REPRODUCTIVE MORPHOLOGY OF ACEROLA (*MALPIGHIA GLABRA* L.)

A THESIS SUBMITTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN HORTICULTURE

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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INTRODUCTION

The fruit of the acerola, Malpighia glabra L., (also called Barbados cherry and West Indian cherry) is one of the richest known sources of natural ascorbic acid, as was discovered by Asenjo and Freire de Guzmon (3) in 1946. Ascorbic acid or vitamin C has been widely recognized as one of the vitamins essential to man and animals, and the unusually high content in acerola has created considerable interest in this plant.

Increased planting of acerola in Puerto Rico, Florida and Hawaii has resulted because of its economic potential as a source of natural ascorbic acid for use in food supplements and additives, and as a potential blender in the fruit juice industry. However, as with most crops, many cultural problems associated with acerola production exist. Among the problems yet to be investigated is that of poor natural seed set and its relationship to gametogenesis.

Previous studies (103, 104) on fruit set requirements of acerola in Hawaii showed that the low percentage of natural fruit set, despite prolific flowering, was due to absence of pollinating agents and thus fruit set could be greatly increased by hand pollination and by growth regulator application. Ledin (52) reported that in Florida natural fruit set is relatively high due to pollination by large numbers of bees.

Hand pollinated flowers of acerola produce relatively low percentages of seed and fruit. A knowledge of gamete and zygote development as related to fruit set is important since clones of acerola have shown various degrees of self and cross incompatibility and natural parthenocarpy. Parthenocarpic fruits, when induced by growth regulator application upon emasculated flowers, contain no viable embryos.

This study was initiated to determine the relative time of floral bud differentiation, gamete and zygote formation and the effects of growth substances upon fruit and seed development, and fruit morphogenesis in terms of normal and parthenocarpic types. Information derived from a study of this nature may be of value to commercial fruit production and to the development of a breeding program.

BOTANICAL DESCRIPTION

The acerola is native to Tropical America and belongs to the family Malpighiaceae and the genus Malpighia, which consists of 30 to 40 species. The acerola is referred to as M. punicifolia L. in publications from Puerto Rico (1, 82) while those from Florida (51, 52) and Hawaii (103, 104) use the binomial, M. glabra L. The correct name for acerola seems to be M. glabra L. since it was first used in 1753 and M. punicifolia L. was not used until 1762 (52).

The botanical description of the acerola plant has been given by several workers (52, 104). The plant is a bushy shrub and reaches an ultimate height of 3.0 to 4.5 meters. Its growth varies from semi-prostrate, to drooping, to upright branching types (Figs. 1 to 3). The leaves are entire, simple, opposite, and slightly pubescent with short petioles. The flowers are 2.0 to 2.5 centimeters in diameter and are borne on simple dichasium inflorescences in the leaf axils. There are 5 green sepals, 5 pink petals, 10 stamens, and 3 carpels fused into a superior ovary.

In Hawaii, flowering cycles occur approximately every 25 days, commencing in April and continuing into late November. Scattered flowering during winter and early spring is quite common. It is not unusual to find both flowers and ripe fruits simultaneously on the plant. Rapid growth of vegetative shoots often occurs simultaneously with the production of flowers a few nodes below the shoot tip (Fig. 5).

PLATE I

FIGURE 1: Semi-prostrate growth type of acerola. Other growth types appear in background.

FIGURE 2: Drooping growth type of acerola.

FIGURE 3: Upright growth type of acerola.

1

2

3

PLATE II

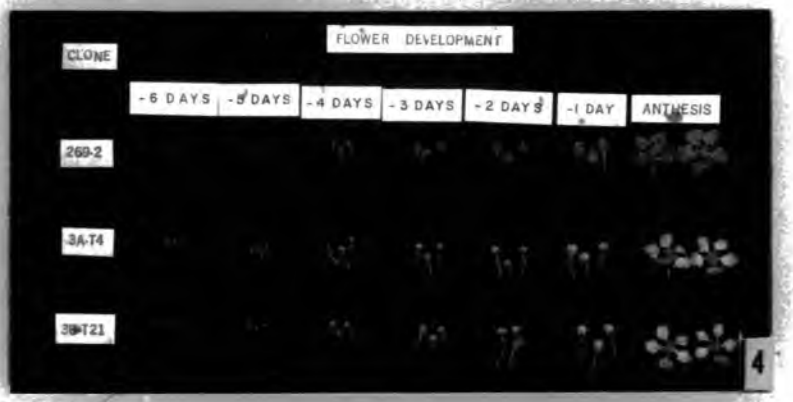
FIGURE 4: Stages of flower development from emergence of bud to anthesis of three acerola clones. 0.45 X.

FIGURE 5: Lateral shoots of clone A in active vegetative growth and producing flowers and young fruits. Arrow shows flower buds at the uppermost axils of fully-expanded leaves. 0.25 X.

FIGURE 6: Stages of fruit development from anthesis until ripe fruits of three acerola clones. 0.25 X.

FIGURE 7: Fruit shape and size of untreated (check) fruits and those induced with auxin and gibberellin on self pollinated flowers of clone C. 0.22 X.

FIGURE 8: Cross sections of untreated (check) fruits and those induced with auxin and gibberellin on self pollinated flowers of clone C. 0.22 X.


FLOWER DEVELOPMENT
CLONE
- 6 DAYS
- 5 DAYS
- 4 DAYS
- 3 DAYS
- 2 DAYS
- 1 DAY
ANTHESIS
269-2
3A-T4
3B-T21
4


5


6


6663,
3B-T21
25-DAY OLD FRUITS
CHECK
AUXIN
GIBBERELLIN
7


6663,
3B-T21
25-DAY OLD FRUITS
CHECK
AUXIN
GIBBERELLIN
8

The fruit (Figs. 6 to 8) is a trilocular drupe having shallow lobes and a thin skin. It is light orange to dark red when ripe. The fruit has a juicy mesocarp at maturity, usually weighs from two to ten grams and ranges from 1.3 to 2.7 centimeters in diameter. It is either sweet or sour in taste when ripe.

REVIEW OF LITERATURE

Gametogenesis

Only limited investigations on gametogenesis and embryogeny of the Malpighiaceae have been reported in the literature. According to Narasimhachar (67) and Rao (77), Braun in 1860 reported polyembryony in Banisteria and Stigmatophyllum as the first of such investigations for this family. Ritzerow (80) mentioned nucellar polyembryony in 3 species of Aspicarpa and said that fertilization did not occur because of the lack of normal pollen and since all the embryos observed were situated at the chalazal end of the ovule. Rao (77) reported that fertilization also did not occur in Banisteria laurifolia, Stigmatophyllum ariatatum and Hiptage madablota because of abnormal pollen and the poor organization and early degeneration of embryo sacs. However, he reported the occurrence of free-nuclear endosperm, which resulted from division of the fusion nucleus, and micropylar embryos of nucellar origin in H. madablota, while B. laurifolia and S. aristatum showed neither embryo nor endosperm formation.

Schurhoff (84) briefly described megagametogenesis in Malpighia coccifera, M. urens and Bunchosia nitida. He said that all three species had 16-nucleate embryo sacs. This has been confirmed by Stenar (89) in M. urens, by Rao (77) in B. laurifolia, S. aristatum and H. madablota and by Narasimhachar (67) in M. punicifolia. Rao reported that embryo sac

development was like the Penaea-form of the Peperomia-type and Narasimhachar reported a Lilium-type of embryo sac development. Development of an 8-nucleate embryo sac of the Allium-type has been reported for Calphimia canadilla (89) and M. glauca (76).

The occurrence of several megaspore mother cells per megasporangium was reported by Rao (77) in B. laurifolia, S. aristatum and H. madablota and by Narasimhachar (67) in M. punicifolia. However, in all cases, only a single megaspore mother cell was found to develop into a mature embryo sac. No polyembryony was observed in M. punicifolia. Investigations in microgametogenesis and embryogeny of the family are fragmentary.

Parthenocarpy

Various degrees of natural parthenocarpy among commercially important plants are quite common. Seedless fruits of certain species or even seedless varieties were reported in the literature as early as 1899 (93).

The occurrence of naturally parthenocarpic fruits has been of much interest to horticulturists since many varieties are self-sterile, which necessitates the planting of more than one variety in an orchard to insure a profitable crop, or since seedless fruits in most cases are preferred for commerce.

Some plants exhibiting vegetative parthenocarpy, in which fruit development is completely independent of fertilization, have been reported for certain varieties of breadfruit (Artocarpus) (32), banana (Musa) (86, 85, 18, 19), cucumber (Cucumis) (65, 38, 100, 92, 59, 2), pineapple (Ananas) (32, 39), Citrus (30, 29, 66, 28), oriental persimmon (Diospyros) (26, 41, 42, 43), tomato (Lycopersicum) (37, 23, 87), tobacco (Nicotiana) (35, 25), date (Phoenix) (69), slender pea (Pisum) (34), peach (Prunus) (90), eggplant (Solanum) (4, 54) and Spanish plum (Spondias) (45).

Other naturally parthenocarpic plants, in which the developing ovules abort before embryo sac maturity, have been reported for Dutchman's-pipe (Aristolochia) (57), Tahitian lime (Citrus) (96), tomato (Lycopersicon) (79, 11) and some varieties of seedless grapes (Vitis) (5, 70, 94, 91, 72, 71, 73).

Stimulative parthenocarpy, in which pollination and germination of pollen tubes take place, but the tubes fail to reach the ovules, are known to occur in certain varieties of pepper (Capsicum) (12, 47), pumpkin (Cucurbita) (20, 10), fig (Ficus) (13, 14), cherry plum (Prunus) (2), pear (Pyrus) (2, 98, 48, 78), apple (Pyrus) (99, 21) and corn (Zea) (58, 7).

While naturally occurring parthenocarpy has been of much interest, conscious attempts to induce parthenocarpy led to the discovery by Fitting (22) in 1909 that pollen extracts could prevent floral abscission and induce ovary swelling. He was followed by Morita (60) in 1913 and Laibach (50) in 1932 who showed that specific (but unknown) substances occurring in pollen extracts were involved in parthenocarpic fruit set. Thimann (95) in 1934 found that pollen contained auxin.

In 1935, Yasuda et al. (105) produced the first mature fruits with a purely chemical artificial stimulus by injecting an aqueous solution of cucumber pollen into cucumber ovaries. In 1936, Gustafson (27) was the first to show that auxin was involved by producing parthenocarpic tomato, squash, pepper, eggplant, Begonia, Salpiglossus and Petunia fruits with external application of auxin. Gustafson (31) found that the ovaries of seedless oranges, lemons and grapes contained larger amounts of auxin than the seeded varieties. Muir (61, 62, 63) indicated that the tip of the germinating pollen tube activates the enzymatic production of auxin in the pistil. In 1960, Gustafson (33) and Crane et al. (16) showed that gibberellin was also involved in parthenocarpy. Numerous workers have

since reported success with many other plants by using auxin (24, 40, 54, 68, 74, 81, 97, 102, 106, 107) and gibberellin (17, 49, 55, 101) to induce parthenocarpic fruit development.

