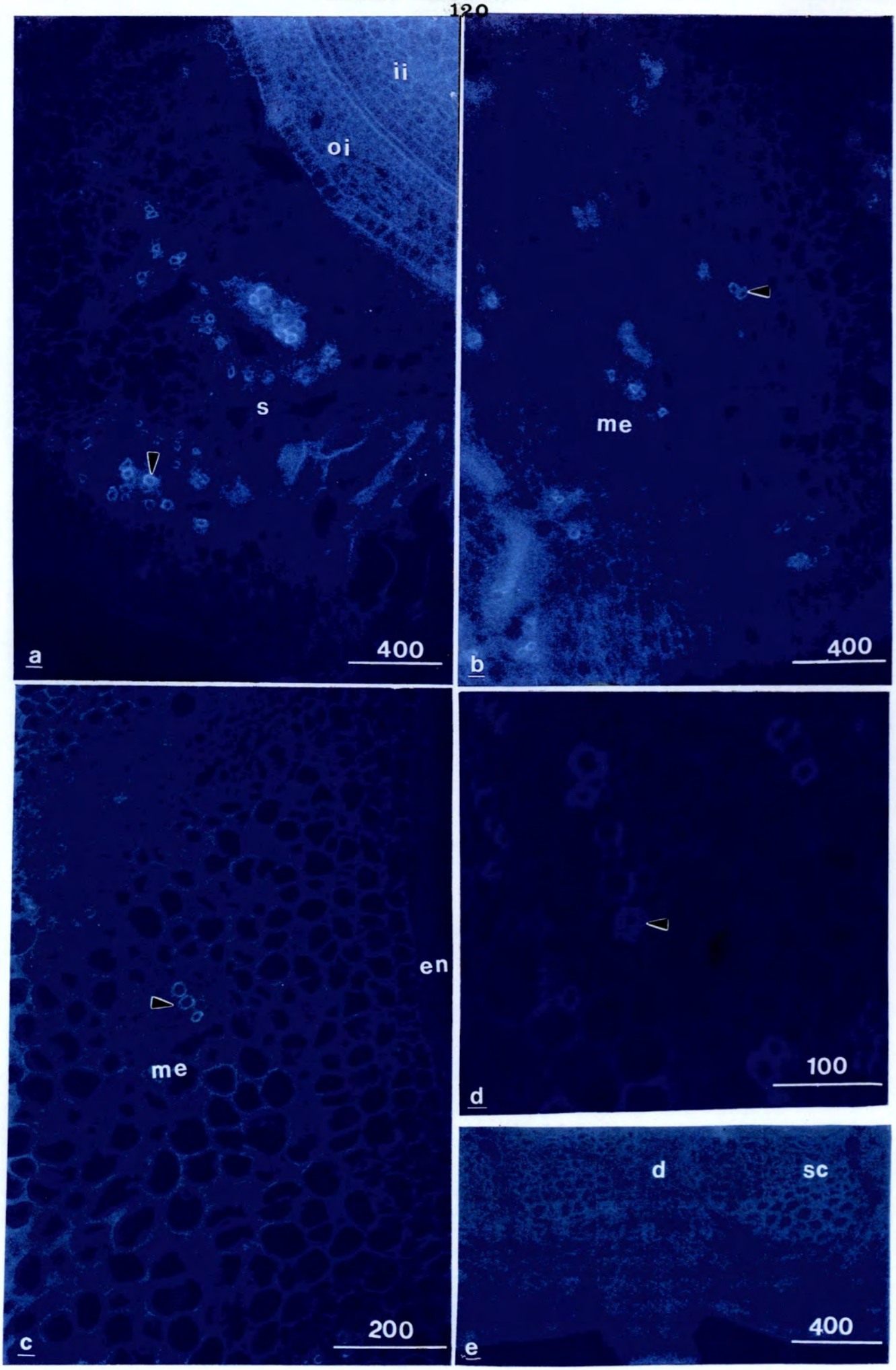


Figure 36

Figure 37

Localization of cellulose on the tissues of fibre of just-fertilized, 3 day and 21 day (AF) fruits.
Cellulose is shown as blue bright blue or bright white.
Transverse sections were stained with Calcofluor and photographed under epifluorescent illumination with UV filter.

(a) Fibre (just fertilized ovary).
(b) Fibre (3 days after fertilization).
(c) Fibre (21 days after fertilization).

e, epidermis; g, guard cells; in, inner integument; f, fibre; oi, outer integument; vb, vascular bundle; dart, cellulose deposition.