MATERIALS AND METHODS

Acerola plants used in this study were clones of sour varieties grown at the Manoa campus and at the Waimanalo Substation of the Hawaii Agricultural Experiment Station, University of Hawaii. The plants were approximately three years old and were propagated from cuttings of seedling selections possessing high ascorbic acid content. Clone 269-2 (USDA PI 209269, selected from 10 seedlings received from the United States Plant Introduction Garden, Glendale, Maryland); clones 6663, 3A-T4, 6663, 3A-T8, and 6663, 3B-T21 (Accession 6663, derived from seeds acquired from the University of Florida); and clone Maunawili (obtained from the Hawaiian Sugar Planters' Association Experiment Station) were used.

Field and laboratory facilities of the Departments of Horticulture and Botany were utilized for this study.

Floral differentiation was studied by determining the growth activity of young branches which were in active vegetative growth. The young branches were of two types, lateral shoots normally forming the upper branches, and induced shoots which were formed when lateral shoot tips were decapitated. The lateral branches will be called "primary" and the induced branches will be referred to as "secondary" as a means of differentiating one from the other. "Emergence" will be used throughout this paper to refer to axillary buds at the time they become distinguishable to the naked eye at a normal viewing distance.

Floral differentiation on actively growing primary branches was studied by labelling the lowermost pair of unexpanded leaves at the tips

of the branches with India ink and by determining the number of days before flowers were produced in their leaf axils. This was followed by daily observations and was done to determine the period of time required for floral differentiation. Nodes of primary branches were collected at various intervals during a flowering cycle and placed immediately in a killing and fixing solution of FAA (23) for histological studies.

Floral differentiation on secondary shoots was studied by decapitating primary branches 20 centimeters from the apex. The growth activity of the resulting secondary shoots and the number of days prior to emergence of the first flower buds in the leaf axils of these shoots were followed by daily observations. This was done to determine the period of time required for floral differentiation on induced secondary branches.

The seed content of mature fruits from three high-yielding clones was determined to establish the degree of natural seed set. These clones were then designated as: low (clone 3A-T4, 0.9 percent of locules contained seed or 0.03 seed per fruit), intermediate (clone 269-2, 3.6 percent of locules contained seed or 0.11 seed per fruit) and high (clone 3B-T21, 16.6 percent of locules contained seed or 0.50 seed per fruit). All three clones were used to investigate the problem of low natural seed set and its relationship to the reproductive morphology in acerola. The three clones will be called: clone A (269-2), clone B (3A-T4) and clone C (3B-T21) throughout this thesis.

Flower development from the time of emergence in the leaf axil to anthesis was followed daily after labelling the tiny calyx of the flower bud with India ink. This was done to determine the number of days between flower bud emergence and anthesis.

Times of anther dehiscence of flowers of clones A (before 7:30 A.M.), B (approximately 1:00 P.M.) and C (by 6:30 A.M.) were determined by rubbing the anthers of some flowers against a black blotting paper to test for the presence of pollen grains and also by viewing the anthers of other flowers under a dissecting microscope. The examinations were made approximately every 30 minutes.

Germination of pollen grains and pollen tube growth were studied *in vitro*. Pollen grains of various ages were sown on artificial medium consisting of one percent agar, 12 percent sucrose and 87 percent tap water. Tap water was used instead of distilled water for two reasons: a, to supplement the medium with minerals; and b, to avoid the excessive bursting of pollen tubes which resulted when the medium was prepared with distilled water (Fig. 54). The medium was pipetted onto micro-slides and allowed to harden. Where distilled water extracts of styles were used, a thin film of the extract was flooded over the hardened medium and the pollen grains sown immediately. Fine bristle brushes were used to sow all pollen. The micro-slides were incubated under high humidity at room temperature in petri dishes lined with moistened filter paper. Percentages of pollen germination were established five hours after sowing by counting the pollen grains in five microscopic fields which were viewed by using a 10X objective.

Attempts were made to trace the growth of pollen tubes down the style and to determine the period of time necessary for pollen penetration into the ovule. Emasculated flowers were hand pollinated and collected after various intervals of time. Eoller's technique (8) for observing pollen tubes in the style of sweet cherry (*Prunus*) using a saturated solution of resorcin blue in 30 percent alcohol and neutralizing the stain with alkaline water was used on carpels which had been dissected longitudinally

with thin dissecting needles or a razor blade. However, the technique was abandoned since no callose plugs of pollen tubes were observed. Carpels were also cleared by boiling in a solution five parts chloral hydrate and three parts distilled water and stained with aniline blue in hopes of viewing callose plugs of pollen tubes in the style. However, no pollen tubes or callose plugs were observed. Further attempts were made by partially macerating carpels in 1 N. hydrochloric acid, neutralizing the acid with alkaline water, followed by staining with tincture of iodine or aniline blue and squashing. This technique did not yield any satisfactory results. One additional attempt to determine the course of the pollen tubes was made by collecting flowers four hours after hand pollination and by making transverse cuts of styles leaving their stigmatic ends intact. Stylar segments with their intact stigmas of one-third, two-thirds and the entire style were studied. They were cultured by placing some of them horizontally on the surface of the sucrose-agar medium and also by standing others upright on the excised end to determine the period of time elapsed before the pollen tubes emerged from the cut end.

In vivo studies on pollen germination and pollen tube growth were made from paraffin sections of flowers, which had been hand pollinated on the day of anthesis and were collected at daily intervals thereafter. The path of the pollen tube and the period of time between pollination and fertilization were determined by this study.

Fruit development from anthesis to maturity was followed by marking the pedicels of the flowers with India ink and making daily observations thereafter. This was done to determine the number of days between anthesis and fruit ripening.

Hand pollination, growth regulator application and the use of water extracts of floral parts and fruits on emasculated and non-emasculated flowers at anthesis were performed to determine their effects on fruit and seed set and other characteristics of fruit morphogenesis such as fruit size and shape.

Self pollination was done by bagging lateral branches bearing numerous open flowers with undehisced anthers with Manila paper sacks to prevent pollen contamination. Self pollination was then accomplished in the afternoon of the same day by removing the paper sacks and by employing a fine bristle brush to transfer the pollen. In clone B, pollen grains from the same flower or from other flowers of the same plant (sibbing) were used because of the low pollen content of some anthers. Sibbing was carried out by grasping excised stamens by their filaments with a pair of forceps and brushing the pollen directly upon the stigmas. The pedicels of the self-pollinated and sib-pollinated flowers were marked with India ink for identification and the paper sacks were replaced for another day before removal.

Emasculation of the female parent for cross pollination was accomplished before anther dehiscence occurred by removing the anthers with a pair of forceps while viewing the flowers with a 25X hand lens. The emasculated flowers were bagged with Manila paper sacks as a preventive measure against pollen contamination. Cross pollination was completed in the afternoon of the same day by following the technique described above for sib pollination.

Growth regulators were used to induce fruit set and were of crystalline forms. PCA (parachlorophenoxyacetic acid, an auxin) and GA (potassium gibberellate, a gibberellin) were used. The growth regulators were weighed by using an analytical balance. The crystals were dissolved in

five milliliters of 95 percent ethanol (the bridging solvent) and distilled water was added in various amounts to make up aqueous solutions of 25 and 50 ppm (parts per million) of PCA and 10 and 25 ppm of GA. A small amount of commercial wetting agent, EM-10 (Pacific Chemical and Fertilizer Company; active ingredients are 25 percent alkyl-benzenesulfonate and 10 percent non-ionic phenols), was added to the distilled water solutions, which were applied with hand atomizers.

Water extracts of floral parts at anthesis were made by adding a small amount of distilled water to excised stamens, styles (which included their stigmas) and ovaries. The excised structures were macerated with morter and pestle. Water extracts of entire flower buds of all stages, flowers at anthesis and fruits three, six, nine and twelve days after anthesis were also made in a similar manner. Emasculated flowers, which were marked with India ink for identification, were treated with the crude extracts. A small pipette was used to apply a drop of extract onto the pistil of the flower. (The flower at anthesis had an ovary of 1.0 mm. in diameter surrounded by a calyx of 5.5 mm. in diameter. The extract completely filled the gap between ovary and calyx and no run-off occurred.)

Fruit set in all treatments was determined six days after anthesis. Ovaries at this age were approximately 3.0 mm. in diameter and were partly surrounded by the calyx which was still about 5.5 mm. in diameter.

Seed set was determined by cutting a cross section of each fruit, 21 to 25 days after anthesis when the exocarp was slightly orange and the mesocarp still firm, using a razor blade. The three locules were examined with the naked eye and the number of fully formed seeds was counted.

Statistical treatment of data following a binomial distribution was analyzed by the 95 percent confidence interval method of Snedecor (83). This yielded the interval range between a lower and an upper limit for the mean of each treatment and was used in Tables II to V to determine significant differences in data of the experiments. The confidence, using this method, is based on a 95 percent probability that the interval range between the lower and upper limits for the mean of each treatment is correct. The percentage value of an individual treatment, making up a mean, is assumed to represent a significant difference if the treatment value is not within the interval range of its mean. Also, the means of the treatments are assumed to represent significant differences if their interval ranges do not have overlapping values.

Floral buds at different stages and various stages of the developing fruits were collected and placed immediately in a killing and fixing solution of Navaschin's Graf III formula (83). Ovules from pollinated flowers were dissected from the stony endocarps in collections made 10 days after anthesis and thereafter, to insure that the fruits contained normally developing ovules, and to facilitate sectioning.

Dehydration was accomplished by the tertiary butyl alcohol method of Johansen (44), and was followed by infiltration with "Parawax" and embedding in "Tissuemat" (melting point 56-58° C.). Paraffin sections were cut at 12 to 15 microns on a rotary microtome utilizing Gillette Blue razor blades in a Spencer razor blade holder. Sections of nodes were stained in Foster's tannic acid-iron chloride-safranin and also in Cross' safranin-fast green as outlined by Brooks et al. (8). Sections of flower buds, flowers and fruits were stained by using Cross' safranin-fast green technique (8) and Mayer's hemalum (83). Paraffin blocks of

nodes and of fruits 10 days after anthesis and older were partially softened by soaking overnight in a mixture of 1 part glacial acetic acid and 9 parts 70 percent ethanol (Gifford, 1950).

Squashes of young ovules from flower buds 3 to 4 days prior to anthesis were made in an attempt to isolate embryo sacs for further study of embryo-sac development. Squashes of root tips and anthers were also made to determine the ploidy of the three acerola clones. Squashes of dissected segments of ovules containing embryo sacs were made to establish the ploidy of the endosperm that was present in some embryo sacs containing immature embryos. All squashes were made with slight modifications following the technique outlined by Kamemoto et al. (46). Modifications included placing the plant material in 0.002M. 8-oxyquinoline solution overnight in a five degrees centigrade refrigerator, which was followed by killing and fixing in Carnoy's fluid (83) for 10 hours or longer at room temperature. After placing a cover glass over the stained material, the cells were loosened and dispersed by gently tapping on the cover glass with a blunt dissecting needle. The slide was covered with a paper towel and was inverted on a flat surface so that the cover glass was situated at the bottom of the slide. The slide was firmly held in place with one hand to prevent movement of the cover glass while the thumb of the other hand was used to squash the cells by pressing down on the slide and by exercising a sideways movement at the same time. Although both aceto-carmine and aceto-orcein were used, the latter was used in preference to the former because darker chromosomes and better results were obtained.