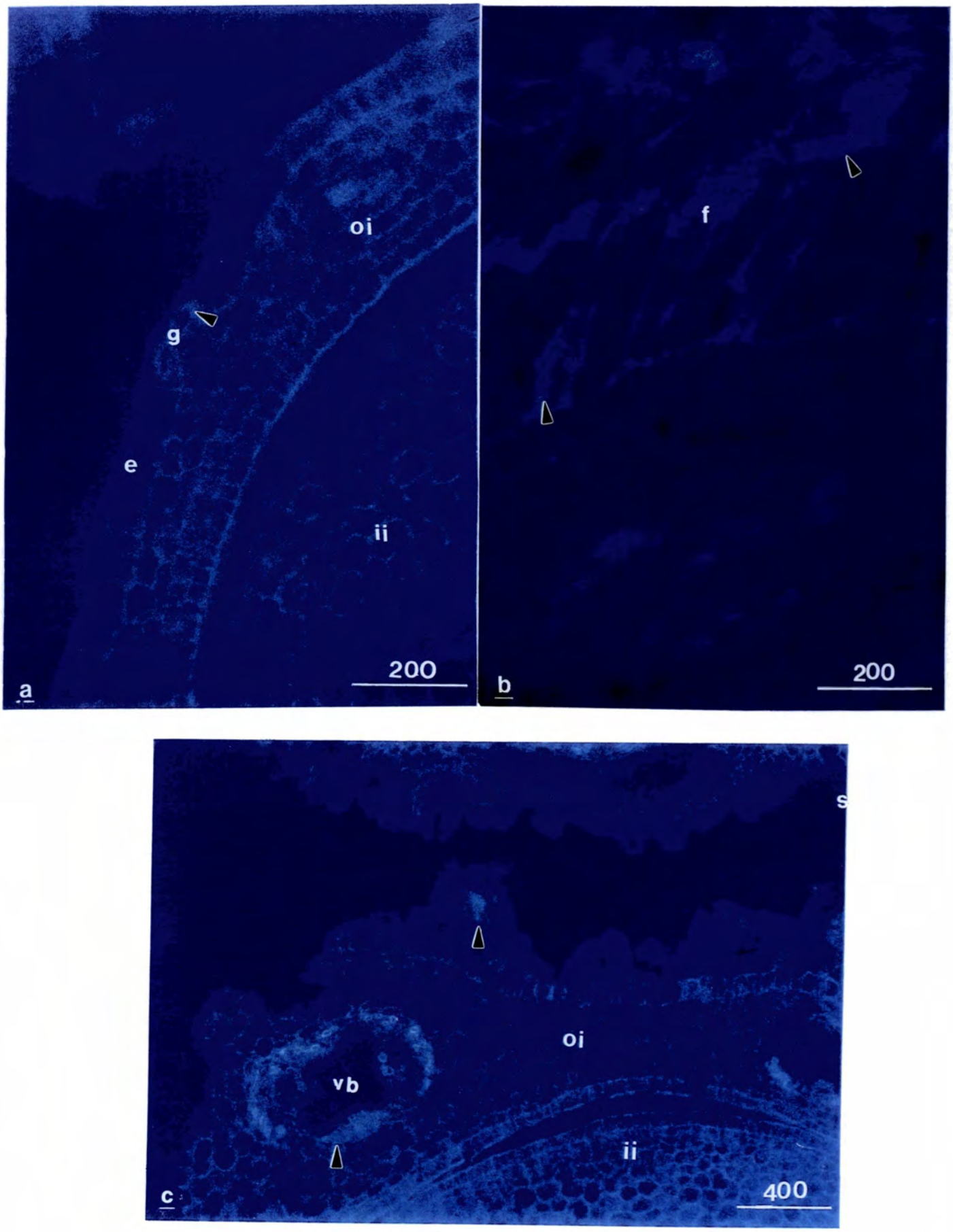


Figure 37

significant binding to the tissue section, however, JIM14 showed strong binding to the cell walls of specific group of cells within the septum (Fig. 38a, c). Interestingly immunolocatization is not restricted to just the cell walls, vesicular and reticulate structures within cells are also stained.

3.5.2 GLYCOPROTEIN ANTIBODIES

The two glycoprotein antibodies JIM11 and JIM16 did not show any significant binding on the tissue to the just fertilized ovary.

3.5.3 PECTIN LOCALIZATION

Two pectin antibodies, JIM5 and JIM7, were used for localisation of pectin within the cell walls. JIM7 showed strong binding to cell walls in all parts of the ovary, although no significant difference seen in binding in cell walls of the different types of tissue. JIM5 did not show any significant binding on the tissues.

3.6 MOLECULAR HISTOCHEMISTRY OF THE OVARY

Cellulase has been implicated in the development and dehiscence of fruit; in an attempt to establish the initial patterns of expression of the cellulase gene we have used *in situ* hybridization to localize the temporal and spatial patterns of accumulation of cellulase mRNAs.

3.6.1 CELLULASE (β 1, 4, GLUCANASE)

The distribution of cellulase mRNA was examined in sections of wax-embedded tissues from fertilized ovaries. After hybridization of a digoxygenin-labelled cDNA probe to the mRNAs, specifically-bound probe was localized by anti-digoxygenin conjugated to a alkaline phosphatase, the latter being detected histochemically as red deposits on the tissues section. Controls, including hybridization with a non-specific cDNA sequence, did not show any red deposition in the tissue sections (Fig. 39b, 40b and 40d).

In the pre-fertilized ovary (20 mm) the highest activity of cellulase mRNA was observed on the outer border of inner epidermis layer of the outer integument, and outer epidermal layer of inner integument of the ovule (Fig. 39a). Staining was also associated with the mesocarp of the carpel wall, although the probe to cellulase

Figure 38

Arabinogalactan localization in just-fertilized ovary, using monoclonal antibody (JIM14). Signal is shown as bright green staining.
After primary incubation transverse sections of just fertilized ovary were stained with FITC-secondary antibody, and photographed under fluorescence illumination.

(a) Septum wall.
(b) Septum wall. (control)
(c) Septum wall.

s, septum wall; dart, showing the signal of antibody (JIM14)

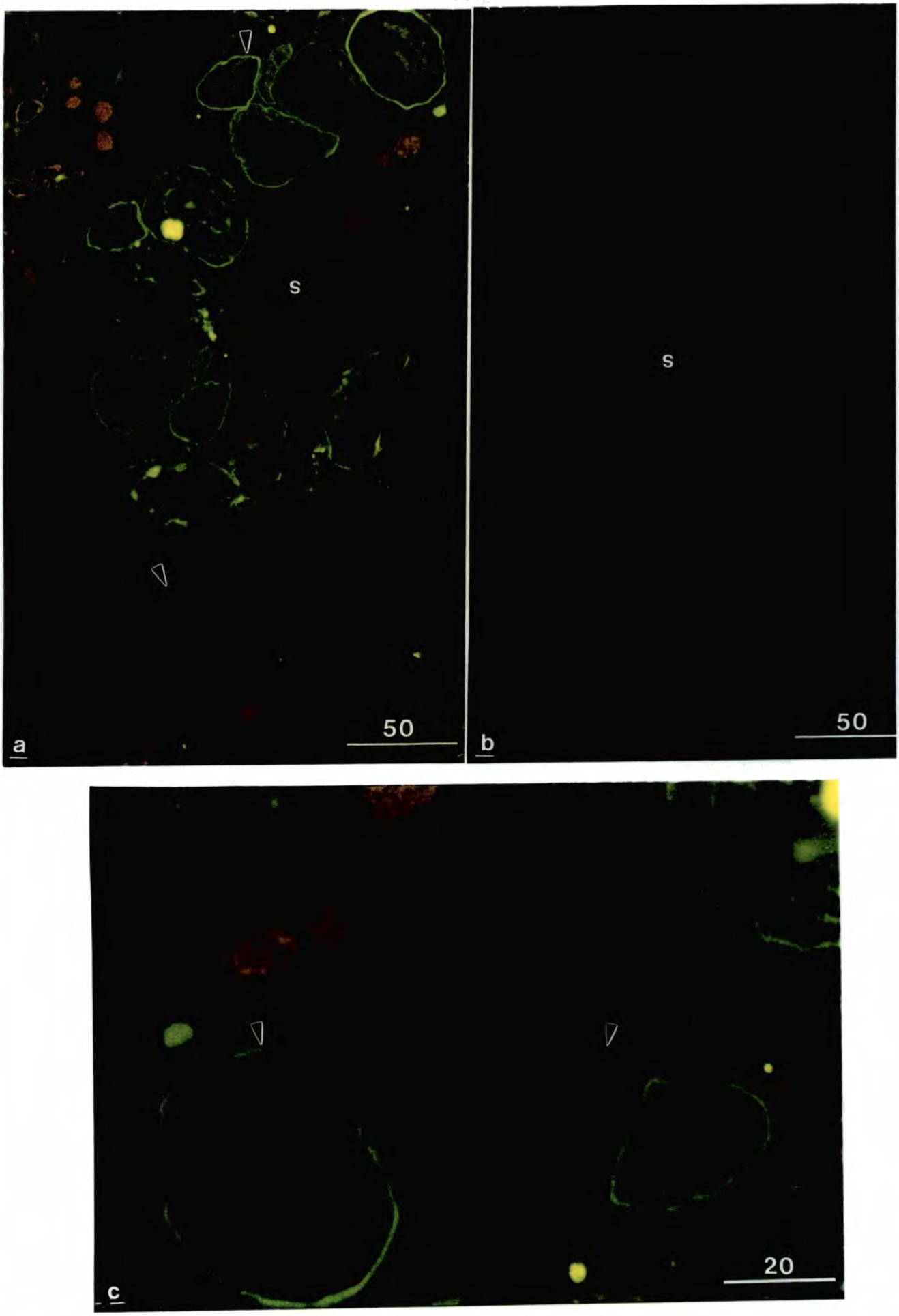


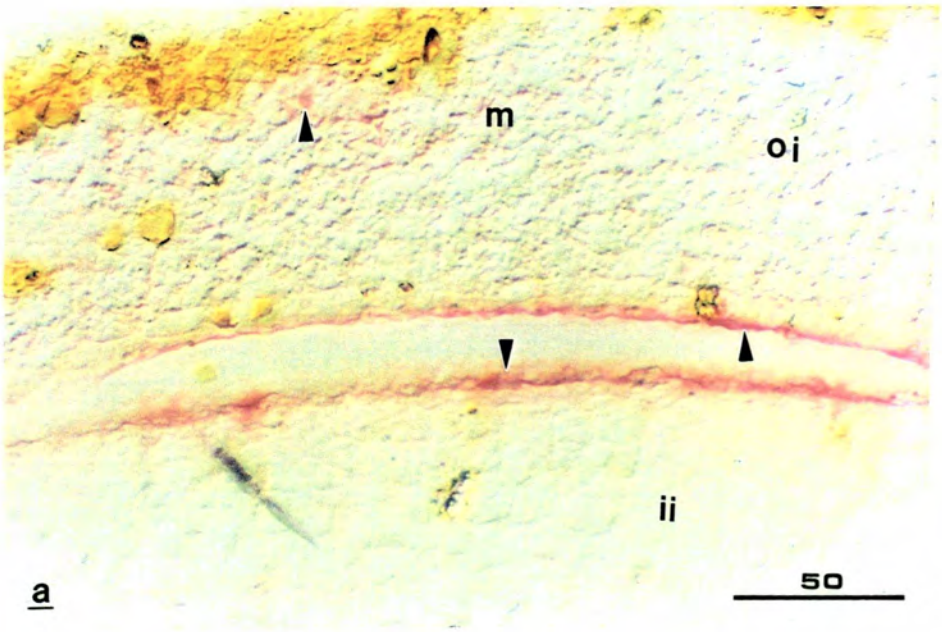
Figure 38

Figure 39

Localization of cellulase mRNA in the tissues of 20 mm ovary (BF) by *in situ* hybridization.
Accumulations of cellulase mRNA shown by red staining. Photographed under Nomarski illumination.

(a) 20 mm ovary.
(b) 20 mm ovary. (control)

ii, inner integument; oi, outer integument; m, mesophyll; nu, nucellus; dart, localization of cellulase mRNAs.



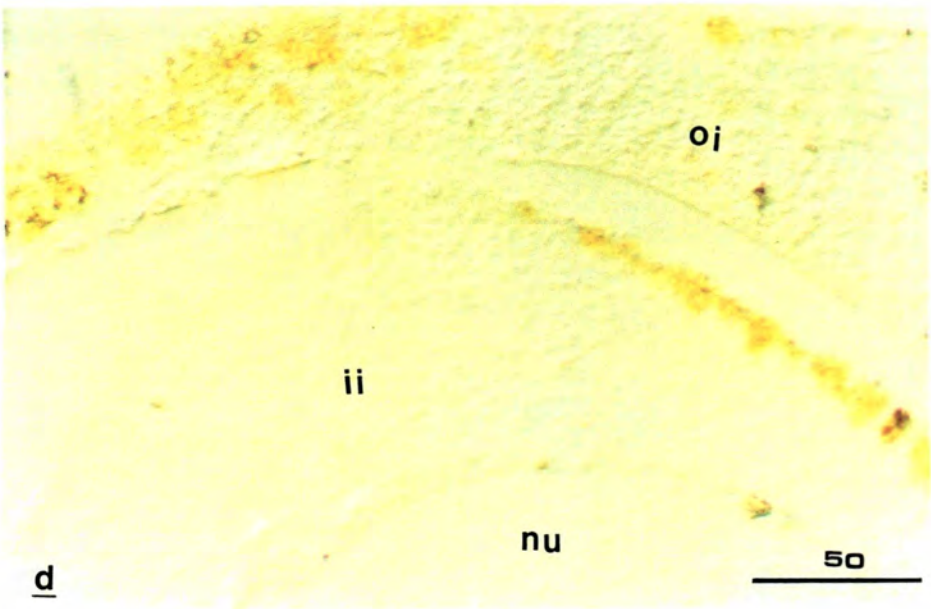


Figure 39

mRNA showed low activity in the tissue of mesophyll of the outer integument of the ovule (Fig. 39a).

Within ovary at 15 days after fertilization highest levels of cellulase mRNAs were found associated with the vascular bundles of mesocarp, and with the exocarp and a few layers of mesocarp (Fig. 40a and 40c), the placenta, and the fibres were also stained. Low activity was observed on septum and the developing dehiscence zone.

At the latest stage examined, 45 days after fertilization, only the seed coat fibres showed high cellulase mRNA activity. Only low levels of cellulase mRNA accumulation were observed in the vascular bundles of the mesocarp, in the endocarp and in the epidermal layer of outer integument of the ovule.

Figure 40

Localization of cellulase mRNA in the tissue of 15 days (AF) by in situ hybridization.
Accumulations of cellulase mRNA shown by red staining. Photographed under Nomarski illumination.