EXPERIMENTAL RESULTS

Floral Bud Differentiation and Development

Floral bud differentiation on primary branches, which were in active vegetative growth, occurred as early as eight to ten days before the first

flower buds emerged in the leaf axils (Fig. 5). In a few instances young flower buds were produced in the leaf axils of the node bearing the youngest pair of fully expanded leaves. Figure 5 shows such a situation and illustrates the earliness of floral bud emergence after initial differentiation. Although a few flowers were produced in the leaf axils of actively growing primary branches, heaviest flowering occurred in leaf axils of numerous short secondary branches. These branches had very short internodes, resembling rosettes, and were the branches where mature fruits normally occurred.

Emergence of vegetative axillary buds was induced by pruning primary branches 20 centimeters from the apex. Emergence of the secondary shoots was mainly confined to the first two or three nodes below the site of decapitation and occurred as early as four days after pruning (Table I). The sequence of emergence of the axillary shoots was basipetal from the cut end. In a few instances, emergence of two secondary shoots, which occurred one above the other, was observed at single leaf axils of the uppermost nodes of the primary branch. Figure 9 shows that there are numerous axillary meristems in a single leaf axil.

The time between initiation of floral bud differentiation on the secondary branches and the peak in a single flowering cycle of the whole plant was closely related. Table I shows that there was a longer period of time, between pruning of primary branches and production of flowers on secondary branches, when pruning was done in February (ending of non-flowering season) rather than in April (beginning of flowering season). Induced secondary branches from the February pruning produced tertiary rosette-like shoots in the leaf axils and the secondary branches resembled the unpruned primary branches when flowering occurred.

TABLE I. FLORAL BUD EMERGENCE ON SECONDARY BRANCHES WHICH WERE INDUCED BY PRUNING PRIMARY BRANCHES OF TWO CLONES OF ACEROLA

Clones	Months of Pruning	Days between Pruning and Emergence of Secondary Branches		Days between Pruning and Flower Emergence on Secondary Branches		Days between Pruning and Peak Flowering of Entire Plant
		Range	Mean	Range	Mean	
6663,3A-T3	February	10-45	19	43-52	47	47
Maumavilli	February	8-20	12	41-60	51	54
6663,3A-T3	April	4-13	11	15-25	19	14
Maumavilli	April	4-15	10	18-34	23	6

Emergence of the first flower buds on secondary branches from the April pruning was observed in the lowest leaf axils of the branches which emerged first. Emergence of the flower buds could be detected as soon as 15 days after pruning in clone 3A-T3, which was pruned 14 days prior to peak flowering of the whole plant, and as soon as 18 days in clone Maumawili, which was pruned six days before the peak in its flowering cycle.

Anatomical preparations of nodes from collections made during a flowering period showed the various stages of floral bud differentiation (Figs. 9 to 14). Axillary buds originated in partly vacuolated tissue which appeared to contain numerous axillary meristems. Figure 9 shows the superposed arrangement of the meristems, the uppermost of which is the oldest. The vegetative apical meristem was dome-shaped and exhibited a tunica-corpus organization. The meristem was subtended by opposite leaf primordia (Fig. 10). The delimitation between the tunica and the corpus was not always clear, since the outer layer of corpus cells were regularly arranged.

As flowering was initiated, the apical meristem of the axillary shoot ceased to produce leaf primordia and gradually flattened at the apex. This phenomenon was accompanied by increased cellular enlargement and vacuolation. The sepals, petals, stamens and carpels were differentiated in acropetal sequence (Figs. 11 to 13). Differentiation of these structures occurred by the time the flower bud emerged in the leaf axil (Fig. 15).

Development of Ovule

There was much variation in ovule development in the three clones studied. The description of what I have interpreted to be normal ovule development from its initiation until fertilization will be given first. Descriptions of abnormal ovule development will follow.

Normally, a single ovule was differentiated in each locule by the time the flower bud emerged from the leaf axil at six to seven days before anthesis

PLATE III

FIGURE 9: Numerous axillary meristems of scerels in superposed arrangement. 13 X.

FIGURE 10: Vegetative axillary meristem. Leaf primordia (l); apical meristem (a). 131 X.

FIGURE 11: Differentiation of sepals (s) and petals (p). 225 X.

FIGURE 12: Differentiation of stamens (a). Sepal (s) and petal (p) development are also shown. 129 X.

FIGURE 13: Differentiation of pistils (k) and development of sepals (s), petals (p) and stamens (a). 56 X.

FIGURE 14: Further growth and development of floral parts. Development of ovules (n) in relation to pistils (k). Young stamens (a) are also shown. 73 X.

FIGURE 15: Flower bud on the day of emergence. Single integument is shown as a short ring of cells (n). Micropyle of ovule is adjacent to the receptacle end of flower bud. Anther is indicated by a. 39 X.

FIGURE 16: Ovule at six days before anthesis showing single megaspore mother cell and palisaded nucellus. Short integument is shown (i). 934 X.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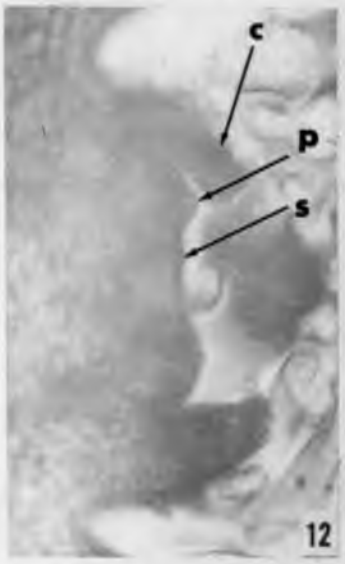

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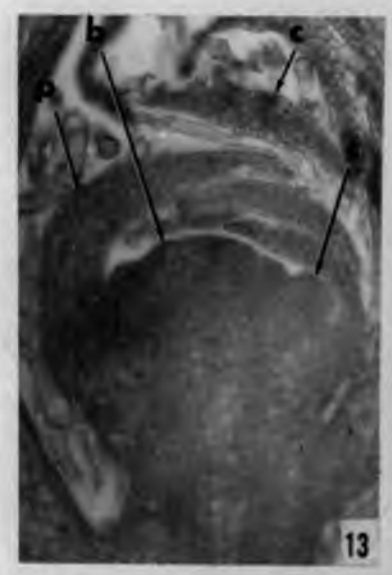

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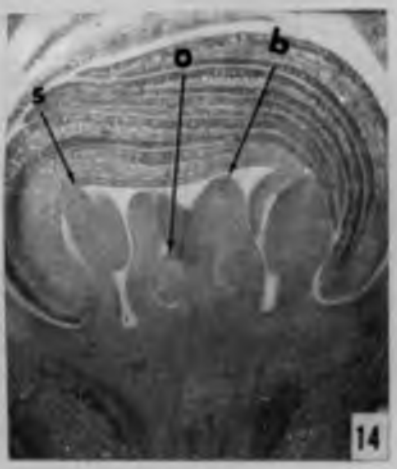

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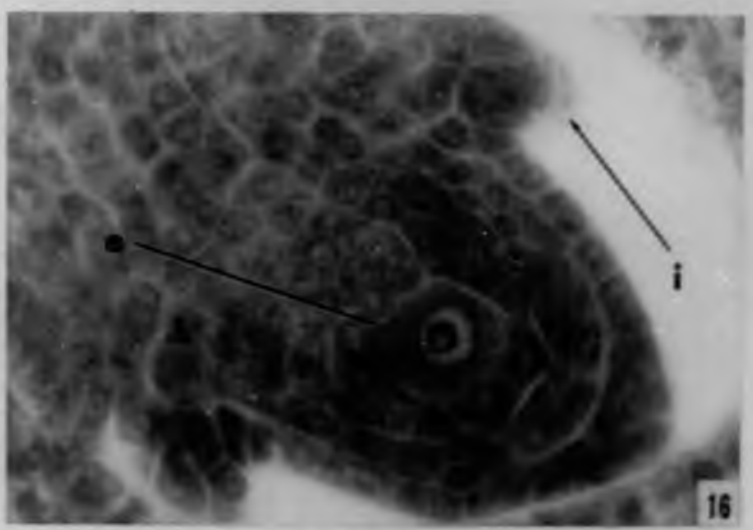

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in the three acerola clones studied. The inner integument was present as a short ring of tissue and extended to about half the length of the nucellus (Fig. 17). The tip of the integument was directed towards the receptacle end of the flower bud at this stage. The outer integument was differentiated as an outer ring of tissue before curvature of the developing ovule was observed (Fig. 20). Unequal growth of the funiculus caused the ovule to turn away from the receptacle end of the bud and become semi-anatropous four to five days prior to anthesis (Fig. 22). Rapid growth of the nucellus and outer integument occurred simultaneously with curving of the ovule so that the nucellus extended well beyond the inner integument, forming a nucellar beak. The outer integument was longest and enclosed the inner integument and nucellus. The ovule almost filled the locule at three days before anthesis and some completely filled locules were also observed. The ovule at this stage was anatropous and showed a short inner integument and a much longer outer integument. The micropylar canal occurred in folds over the nucellus (Fig. 25) or was more or less straight. The inner integument continued growing slowly, but the micropyle at anthesis was formed by the outer integument only. The cells of the nucellus began disintegrating from the chalazal end at two days after anthesis and the embryo sac became pear-shaped with increase in size.

Figure 28 shows the ovule on the day of anthesis. The disintegration of nucellar cells at both ends of the ovule is shown.

Abnormal development of the ovule, in which the cells forming the nucellar beak began disintegrating, was observed in flowers collected on the day of anthesis. Counts of 36 ovules from 12 ovaries of clone A at anthesis yielded 33.3 percent of ovules or 1.0 ovule per ovary having this abnormality. The disintegrating cells (Figs. 26 and 28 to 31) were much larger than the other nucellar cells and had thinner cell walls. Many of

PLATE IV

FIGURE 17: Single sporogenous cell (s) at six days before anthesis. Short integument (i) occurs at this stage. 961 X.

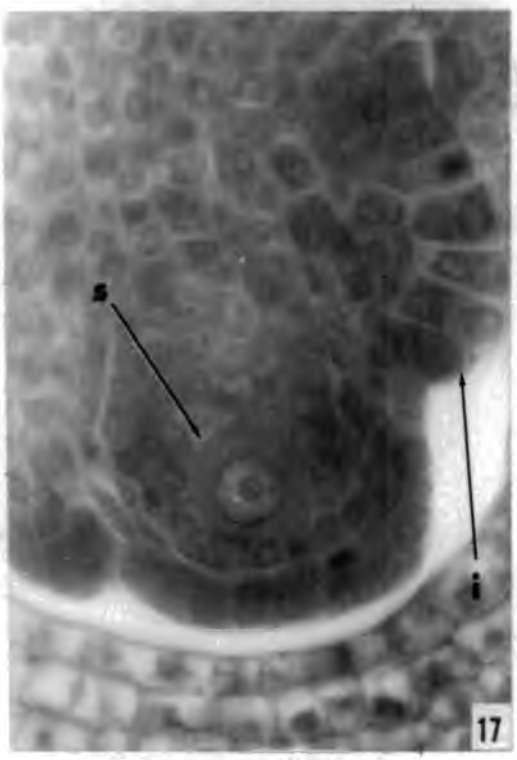
FIGURE 18: Ovule at six days before anthesis shows at least three sporogenous cells (s). 667 X.

FIGURE 19: Two sporogenous cells (s) in ovule at anthesis minus six days. 97 X.

FIGURE 20: Young ovule with single sporogenous cell (s) and showing differentiation of an outer integument (e) at five days before anthesis. Inner integument (i) is short at this age. 194 X.

FIGURE 21: Two-nucleate sporogenous cell (s) at four days before anthesis. 342 X.

FIGURE 22: Extension of nucellus beyond inner integument (i) forming a nucellar beak (n). Outer integument (e) is longer than nucellus. Ovule is semi-anatropous and funiculus (f) and a two-nucleate sporogenous cell (s) are shown. 378 X.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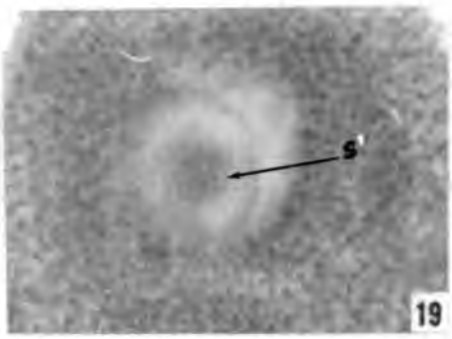
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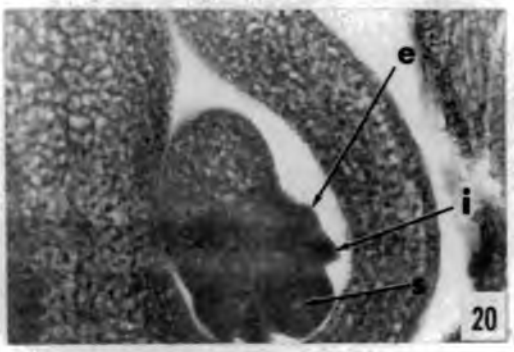
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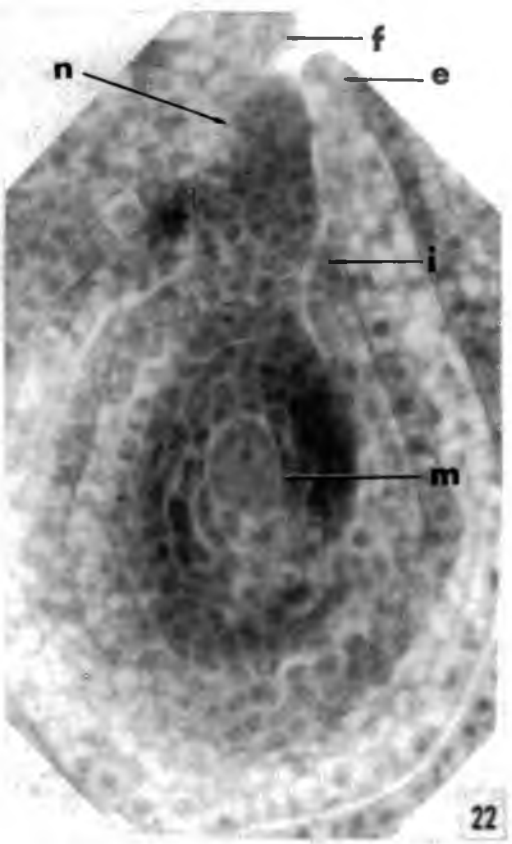
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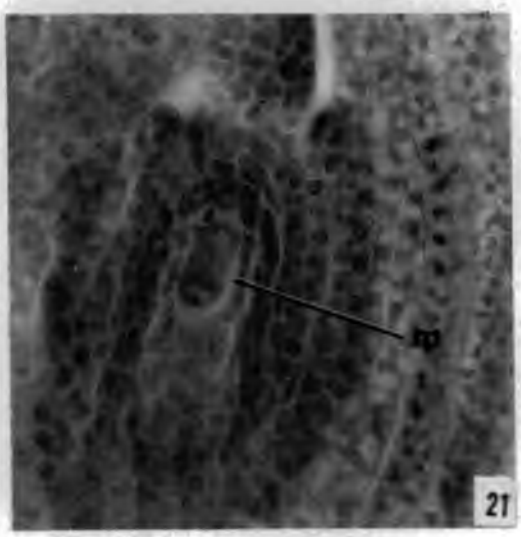
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the larger cells had large vacuoles before disintegrating. At three days after anthesis the nucellar cells in this type of abnormality were almost completely disintegrated and abortion of the embryo sac was observed (Fig. 26). Figure 31 shows evidence of disintegrated nucellar cells at the chalaza and also what appear to be two pollen tubes that have penetrated the micropyle. Fertilization cannot possibly occur in these types of ovules and the disintegration of nucellar cells before fertilization is one of the causes of low seed set in acerola.

The formation of other abnormal ovules are shown in Figures 32 to 40. In some of the cases studied, two ovules were differentiated in some locules early in floral development (Fig. 32) of clones B and C, but each locule of clone A contained a single ovule only. Determination of the frequency of this abnormality and the subsequent development of the abnormal ovules was made by examining the carpels of 100 mature fruits of each of the three acerola clones. Clone A showed but a single ovule per locule in all of the fruits examined. Clone B showed 50 percent of its fruits with a single ovule in each locule and 50 percent with two ovules in only one locule; i.e. of a total of three locules per fruit, only one locule had two ovules. Locules of fruits of clone C showed that 72 percent had one ovule, 24 percent had two ovules and four percent had two each within two of the three locules.

None of the locules bearing two ovules contained fully-formed seeds at maturity. The ovules had aborted and were collapsed. Figure 35 shows the orientation of the two ovules from a young ovary three days after anthesis. The micropyle of ovule "a" is at the basal end of the locule and that of the anatropous ovule "b" points between the funiculus and placenta. The orientation of both ovules is such that pollen tube penetration would seem unlikely in acerola in most cases. Based on the evidence of aborted ovules

PLATE V

FIGURE 23: Four megaspore stage at anthesis minus three days. 438 X.

FIGURE 24: Camera-lucida drawing showing an eight-nucleate embryo sac at three days before anthesis. 806 X.

FIGURE 25: Long outer integument forming a folded micropylar canal (m). 250 X.

FIGURE 26: Disintegration of nucellus and embryo-sac abortion. 109 X.

FIGURE 27: Camera-lucida drawing showing a sixteen-nucleate embryo sac in four nuclear groups at two days before anthesis. 625 X.

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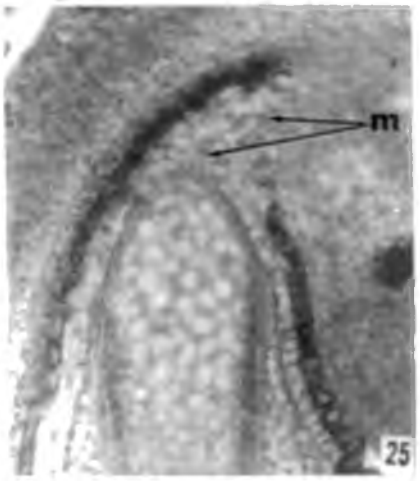

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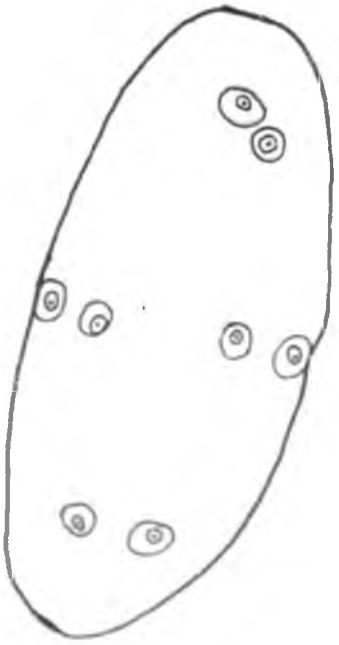

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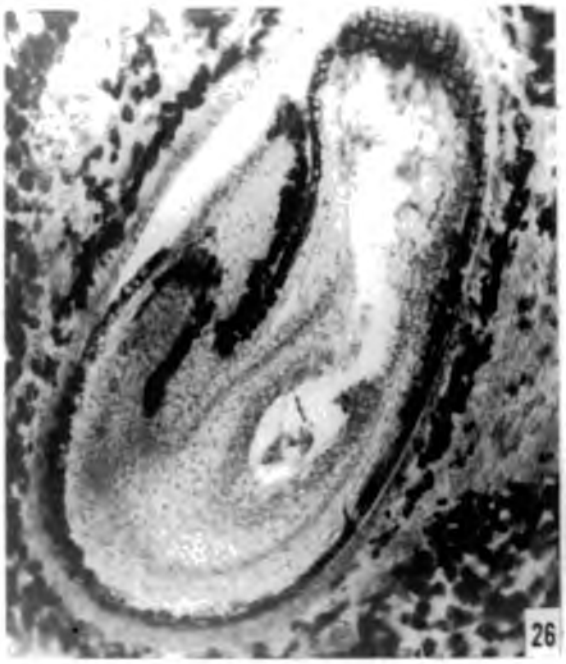

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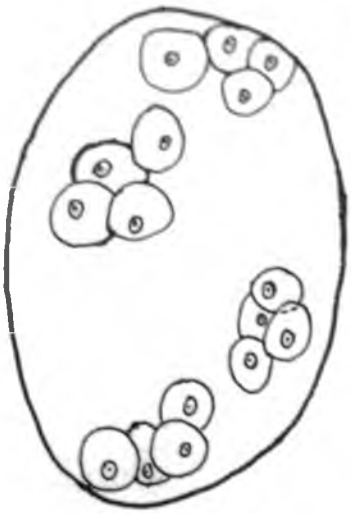

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PLATE VI

FIGURE 28: Ovule abortion on the day of anthesis. Disintegration of nucellar cells in this figure is shown at both the micropylar and chalazal ends. 111 X.

FIGURE 29: Ovule disintegration on the day of anthesis. 94 X.

FIGURE 30: Disintegrated nucellus at three days after anthesis. 94 X.

FIGURE 31: Disintegrated ovule at three days after anthesis. Two pollen tubes are shown at the micropyle by p. Embryo sac has completely disintegrated (e). 104 X.

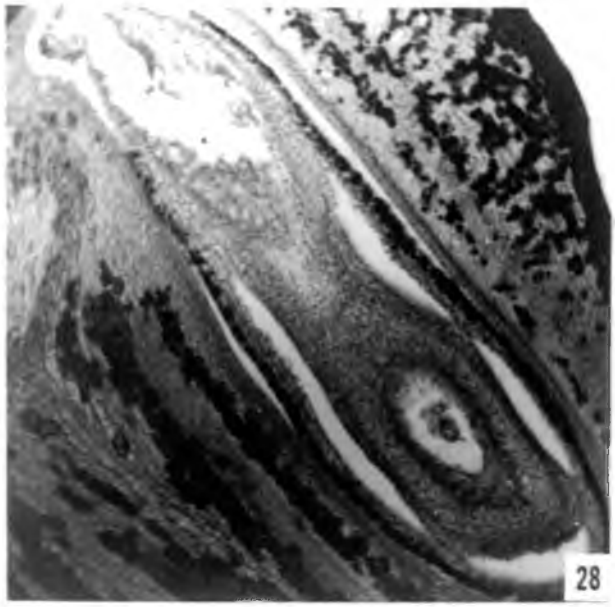

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PLATE VII

FIGURE 32: Cross section of ovary showing two ovules (o) in one locule and a single ovule in the other two locules. 77 X.