(a) Exocarp of the carpel wall
(b) Exocarp of the carpel wall. (control)
(c) Vascular bundles of the mesocarp.
(d) Vascular bundles of the mesocorp. (control)

ex, exocarp; me, mesocarp; m, mesophyll; vb, vascular bundle; dart, localization of cellulase mRNAs.

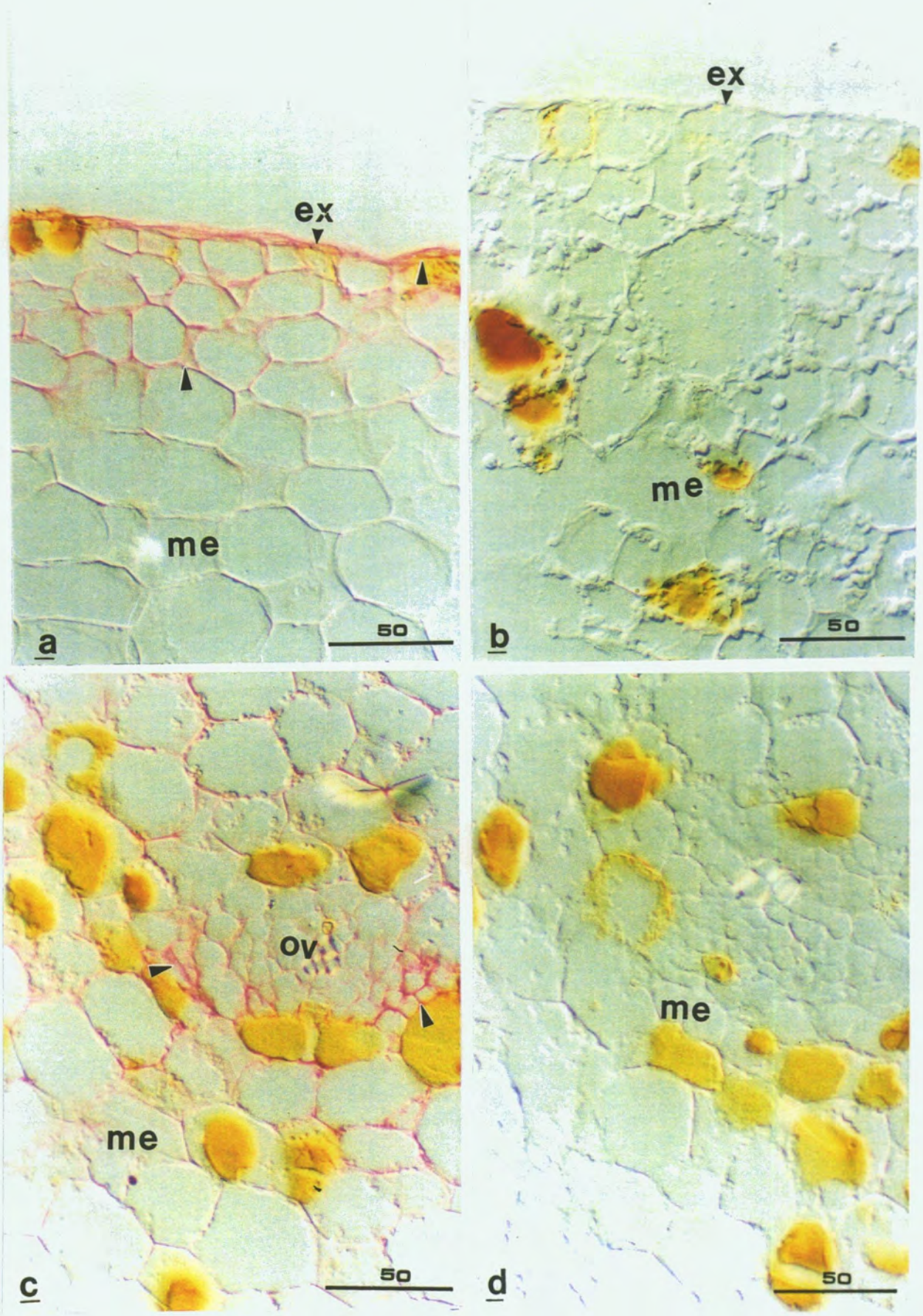


Figure 40

DISCUSSION

Although Durham is not within the normal geographical range for growth of cotton, within the greenhouses plants grew in Durham to a normal stature and at a normal rate. Some problems with infestation by White fly and Spider mite were, however, encountered and samples were taken only from the healthiest plant which showed development of flowers and boll on normal plants.

4.1 Floral development

Doak (1928) and Gore (1935) described the general pattern of initial development of the floral organs. The primordia of the calyx arise as an undulating ring of meristematic tissue.

Results presented here show that with further development the calyx develops distinct outer and inner epidermal layers of the isodiametric cells. At later stages the calyx contains vascular bundles, with thick-walled xylem cells, and resin ducts. The surface of the outer epidermis of the sepals contained multicellular hairs, whilst the inner surface remains smooth.

The primordia of the petals arise from meristematic tissues after the calyx primordia, initially developing as an apical 'notch'. With further development of the petals, the two halves grow at equal rate. Joshi *et al* (1967) reported that the floral organs are covered with unicellular stellate and multicellular hairs. Herman (1951) reported both sides of the longer half of the petals covered by the stellate hairs, while the shorter half remains glabrous. In the lines examined in our studies the longer half of the petals contained stellate hairs on the both side, while shorter half remains glabrous.

The outer and inner epidermis contained a single row of isodiametric cells with interspersed gland cells. The petal wall contained vascular bundles and resin ducts.

The staminal-column primordia arise after corolla primordia. With further development of staminal-column, the staminal-column develops numerous stamens and vascular tissues. The stamens contained several small anthers.

The results presented here generally give more detailed information than previous histological studies of cotton flower development. These have described isolated features of the developing floral anatomy, with the aid of line diagrams. The use of aldehyde-fixed and resin-embedded tissue to provide 1µm sections allows examination of the cellular structure in much greater detail.

Histochemical study of the early flower showed that the highest esterase activity was detected in the vascular bundles of the sepal and vascular tissues of the staminal-column of the 18 mm ovary (B.F.), but it was absent from the 3-4 layers of the inner and outer border of sepal wall and staminal-column.

Esterases include a broad spectrum of enzymes that are widely distributed in plant tissues and are capable of hydrolysing the carboxylic acid esters of alcohols, phenols and naphthols

$$R\text{-}OOR' + H_2O \rightleftharpoons R\text{-}COOH + R'OH$$

At present five groups of esterases can be considered in cytochemistry

1) carboxylesterase - which preferentially hydrolyse aliphatic and aromatic esters. They are inhibited by organophosphates but not by eserine.