FIGURE 33: Aborted nucellar cells and completely disintegrated chalaza at three days after anthesis. 83 X.

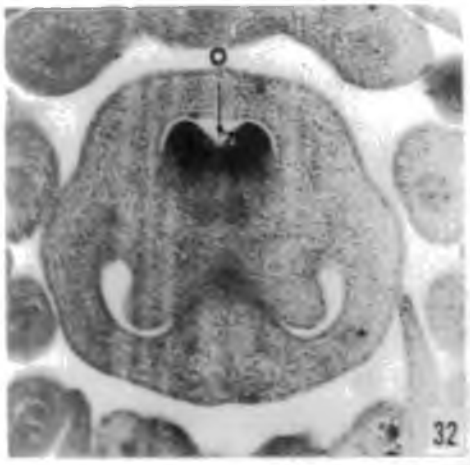
FIGURE 34: Embryo sac situated at center of nucellus. Chalaza (c) and embryo sac (e) are shown by arrows. 43 X.

FIGURE 35: Two ovules per locule showing abnormal positions of the micropyles (m). That of one ovule (a) is adjacent to the receptacle end of the locule and that of the other ovule (b) is semi-anatropous but faces the attachment of funiculus and placenta (f). 46 X.

FIGURE 36: Two locules showing the abnormal positions of ovules. The ovule in the left locule is upside down and the one in the right locule is curved coward the attachment of funiculus and placenta. 31 X.

FIGURE 37: Embryo sac situated at the chalazal end of the ovule. Nucellus shows constricted central portion and may abort later. Embryo sac (e) shows three peripheral nuclei; the densely stained central portion may be due to disintegrated nuclei. 111 X.

FIGURE 38: Embryo sac (e) situated at micropylar end of ovule. The pollen tube is interpreted to grow down the ovary at p and enters the locule to the right of the funiculus (f). The micropyle (m) is shown between the axil of funiculus and placenta. 97 X.



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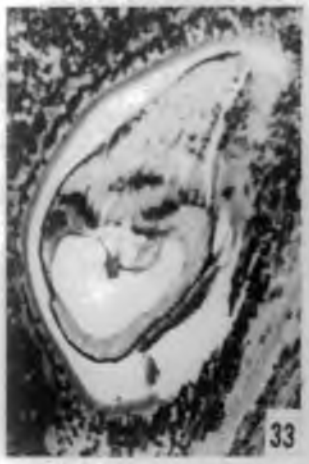
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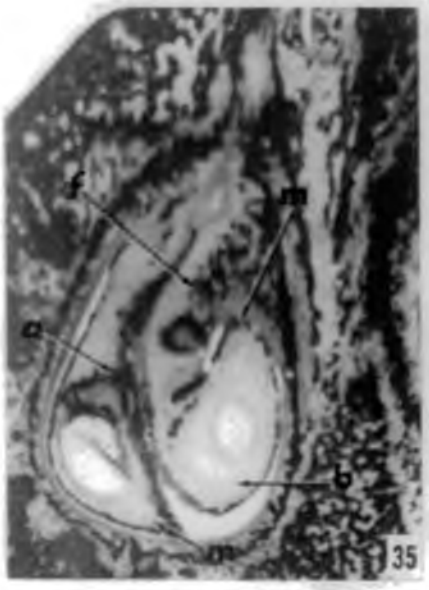

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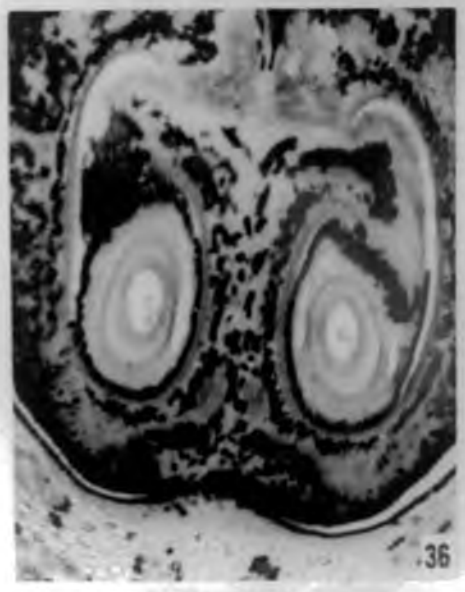
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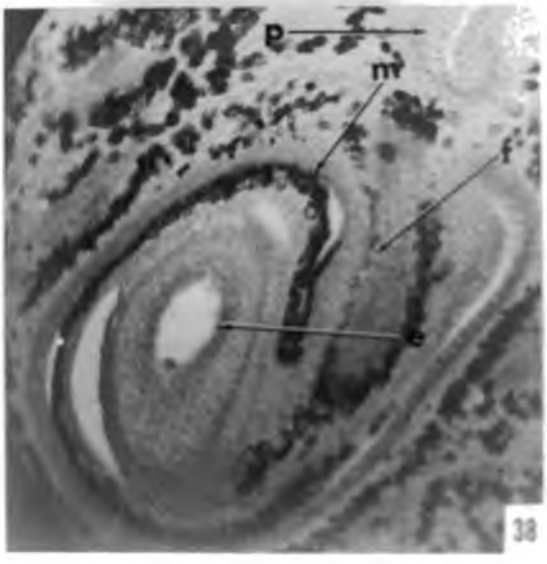

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in mature fruits and the abnormal orientation of the ovules, the production of two ovules in one locule of an ovary appears to be one of the contributing factors in low seed set in acerola.

Further evidence of the abnormal ovule orientation in relation to pollen tube growth is shown in Figures 36 and 38. Figure 38 shows a semi-anatropous ovule, but the micropyle occurs between the axil of funiculus and placenta. Figure 36 shows two locules, each of which contains an inverted ovule with the micropyle opposite the receptacle-end of the fruit. Counts of 84 ovules within 28 ovaries of clone A showed that the frequency of inverted ovules was 8.3 percent or 0.25 per ovary.

Megagametogenesis and Embryo-Sac Development

The development of the embryo sacs within the ovules showed much variation and abnormality in all three clones studied. The sporogenous cells of flower buds six to seven days prior to anthesis consisted of either a single large megaspore mother cell (Fig. 17) or showed several sporogenous cells of different sizes and arrangements (Figs. 18 and 19). In the latter, the largest megaspore mother cell was the only functional one.

Normally, only a single sporogenous cell developed within an ovule and formed a 16-nucleate mature embryo sac of the Penaea-type. However, in one ovule, from a flower bud collected at anthesis minus three days, two developing embryo sacs were observed (Fig. 40). These may have originated from two adjacent megaspore mother cells.

Nuclear division of the megaspore mother cell occurred at various times, and the first reduction division was not observed until four or five days prior to anthesis (Fig. 21). The cytoplasm of the megaspore mother cell, between the two resulting nuclei, appeared fibrillar. Megasporogenesis was completed three to four days before anthesis when a four-nucleate cell was present, in which each of the nuclei is interpreted as a megaspore.

The four nuclei were situated towards the periphery of the embryo sac and were obliquely connected by fibrillar cytoplasm (Fig. 23).

Megagametogenesis was evidenced three days before anthesis with the formation of an eight-nucleate embryo sac, which resulted from nuclear division of the four megaspores. The eight nuclei of the embryo sac were arranged in four pairs towards the periphery of the embryo sac (Fig. 24). Two to three days prior to anthesis, a further nuclear division occurred and a 16-nucleate embryo sac was produced. The nuclei were arranged in four groups of four nuclei each (Fig. 27) and were enclosed in peripheral cytoplasm.

Upon maturity, the embryo sac consisted of five nuclear groups. One nucleus from each of the four peripheral groups migrated to the center of the embryo sac and formed a four-nucleate polar group (Fig. 42). The embryo sac appeared mature on the day of anthesis. Polarity in embryo-sac development was weak and there was considerable variation in nuclear arrangements. The nuclei of the mature embryo sac were approximately the same size and the egg was not distinguishable.

The embryo sac occurred in various places within the nucellus. Figures 34 and 37 to 38 show that the embryo sac at anthesis may lie deep within the nucellus or closer to the micropyle. The micropylar-end of the embryo sac at anthesis was enlarged and the sac appeared to be pear-shaped. Further enlargement of the micropylar end occurred and the embryo sac at three days after anthesis had a sharply constricted chalazal end. Usually the embryo sac at anthesis was situated deep within the nucellus. The location of the embryo sac within the nucellus might be a determining factor influencing the time and success of fertilization.

Various types of abnormalities were frequently observed in embryo-sac development in the three clones studied. Figures 28 to 31 show some of the abnormalities that may ultimately result in ovule sterility. The abnormalities ranged in time of appearance from very early in megasporogenesis, where the megaspore mother cell failed to undergo meiosis, to later stages when nuclei disintegrated (Figs. 28 and 37) and embryo sacs aborted (Figs. 26 and 31). At the present time I am unable to interpret these abnormalities or to speculate on the causal factors. However, failure of embryo sacs to mature undoubtedly also contributes to the low percentage of seed set in acerola.

Development of Anther and Pollen

Development of anthers and pollen appeared to be normal in all three clones studied. However, two exceptions were observed during this study and will be described first. These exceptions were of local occurrence (Figs. 41 and 43) and were not observed in other cases during this study. Figure 43 shows numerous aborted or collapsed pollen grains within one anther sac only of clone B at three days before anthesis. Normal pollen grains were observed in the adjacent anther sacs. Occasionally, a few aborted pollen grains were observed in other anthers, but the number of aborted grains was relatively low. Figure 41 shows pollen germination and pollen tube growth within an undehisced anther sac of clone A at anthesis. Germination of pollen grains in undehisced anther sacs has been reported in cleistogamous flowers. Maheshwari (106, p. 183) gives four references to this occurrence. However, I do not know of any reports in the literature of this phenomenon in plants whose flowers are produced well above ground and open normally at anthesis.

In cross section, the anther appeared as a structure with two larger lobes, each of which consisted of two smaller lobes, at the time the bud

PLATE VIII

FIGURE 39: Aborting ovule at anthesis showing disintegration of nucellus (b) and degeneration of nuclei in the embryo sac (d). Constriction (c) of ovule is shown. 97 X.

FIGURE 40: Abnormal development of two ovules in one locule. The left ovule contains a single sporogenous cell (g). The right ovule shows the development of two embryo sacs (e) arranged one above the other. The outer integument (i) and micropyle (m) are facing the receptacle end of the locule. 157 X.

FIGURE 41: Pollen germination in an undehisced anther of clone A at anthesis. 196 X.

FIGURE 42: Formation of polar nuclear group at one day before anthesis. Two peripheral nuclear groups are shown and one nucleus (n) from each group appears to be migrating toward the center of the embryo sac. 174 X.

FIGURE 43: Aborted pollen grains in anther sac of clone B three days before anthesis. 286 X.

FIGURE 44: Cross section of anther at six days before anthesis. There are two larger lobes (L) and each larger lobe consists of two smaller lobes (l) adaxial to the connective (c). 87 X.

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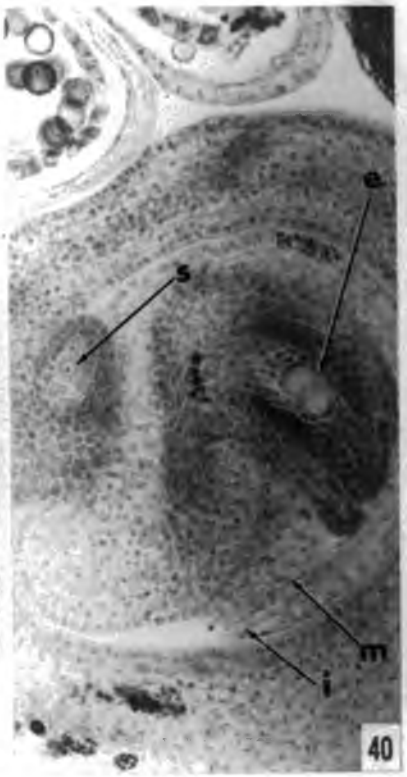

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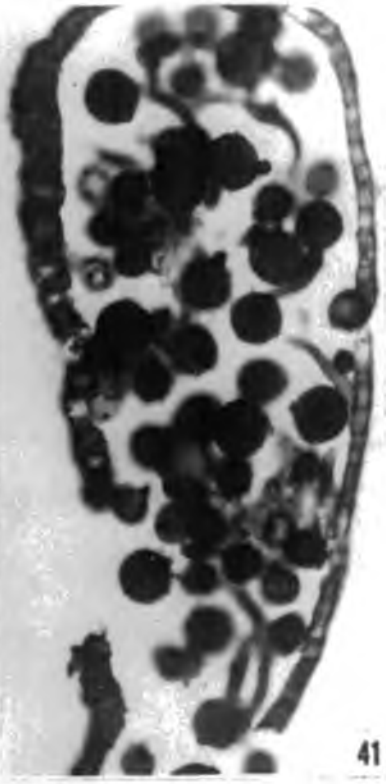
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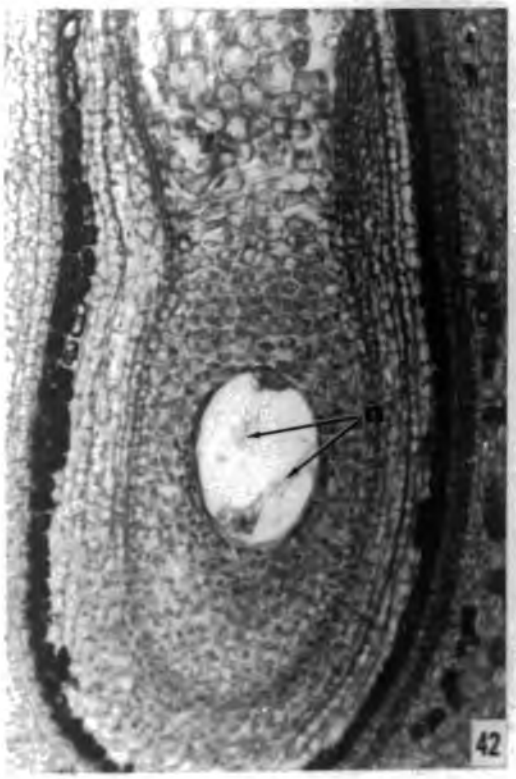

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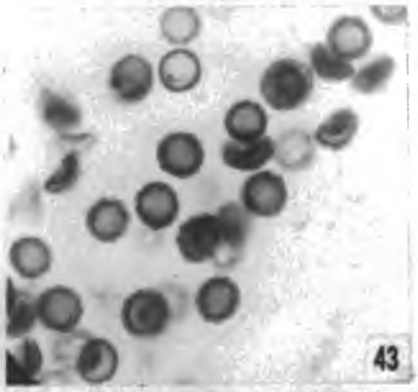
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PLATE IX

FIGURE 45: Two-celled stage in microsporogenesis at five days before anthesis. Dyads (d) and tapetal cells (t) are shown by arrows. 214 X.

FIGURE 46: Tetrads of microspores at four days before anthesis. 192 X.

FIGURE 47: Separation of microspores from tetrads in pollen grain formation at three days before anthesis. Disintegration of tapetal cells (t). 267 X.

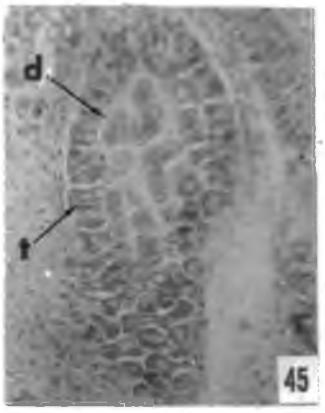
FIGURE 48: Anther at three days before anthesis showing four anther sacs and morphologically mature pollen grains. Larger lobe (L), smaller lobe (l) and connective (c) are shown by arrows. 65 X.

FIGURE 49: Anther at two days before anthesis showing rupture (r) of the partition separating the two smaller lobes of a larger lobe. 132 X.

FIGURE 50: Anther dehiscence at anthesis of clone C. 58 X.

FIGURE 51: No *in vitro* pollen germination of grains one day before anthesis. 98 X.

FIGURE 52: No pollen germination of grains two days after anthesis. 98 X.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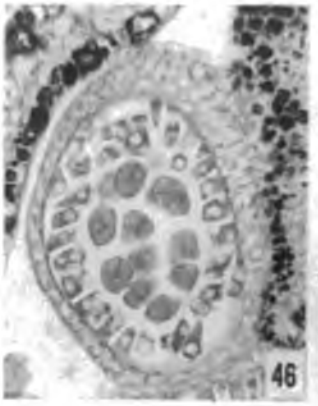
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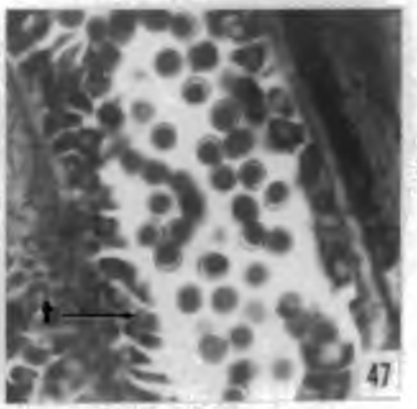
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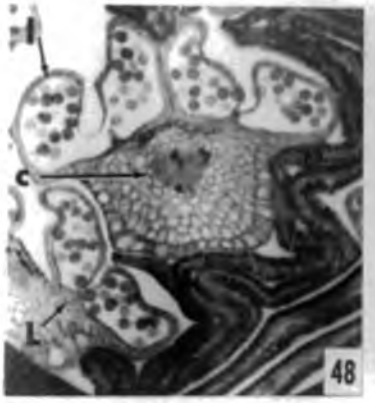
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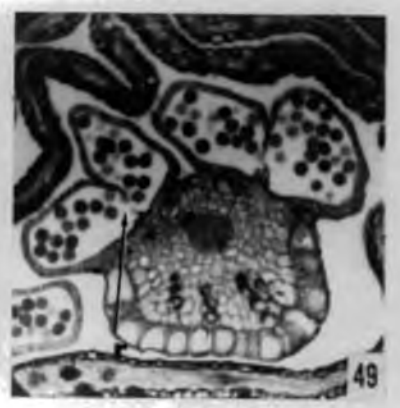
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emerged in the leaf axil six to seven days before anthesis (Fig. 44). Each of these four smaller lobes contained a single pollen sac (microsporangium) in which several layers of sporogenous cells (pollen mother cells) formed a central cylinder. The sporogenous cells were surrounded by a layer of radially elongated tapetal cells, outside of which were two middle layers and an endothecium and epidermis. The two larger lobes were located side by side and adaxial to the connective.

Reduction division of pollen mother cell began five days before anthesis, at which time a two-celled stage was observed (Fig. 45). Tetrads of microspores (Fig. 46) were formed four days before anthesis, and at this time, the tapetum began disintegrating. The tapetal cells had disintegrated completely by anthesis minus three days (Fig. 47). Pollen grains, which were formed by the separation of the tetrads of microspores, possessed single nuclei at anthesis minus three days, but had increased in size and had developed reticulately thickened walls. At anthesis minus two days, a second nucleus was observed in each pollen grain, which was not morphologically distinguishable from the mature binucleate grain at anthesis.

The four pollen sacs remained distinct until three days before anthesis. At this time growth of the anther resulted in each of the two larger anther lobes being moved to slightly lateral positions in respect to the connective. Two days before anthesis, the partition separating the two pollen sacs within each larger anther lobe ruptured (Fig. 49), and it disintegrated one day before anthesis. Thus, in effect, each larger anther lobe contained a single chamber filled with morphologically mature pollen grains one day before anthesis.

During the day of anthesis, the pollen was liberated. However, anther dehiscence showed considerable variation in both degree and time among the clones. The anthers of clone C normally dehisced by 6:30 A.M.; those of

clone A before 7:30 P.M.; while the anthers of clone B either initiated dehiscence around 1:00 P.M. or completely failed to dehisce in observations made of newly opened flowers on five separate days. The temperature range for this period was from a mean low temperature of 21.1° C. to a mean high temperature of 30.8° C. Anther dehiscence of intact flowers seemed to be associated with atmospheric conditions, particularly temperature and to a lesser degree with precipitation. Higher morning temperature with some rains during the previous evening seemed to promote anther dehiscence. Poor anther dehiscence of other acerola clones and the lack of effective pollinating agents in Hawaii have been reported by Yamane and Nakasone (104) and may be a contributing factor in low natural seed set.

Pollen Germination and Pollen Tube Growth

In Vitro Studies. Data on pollen germination *in vitro* are presented in Tables II and III and pollen tube growth is illustrated in Figures 53 to 73. Germination of pollen grains was found to be related to the time of natural dehiscence of anthers, and the pollen was germinable for only a short time. Pollen grains, which appeared to be mature, were dissected from anthers one to three days prior to anthesis and sown immediately on a sucrose-agar medium. No germination was observed even after a period of 48 hours. Pollen grains from naturally dehisced anthers at anthesis showed significant increase in germination. Peak pollen germination occurred in the afternoon hours as shown in Table II. Pollen germination declined at the end of the day (Figs. 58 to 60) and pollen grains from day-old flowers (Fig. 53) showed almost no germination (2.3 percent for clone C). Pollen obtained from flowers two days after anthesis showed no germination (Fig. 52). A significantly low percentage of pollen germination was observed in clone B (Table II). Pollen germination of groups of pollen grains was observed as early as 30 minutes after sowing while germination of isolated

pollen grains was not observed until approximately two hours after sowing.