2) arylesterases - which hydrolyse aromatic esters, especially acetates. They are inhibited by sulphydryl agents, but not by organophosphates.

3) acetylesterases - which hydrolyse both aromatic and aliphatic acetyl esters. They are not inhibited by eserine sulphydryl agents or low concentrations of organophosphates.

4) acetylcholinesterases - which hydrolyse acetylcholine at a higher rate than either aliphatic or aromatic esters. They are inhibited by low concentrations of eserine or organophosphates.

5) cholinesterase - which hydrolyse preferentially butyrylcholine and aromatic butyrate esters. They are inhibited by eserine and organophosphates.

These esterases can be grouped by their substrates, including a- naphthyl acetate (1,2,4 and 5). Naphthol-AS-D acetate (1-3), indoxy acetates (1-5), thiolacetic acid (1-5), acetylthiocholine (3 and 4), butyryl thiocholine (5) and myrystylthiocholine (3). The reaction with a-naphthol acetate and naphthol AS-D acetate are by the azo-dye method and indoxyl acetate method, and the remainder by the metal salt technique. The azo-dye method can be used for optical and electron m microscopy, although naphthol AS-D acetate has also been used with plant tissues (Gahan and Mclean, 1969; Livingstone *et al.*, 1969; Knox and Heslop-Harrison, 1970).

Each of the main groups contains a number of enzymes (Gahan, 1981), which have in plant tissues been associated with the differentiation of vascular tissues.

Gahan (1981) working with *Vicia faba* and *Pisum sativum* reported that esterase activity to be present in the stele, root cap and rhizodermis, but almost completely absent from the developing cortex and quiescent centers. The meristem cells giving rise to the cortex were almost negative as long as those giving rise to the stele were positive for esterase activity. It was proposed that the esterase activity may be used as an early marker of commitment to differentiation into stele in roots of dicotyledonous plants.

Gahan (1981) also suggested that the cytochemical test for esterase activity may be used as an early marker of commitment to differentiation in both primary meristem of roots and the vascular cambia of dicotyledenous plants.

Cytochemical study of the distribution of some hydrolyses in root of *Vicia faba* shown esterase activity to be high in the rhizodermis and stele, but low in the developing cortex (McLean, 1969; McLean and Gahan, 1970).

The presence of esterase is however not just limited to developing vascular bundles. In a recent study (Vercher personal communication) esterase was localized to the endocarp of developing pea pods. These tissues also lignify during their differentiation, however when the substrates were supplied to sections of pods in which endocarp lignification does not occur strong histochemical localization was still observed. Analysis of the mutants lines, and re-examination of the lignifying endocarp tissues, indicated that the presence of esterase is associated with the period of cell elongation prior to the later phase when lignification occurs. In the mutant lines the endocarp cells elongate but do not lignify.

The localization of high esterase activity to the early cotton fibres, which do not lignify at any stage, confirms that this activity is associated with cell elongation and not, as originally suggested, only with lignification of the vascular elements.

4.2 Ovary development

Size parameter has been used for staging the initial development of the ovary prior to fertilization. Stages after fertilization have been measured by time because of the uneven growth patterns during this period.

Histological studies showed that the margins of carpels were never joined at the centre of ovary before the stage of 18 mm (B.F.). At the initial stage (8 mm ovary) the ovary

develops four regions of meristematic tissue, later these meristematic tissues formed locular ridges which initially progressed and produce the locular space. As the carpels increase in size the internal margins of the carpel grow centripetally and upward, and original cavity divided into four locules. The results are in agreement with the general pattern reported by Herman (1951) and Gore (1935) who studied only the development prior to fertilization and presented only histological diagrams but none of the cytological detail presented here.

With continued growth of the reflexed margins of the carpel, each formed placentae. The placentae developed ovules, which are attached to the placenta by a funicle. At the 18 mm stage of development the ovary contains septa, with primary vascular bundles in both carpels and septa. Mucilage glands were observed at the septum wall as previously reported by Joshi *et al.* (1967).

4.3 Ovule development

Primordia of the ovules arise on the placenta as rounded masses of meristematic cells at about the time the locules becomes closed. During the development of integument, and as the apex of the nucellus begins to curve, a second fold is formed on the outer integument. The outer integument soon overgrows the inner integument, and both continue their growth until they cover the nucellus, and the ovule becomes anatropous. At the time of flowering the inner integument of the ovule contained 10-12 layers, and outer integument 6-8 cell layers. The results are in agreement with histological diagrams presented by Gore (1932), Joshi *et al.* (1967) and micrographs of Linthac and Jensen (1974).

Twenty-four hours before pollination, the epidermis of the outer integument develops stomatal guard cells, ordinary epidermal cells and fibre cells. Initially the fibres develop at the chalazal end of the ovule. These observations are similar to those as previously reported by Stewart (1975) and Ryser (1992).

Additional results presented here, from histochemical analyses, indicate that the highest esterase enzyme activity was detected in the nucellus area of the ovules of the ovary of 15 mm and 18 mm (B.F.). The other parts of the ovule (inner and outer integument) did not show esterase activity. Calcofluor staining showed a uniform pattern of cellulose cell walls although the guard cells of the outer epidermis of the outer integument showed particularly strong staining due to the deposition of additional cellulose to the thickening cell walls. Immunocytological studies with the JIM antibodies did not show any patterns of differential staining, indicating that cell wall composition was generally uniform within the different layers of the developing ovule prior to fertilization.

4.4 Seed coat development

By two days after fertilization the outer integument of seed coat may be distinguished into three zones, (a)outer epidermis, (b) outer mesophyll zone, (c) inner epidermis. As the seed matures the cells enlarge considerably. The outer and inner epidermis remain single-layered and the mesophyll 4 or 5-layered. The cells of the outer epidermis enlarged to several times their original size and became filled with tannin which may play a role in protection against attack by some plant pathogens. The cells of the mesophyll layer accumulate vacuolar tannin or starch in the peripheral cytoplasm. The patterns of deposition are quite distinct with different products being accumulated within the different layers. Such patterns have been reported by Joshi et al. (1967) and represent regulation of expression of different genes within the different tissue layers of the outer integument.