Studies on the effect of stylar extracts on pollen germination showed that the addition of stylar extracts did not increase pollen germination significantly except in the case of clone B (Table III). In clone B addition of its own extract significantly increased its pollen germination as shown in Table III. However, percentage of pollen germination of clone B was still significantly lower than those of the other two clones studied.

Although addition of stylar extracts showed some beneficial effect on percentage of pollen germination, the subsequent pollen tube growth appeared to be inhibited (Figs. 61 to 69). Pollen tubes of clone A measured five hours after germination showed tube lengths of over 1.80 mm. with no extract; as long as 0.71 mm. with its own extract, and up to 0.15 mm. and 0.50 mm. with the stylar extracts of clones B and C respectively. Similar trends of pollen tube growth were observed in the other two clones. Figures 61 to 69 illustrate the relative pollen tube lengths after different treatments.

Weakest inhibition of pollen tube growth was shown when the extracts and the pollen were from the same clone. Stylar extracts of clone B, which showed beneficial effects upon pollen germination, appeared to show strongest inhibition in pollen tube growth of pollen grains from clones A and C.

Additional *in vitro* studies on pollen germination and pollen tube growth using decapitated stylar segments were made. Decapitated segments of styles, from flowers hand pollinated four hours earlier, were cultured on sucrose-agar medium. Observations of cultures five hours later showed a few pollen grains on the stigmas (Figs. 70 to 72). There was also an abundance of pollen tubes from grains which were deposited on the medium from the stylar segments. This made it almost impossible to tell whether or not the pollen tubes which were observed at the cut ends represented those that had grown through the lengths of the styles.

PLATE X

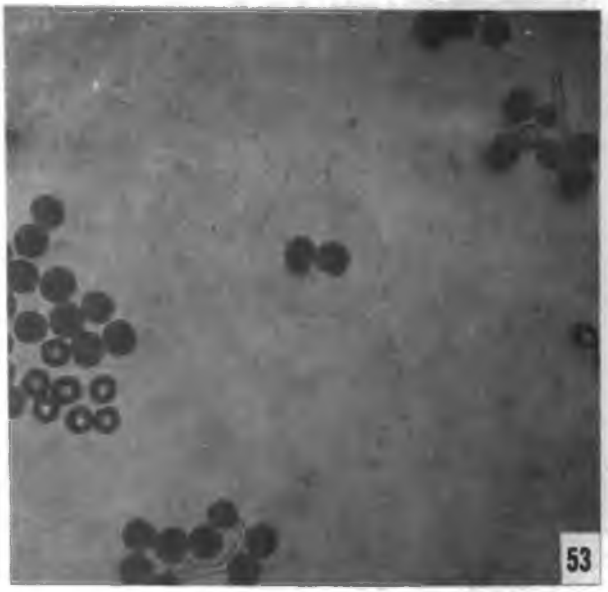
FIGURE 53: Almost no pollen germination of grains one day after anthesis. 98 X.

FIGURE 54: Bursting of pollen tubes using distilled water as the solvent in the sucrose-agar medium. Pollen grains (clone A) were sown at 7:30 AM. 98 X.

FIGURE 55: Pollen grains sown at 1:30 PM (clone A). Shows germinated pollen grains and long pollen tubes. 98 X.

FIGURE 56: Pollen germination and pollen tube growth of clone B from pollen sown at 1:30 PM. 98 X.

FIGURE 57: Pollen germination and pollen tube growth of clone A pollen sown at 7:30 PM. Shows the decrease in pollen germination. 98 X.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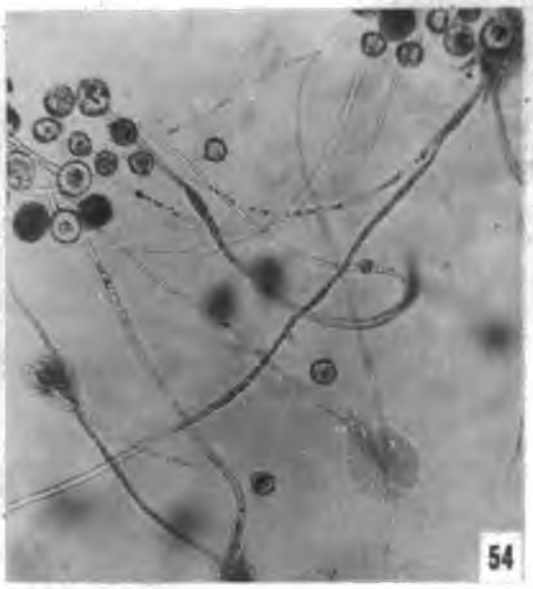
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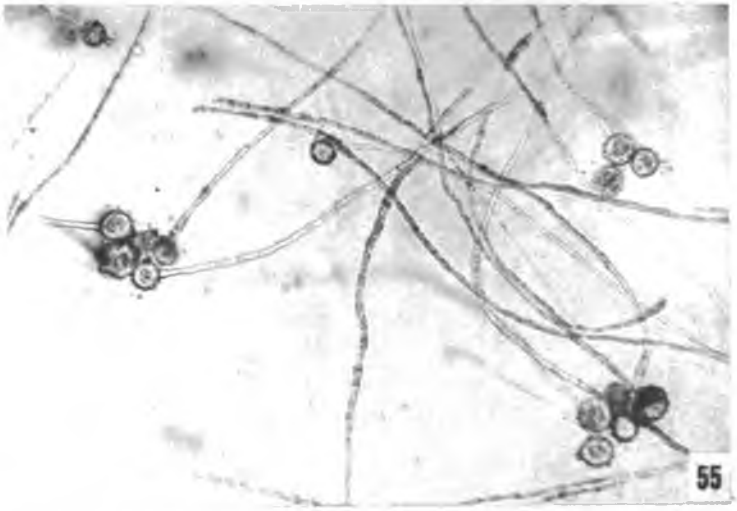
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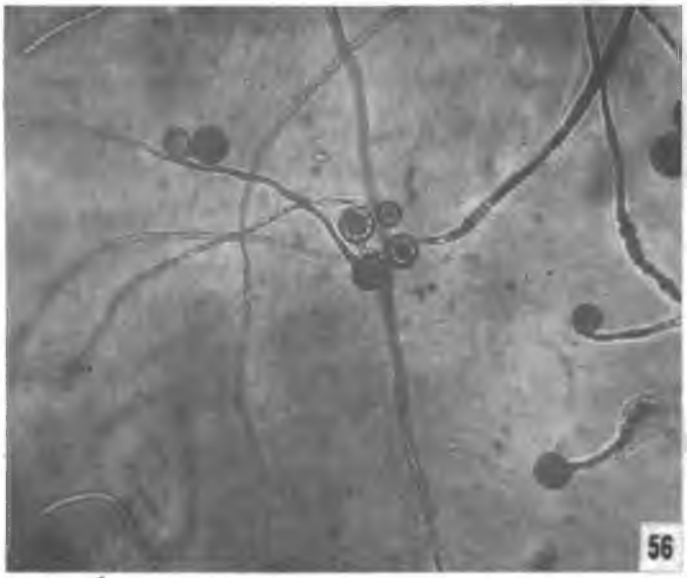
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PLATE XI

FIGURE 58: Pollen germination and pollen tube growth of clone C from pollen sown at 7:30 AM. 98 X.

FIGURE 59: Pollen germination and pollen tube growth of clone C from pollen sown at 1:30 PM. 98 X.

FIGURE 60: Pollen germination and pollen tube growth of clone C from pollen sown at 7:30 PM. 98 X.

FIGURE 61: Effect of clone A stylar extract on pollen germination and pollen tube growth of clone A. 98 X.

FIGURE 62: Effect of clone B stylar extract on pollen germination and pollen tube growth of clone A. 98 X.


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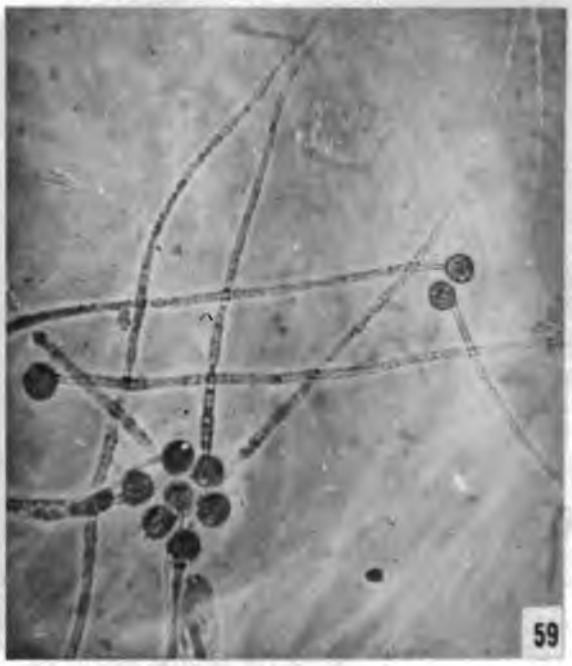
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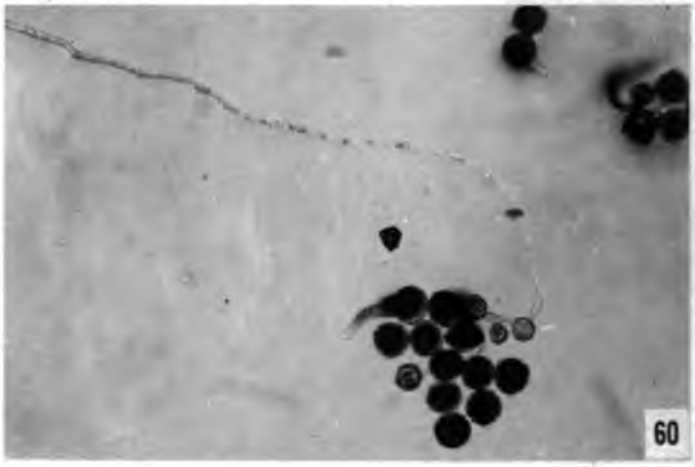
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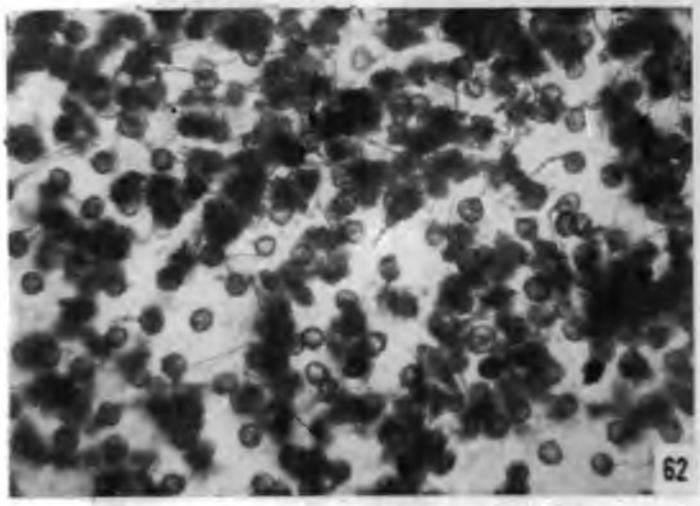
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PLATE XII

FIGURE 63: Effect of clone C stylar extract on pollen germination and pollen tube growth of clone A. 98 X.