Prior to fertilization the inner integument contains 14-16 layers. Seven days after fertilization no conspicuous change occurred except some cell enlargement including radial elongation of the outer epidermal cells. By 15 days AF these cells had elongated

to many times than their original size, and their nuclei were restricted to the outer ends. The inner integument is distinguishable into four zones; (a) an outer layer, described by Joshi *et al.* (1967) as a 'palisade layer' because of the cell shape and arrangement, although it is dissimilar to the typical leaf palisade type; this layer is derived from the outer epidermis layer. The other regions include (b) tanniferous and starchy zone of inner mesophyll containing 5-6 layers of cells, (c) an inner, 'colourless' zone of mesophyll containing 9-10 layers, and (d) the 'Fringe' layer (Joshi *et al.* 1967; Reyser *et al.* 1988) derived from the inner epidermal layer. A system of interconnected ridges oriented longitudinally with the fringe cells, and a large number of knob-like protrusions are the typical elements of the inner surface of the inner integument.

At early stage after fertilization the xylem cells in vascular bundle of the outer integument of the seed show primary deposition of cellulose. Later they develop their secondary growth due to additional deposition of the cellulose (as shown by calcofluor staining), but acridine orange staining indicates that there is little lignification within the vascular bundles of the outer epidermis even late in seed development.

Within the developing seed esterase activity was found associated with the outer layer of the outer integument, which develops into the fibres, and associated with the nucellus. Neither of these tissues shows any lignification although both involve cell expansion. However the development of the outer layer of the inner integument, the 'palisade' layer also involves considerable cell expansion (elongation) after fertilization but little evidence was found of any esterase activity at this stage, although esterase activity is localized to this layer prior to fertilisation, long before any cell expansion. The lack of accumulation of blue stain reaction product in post-fertilization samples may have been a consequence of the extensive reactions of the tannins of the adjacent cell layers which resulted in the deposition of a dense brown / red product even in no-substrate controls.

4.5 Fibre development

The outer epidermal layer of the outer integument develops two kinds of fibre, one is lint fibre, and the other fuzz fibre. At the day of fertilization the ovule develops only lint fibre; fuzz fibre is not initiated until 7-days after fertilization. At the initial stage of expansion of lint fibre cells the enlarging dome is filled with cytoplasm, a vacuole is not apparent. By contrast, at the early comparable stage of fuzz fibre development, which occurs several days later, the expanding dome is characterised by a large, central vacuole, with the nucleus and thin cytoplasmic layer restricted to the cell perifery. Other authors who have examined cotton fibre development include Joshi *et al* (1967), Peeters *et al* (1987) and Ryser (1985), however, they did not examine the very early stages of cell development and were thus unable to distinguish the initial events in lint and fuzz fibre development as described here.

The results presented here are in agreement with the work of Beasly (1975) and Ryser (1992) who reported that fibres complete their secondary thickening within 48-69 days after anthesis. The lint fibres are large, about 26 to 40 mm long. At maturity they become twisted ribbon like. Fuzz fibres are much shorter, about 2-8 mm, and do not become twisted; they remain adhered to the seed coat after ginning. Similar findings were recorded by Joshi *et al.* (1967) and Basra and Malik (1984).

The primary cell walls of fibre, composed largely of cellulose (Timpa and Triplett 1993; Ryser 1985), continues to be deposited throughout the period of cell expansion, during which several layers may be developed. The deposition of secondary wall starts when cells have ceased expansion (28 - 35 days after fertilization) and involves additional cellulose deposition. The fibres usually complete their secondary wall within 70 days after anthesis.

As well as using calcofluor to follow the deposition of the cellulose cell wall in the developing fibres, studies were carried out for the localization of the esterase enzyme

activity in developing fibre at different stages. The levels of esterase activity detected on fibres increased up to 7-days after fertilization, and remained mostly constant at a high level up to 21-days. The level then declined gradually until by 35 - 40 days esterase activity was not detectable. The levels correlate very well with the period of fibre cell expansion.

4.6 Embryo development

Micrographs presented here show that the ovule develops two synergids within the nucellus by twenty-four hours before fertilization. These synergids start to degenerate after pollination. Twenty-four hours after pollination the pollen tube enters into the micropyle of the ovule, and grows for a short distance between the nucellus of the ovule, and reaches the embryo sac. The results are in agreement with findings of Balls (1907), Gore (1935), Joshi, *et al.* (1967), Jensen (1965) and Jensen and Fisher (1968). After forty-eight hours the pollen tube grows into the degenerating synergid through a filiform apparatus and discharges the sperm into the synergid by the release of a small pore, or discharging plug (Jensen 1963; 1964; 1965).

After fertilization the zygote first divides periclinally, then anticlinally, to form a four-celled embryo. The embryo increase its size by subsequent cell division and expansion to attained its globular stage 15 days after fertilization. Twenty one days after fertilization the embryo reaches a heart-shaped stage, when the embryo is flatten on the top and has formed cotyledonary primordia. The result are similar to those of Pollock and Jensen (1964).

With further development of the embryo the cotyledons expand, their margins are curved inward and by 45 days after fertilization they coil around each other. The cotyledons contains several layers of mesophyll, which are not clearly distinguished into palisade and spongy layers, and vascular elements and resin ducts.

4.7 Fruit wall development

Early in development the fruit wall consists mainly of parenchyma cells with some vascular tissue; the outer surface is covered by a thin-walled cuticle. Three characteristic layers are apparent, an outer exocarp, a mesocarp and an inner endocarp. As the ovary develops into the fruit striking histological changes take place in the fruit wall. Although several authors have described features of the mature fruit (Joshi, *et a.* 1967; Morris, 1964b; Pickard and Baehr 1973), the development of the ovary wall and the patterns of cell differentiation after fertilization appear not to have been described in detail before.

4.7.1 Exocarp

At the early stage the exocarp contains a layer of isodiametric cells, these expand progressively during ovary development and develop multivacuolate protoplasts.

After fertilization major changes were observed in the exocarp of the developing developing fruit. Although the exocarp remains as a single cell layer with no evidence of periclinal division to produce an inner layer, the outer surface of the exocarp develops a thick cuticle with numerous small, spiny projections. The inner radial walls also thicken and there is some filling of the intercellular spaces between the exocarp and the mesocarp. The radial walls, however, remain relatively unthickened until quite late in the development of the fruit wall.

Staining with acridine orange indicated that there was no general lignification of the cells of the exocarp, except in the region of the valve at the outer side of the dehiscence zone, were a small amount of lignified tissue accumulates. Staining for esterase showed no activity in the exocarp layer at any stage.

4.7.2 Mesocarp

The mesocarp is the largest layer of the pericarp. The mature mesocarp (45 days after fertilization) contains 34-36 layers of parenchymatous cells traversed by a network of vascular strands. Resin ducts are distributed in the outer layers, while many cells contain tannins.

The outer border of the mesocarp is covered by the exocarp and the inner portion bordered by the heavily lignified endocarp. The vascular bundles contains highly lignified sclerenchyma fibre. Parenchyma develops large air-space near the endocarp. Similar observations were recorded by Joshi *et al.*(1967) Pickard and Baehr (1973).

Additional results presented here indicated that even at the earliest stage examined (10 mm) the mesocarp contains 34-36 layers of slightly irregular parenchymatous cells. Numerous cell division figures are seen and in many of the cells the nucleus contained many nucleoli. At this stage the mesocarp develops pro-vascular bundles and by 15 mm AF develops primary vascular bundles.

Later (after 18 mm) the mesocarp develops primary vascular bundle containing thin-walled lignified xylem cells. The mesocarp at this stage contains two type of parenchymatous cells which are spread through out whole layer, (a) tannin-filled parenchyma cells that stained dark-green with toluidene blue, (b) colourless parenchyma cells, that did not stain with toluidene blue.

After fertilization the mesocarp still contained 34 to 36 layers, and no changes occurred in the cell-layers, except cell elongation. The mesocarp first develops many of chloroplast-containing parenchyma cells. These cells are different from tannin containing parenchyma cells due to their staining pattern. Calcofluor staining

indicated that the xylem cells in the vascular bundles start their secondary growth by depositing additional cellulose. With further development of ovary from 15-30-days (AF), the mesocarp contains quite large parenchyma cells which develop large intercellular space near the endocarp. The xylem cells deposit extra cellulose and become heavily lignified.

The highest esterase activity detected in the vascular bundles of the mesocarp layer was found at 7 days AF. Mostly it remains constant through the two stages examined (15 days and 21 days after fertilization) and then declined gradually by maturity. Thaker *et al.* (1987) and Spence (1992) have reported that the esterase activity degrading at the maturity of the fruit.

4.7.3 Endocarp

The endocarp is the inner layer of the fruit wall; it is proposed that this layer may play an important role in boll dehiscence.

By the time of maturity the endocarp has developed heavily lignified sclerenchyma fibres which border the large area of the locules. The lignified layer may prevent loss of the moisture into the locular area and provides favourable condition for boll dehiscence, and may ensure that tensions developed with moisture loss from the mesocarp and exocarp result in an outward curling of the carpels as they separate.

The endocarp was examined at eight different stages of development, four before fertilization and four after fertilization, to follow the major changes during development. During the first two stages examined the endocarp contains a single layer of cells, but their form appears quite different from other cell types. Initially isodiametric, with large nuclei containing many nucleoli, the cells developed to slightly more irregular forms. Periclinal cell divisions took place in many cells and the

endocarp of 18 mm (B.F.) contained two, and endocarp of 20 mm (B.F.) developed four layers of elongated cells.

During the first two stages examined after fertilization (7 and 15 days AF) the endocarp did not show any major developmental changes, except some elongation of the cells. At 30 days the endocarp starts secondary growth, and after cell wall thickening the cell showed initial lignification, demonstrated by staining with acridine orange. At maturity (45 days after fertilization) the endocarp is seen to have 4-5 layers of heavily lignified sclerenchyma fibre. The mature structure has been reported previously (Joshi *et al.* 1967 ; Pickard and Baehr 1973), although these authors gave no indication of the development of the tissues, or the degree of detail presented in this work. Additionally, results presented here show that the low esterase activity was detected in the endocarp at early stages of 15 mm and 18 mm (B.F.), but was absent from the later stages; indicating association of this activity with the phase of cell division and expansion but not with the phase of lignification.

4.8 Septum wall development

The septum is also a basic unit of the fruit wall, and may play an important role in the boll dehiscence. By time of the maturity it divides into halves, the outer side of each half is covered by the highly lignified sclerenchyma fibre of the extension of the endocarp. The longitudinal hole which develops within the septum is, however, not lignified. The highly lignified layer of the septum prevents the moisture in the locular area. The regulation of the loss of moisture may be a cause of active dehisce by establishment of differential stress within the septum and carpels.

The results presented here are in general agreement with the findings of Abraham (1934) and Morris (1964b) who reported that the septum is a dorsal carpellary bundle which dehisces into forked branches at maturity. These branches develop thick-

walled sclerenchyma fibres which run parallel from the base of the carpel, passing through the center, to reach on the top of the carpel wall (apex). Meakin and Roberts (1990) worked with dehiscence of fruit in oilseed rape (*Brassica napus* L) and report that the loss of moisture is a cause of the active dehiscence. Although brassica have a pod-shaped fruit it contains a central (pseudo) septum and shows a similar pattern of lignification of the endocarp and extension of the endocarp over the outer surface of the septum.

Additional results presented here indicate other, previously unreported changes which occur during the histological development of the septum. During the first two stages examined (10 and 15 mm) there was not much difference in tissue development, although at the 15 mm stage primary vascular bundle had started to develop. By the next stage (18 mm) the septum develops mucilage glands in its center; these glands remains in the septum wall until the 7-days after fertilization, but from that time mucilage is lost progressively from the inner part of the septum. In the initial stages examined after fertilization little difference in structure was observed except in the vascular bundles. By 21 days stage this had developed heavily lignified xylem cell in the vascular bundle and septum divides into two portions (branches). Calcofluor staining illustrated the initial accumulation of thick cellulose walls in xylem cells in the vascular bundles of the septum. Additional results showed that the esterase activity detected in the septum of the 7-days and 15-days (AF) was associated with these cells in the developing bundles, and also with the expanding cells on the outer surface of the septum wall; these later become lignified. Esterase activity was not apparent in any of the septum in the subsequent stages of development.

4.9 Capsule dehiscence

On the dorsal side of the cotton boll the suture regions lie longitudinally between each of the adjacent carpels. However, on the lateral side the split develops between two

septum walls. Initially the parenchyma of the dehiscence zones joints both carpels firmly, but at the time of boll opening breakdown of the suture parenchyma cells results in separation of the carpels and exposure of the formerly-enclosed seeds. The dehiscence area of cotton was studied in 6 sequential stages of fruit development; three before fertilization and three after fertilization. The dehiscence area was already initiated at 10 mm (B.F.) stage and did not show significance differences in cell structure and development at the 15 mm stage. However, cell differentiation in and around the dehiscence zone of 18 mm ovary is significantly different from the previous stages, resulting in the formation of a distinctive zone.

Dehiscence has begun, internally, by 15 days after fertilization by splitting of the suture parenchyma cell near the endocarp. The mesocarp develops vascular tissues in both sides of the developing dehiscence zone, and by the next stage examined (30 days AF) the vascular tissue had developed thick-walled sclerenchyma fibre. At the mature stage (45 days AF) the vascular tissues had accumulated heavily lignified sclerenchyma fibre.

Baehr and Pickard (1970) and Pickard and Baehr (1973) report that the dehiscence in cotton boll is complete internally by 40-45 days after anthesis, but possible separation of suture parenchyma cells is prevented by the outer thick-walled cuticle. The breakdown of cuticle takes several more days, depending on physiological factors, temperature and the variety.

The present study, however, shows the valve tissues playing a very important role in the break down of the cuticle. The valve tissue contains lignified cells which prevent moisture from getting into the outer layers of mesocarp. Within the valve tissues breakdown occurred resulting in a tangential arc of separation running either side of the outer region of the radial dehiscence zone (see Figure 29a). Moisture stress is one

of the major causes of quick dehiscence (Abraham 1938; Morris 1964b; Meakin and Roberts 1990).

4.10 Histochemistry

Several histochemical techniques have been applied to give additional information about cell differentiation involving expansion, cell wall thickening and lignification. Esterase has been implicated in the early stages of xylogenesis (Gahan 1981) although recent work has suggested that it may also be associated with cell expansion which is not followed by lignification (Vercher and Harris personal communication). Results presented above show that esterase activity is associated with many of the tissues which subsequently lignify, the differentiating fibres and endocarp for example, but is also present in the expanding seed coat fibres. These cells do not lignify at any stage but do have a period of very rapid cell expansion when loosening of existing cell wall components is accompanied by deposition of massive amounts of new wall material; esterase activity may play a role in the loosening of the intermolecular bonds which link the components of the cell wall during the period when rapid expansion of the primary wall occurs.

The deposition of the cellulose was studied through the different stages of the ovary development. The just-fertilized ovary showed the deposition of the additional cellulose, over the levels in the non-differentiated parenchyma, in cells of the vascular bundles within the carpels, septa, and outer integument, and to walls of the guard cells within the epidermal layers. The xylem of the vascular bundles develops secondary growth due to additional deposition of the cellulose and then became highly lignified at a later stage.

Initially fibres develop thick primary cell walls by depositing of the cellulose. At about 40-45 days after fertilization the fibre develops secondary growth due to additional deposit of the cellulose, which was then lignified.

4.11 Immunocytochemistry

During cell differentiation significant changes occur to the wall in both its structure, as shown by microscopy, and its chemical composition (Carpita and Gibeaut 1993). In an initial study, to look for immunological markers that might be useful for screening changes in cell wall differentiation within the developing cotton boll, a series of antibodies were used.

Ten monoclonal antibodies raised at the John Innes Institute (Norwich) were used for localization of specific cell wall antigens; these included arabinogalactans, glycoproteins, and pectins. JIM14, which is known to bind to arabinogalactans, showed strong binding on the cell walls of the septum; the rest of antibodies of this group showed little or no specific binding. JIM7, which binds to a cell wall pectin component, did not show significant difference in the binding of cell walls at different type tissues, indicating a similarity in the levels of this component within the cell walls. The anti-glycoprotein antibodies did not show any binding on the cell walls of the cotton tissues examined, suggesting that different types of glycoproteins are present in cotton to those from the cell wall fragments used to raise the monoclonals.

The labelling pattern with JIM14 showed both cell wall labelling and labelling within the protoplast. The latter, which was seen as discrete vesicular and reticulate patterns and associated with the plasmamembrane indicates that the component labelled is synthesised within the protoplast, probably within the endomembrane system, and exported to the cell wall.

4.12 Molecular histochemistry

Cellulase has been implicated by several researchers in the dehiscence mechanism (eg. Meakin and Roberts 1990). To study its potential role in the dehiscence of cotton boll *in situ* hybridization was used to follow the accumulation of mRNAs in the various tissues of the developing fruit.

Results indicate that cellulase mRNAs are present in developing fibres upto maturity (45 days after fertilization), but in other tissues where they were present they declined gradually from 15 days after fertilization. The highest levels of cellulase mRNAs were observed in the vascular bundles of the mesocarp and exocarp along with few layers of the mesocarp at 15-days after fertilization. The ovary of 15-days showed low distribution of cellulase mRNA seen in the placenta and septum wall.

Generally, however, there was no evidence of a direct correlation between high levels of cellulase mRNAs and subsequent breakdown of tissues within the dehiscence zones.

4.13 Summary of conclusions

The work presented in this thesis pertains to the cytological development of the major tissues of the developing cotton ovary, particular attention has been paid to the development of the dehiscence mechanism. Light microscopy results showed that the sclerenchyma fibres in the mesocarp lignified heavily as did the endocarp, after periods of cell division and cell expansion. The extensions of the endocarp around the septa also lignified, and thick-walled cells developed in 'hinge' regions associated with the dehiscence zones. The combination of these changes results in the

development of predictable patterns of stress building up within the fruit walls as the tissues dry out; undifferentiated parenchyma cells in the tissues will collapse whilst the thickened and lignified cells do not. The differential stresses result in the dehiscence of the cotton boll.

4.14 Future work

Good boll opening is of great value in cotton production, also the cotton growers in Pakistan are desperately in need of the early varieties of cotton, in which boll opening is not delayed, so that they can plant their succeeding wheat crop as soon as possible after the cotton harvest.

Pakistan has a variety of genotypes which are either early or late with regard to boll opening. These include varieties like CIM-70, NIAB and Cris-9 which are early varieties and Qalandri, Sarmast and MNH-93 which are late opening. The late varieties are most hardly against drought conditions, tolerant to insect pests and more or less good in terms of yield. These varieties are avoided for planting by growers only because of their late maturing which ultimately hinders the following crops such as wheat, Rabi, oilseeds and pulses etc.

The older conventional varieties have been rejected in recent years but may be of value in an extension to this project in addressing the question of late maturity. Histological screening may be a relatively easy approach to explain why maturity / boll opening is delayed, and identify lines in which only one or two phenotypic features may be changed to produce earlier opening whilst retaining the other desirable genetic traits. To my perception this research would not only be a beneficial to the cotton growers but might also contribute to application to induce earliness in other crops as well.

This project could thus be extended by further detailed studies, by light and electron microscopy, for an understanding of the dehiscence mechanism and the basis of early and late opening involved in the genetically diverse genotypes. The results of the forthcoming studies would also be of great value for selecting early opening varieties of cotton by exploiting the techniques developed after studies associated with plant breeding for crop improvement.

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