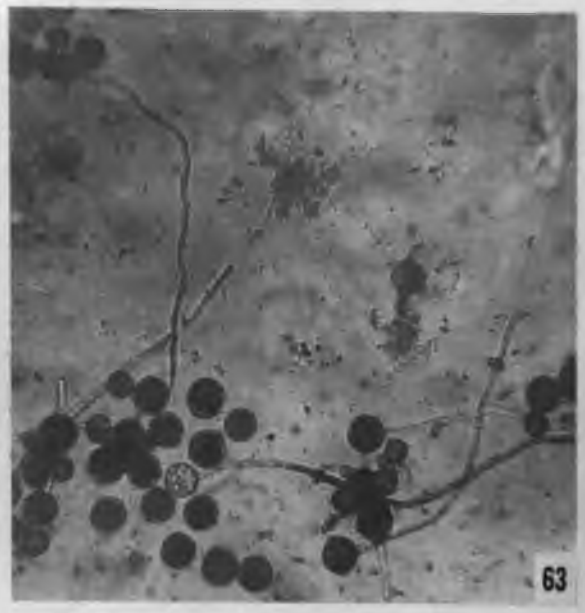
FIGURE 64: Effect of clone B stylar extract on pollen germination and pollen tube growth of clone B. 98 X.

FIGURE 65: Effect of clone A stylar extract on pollen germination and pollen tube growth of clone B. 98 X.

FIGURE 66: Effect of clone C stylar extract on pollen germination and pollen tube growth of clone B. 98 X.

FIGURE 67: Effect of clone C stylar extract on pollen germination and pollen tube growth of clone C. 98 X.

FIGURE 68: Effect of clone A stylar extract on pollen germination and pollen tube growth of clone C. 98 X.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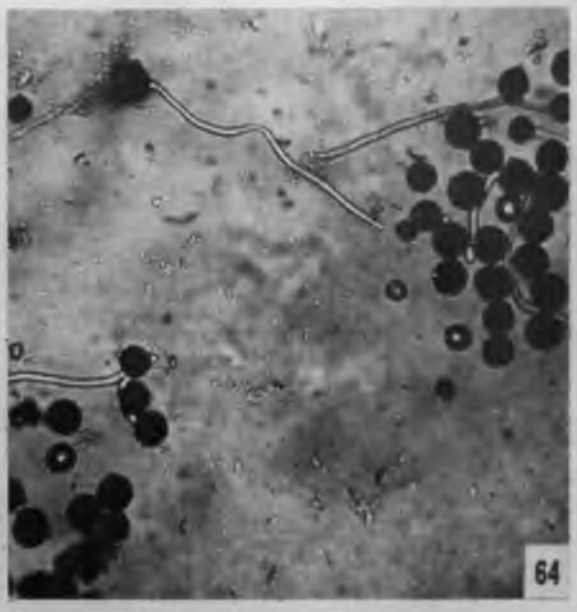
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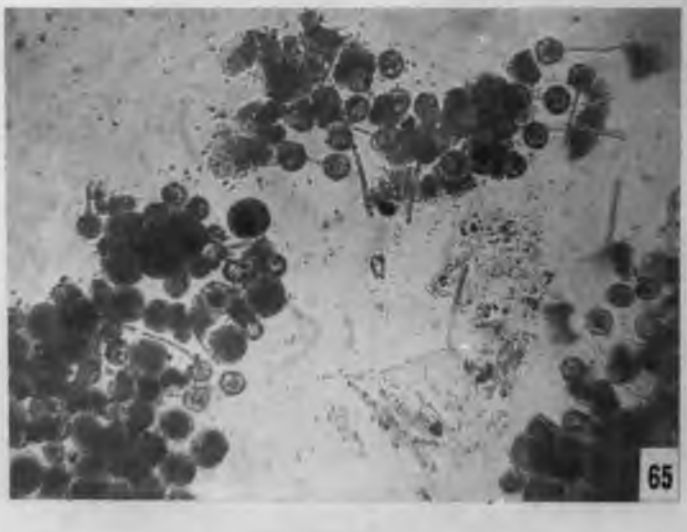
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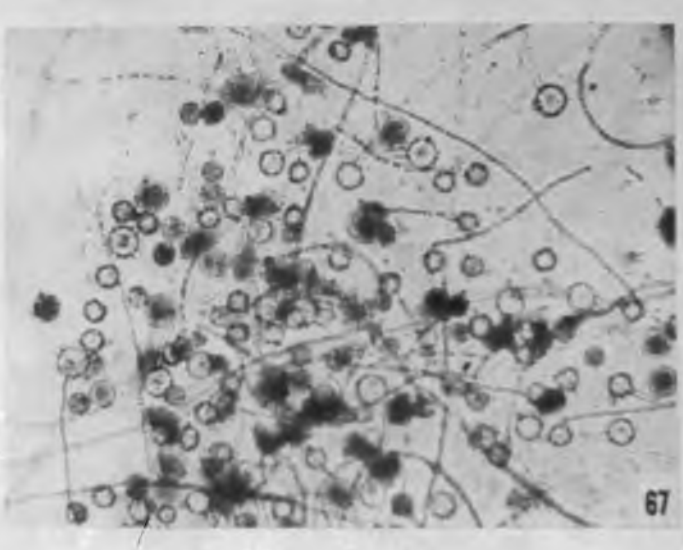
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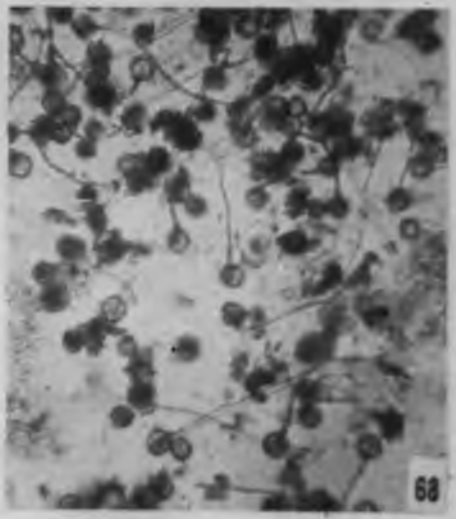
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PLATE XIII

FIGURE 69: Effects of clone B stylar extract on pollen germination and pollen tube growth of clone C. 98 X.

FIGURE 70: Chemotropic response in growth of clone C pollen tubes (p) away from the stigma (s) of clone B in in vitro culture. 97 X.

FIGURES 71 and 72: Chemotropic response in growth of clone C pollen tubes toward the stigmas and styles of clone B in in vitro culture. 97 X.

FIGURE 73: Pollen tube (t) growth of clone C pollen (p) through excised style of clone B. 97 X..

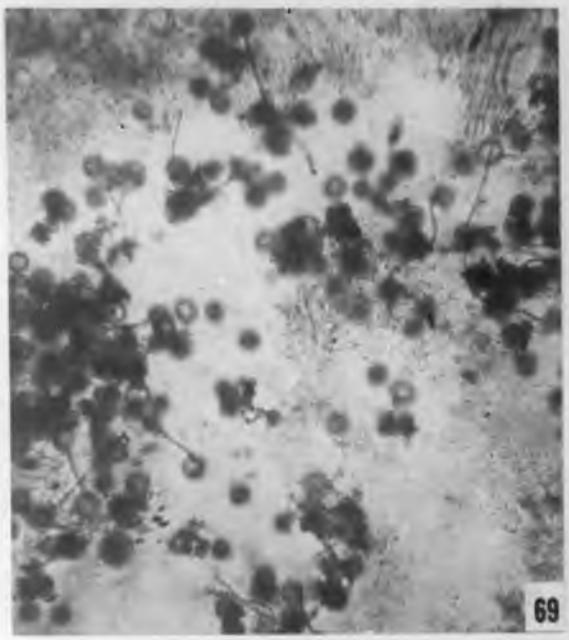
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TABLE II. PERCENTAGE OF POLLEN GERMINATION OF THREE ACEROLA CLONES FIVE HOURS AFTER SOWING ON THE DAY OF ANTHESIS.

Clones	Time of Anther Dehiscence	Time of Sowing 7:30AM	1:30PM	7:30PM	Mean Percentage Germination of Clones	Confidence Limits (95% Level)
		Percentage of Germination				
A	7 AM	37.9*+	62.6*+	32.0*	47.0	39.6-54.4
B	1 PM	09.0*+	30.2*+	7.7+	11.1*	7.1-15.8
C	6 AM	55.6+	67.8*+	37.2*+	59.2	44.0-56.0
Mean Percentage Germination of Treatments		14.8+	53.7+	26.8+		
Confidence Limits (95% Level)		9.5-21.0	48.1-59.9	21.2-32.8		

*Significantly different percentage values for clones.
+Significantly different percentage values for time of sowing.

TABLE III. PERCENTAGE OF POLLEN GERMINATION WITH THE ADDITION OF STYLAR EXTRACTS ON THE DAY OF ANTHESIS OF THREE ACEROLA CLONES.

Stylar Extracts of Clones	Pollen of Clones A	B	C	Mean Percentage Germination With Extracts	Confidence Limits (95% Level)
	Percentage of Germination				
A	56.5	32.4*	64.6*	52.7	43.8-62.2
B	67.6*	50.0+	71.9*	64.2	47.1-67.5
C	64.7	43.6*	69.4	59.8	50.6-69.4
None	62.6*	30.2*	67.8*	55.0	49.0-61.0
Mean Percentage Germination of Clones	62.5	37.2+	67.7		
Confidence Limits (95% Level)	54.9-69.1	29.6-44.9	61.2-74.6		

*Significantly different percentage values for effects of stylar extracts.
+Significantly higher percentage value for clone B pollen.

Various chemotropic responses were observed of the elongating pollen tubes of the scattered grains which were deposited on the medium (Figs. 70 to 72). Growth of these pollen tubes appeared to be directed towards the stylar segments in most cases. However, these pollen tubes did not show any definite chemotropic responses of growth towards the stigmatic surfaces as might be expected if some growth promoting substance was secreted by the stigmas after pollination as in certain grasses (107).

Since hand pollination of intact flowers using conventional methods caused a wide scattering of pollen which were deposited on the medium and germinated along the styles, flowers of clone B bearing undehisced anthers were hand pollinated with clone C pollen in a special way. Flowers of clone B were removed from the plant and were held upside down while the stigmas were gently touched to the pollen source. This procedure insured that pollen would be placed only on the stigma. The pedicels of the pollinated flowers were immediately placed in water and stylar decapitations were made after four hours. Figure 73 shows a pollen tube emerging from the cut end of a stylar segment 16 to 17 hours after pollination. The stylar segment was 1.03 mm. in length and the length of the pollen tube was 1.05 mm. Measurements of the lengths of 15 styles from the stigmatic tip to the point of attachment on the ovaries yielded an average length of 3.26 mm. for clone B. At this rate of growth, the pollen tubes would take approximately two to three days to grow through the entire lengths of cultured styles. (In vivo studies on pollen tube growth, to be presented later, showed that pollen tubes were approximately one-third to one-half the lengths of styles from flowers collected 24 hours after pollination.)

In Vivo Studies. Examinations of sections of flowers showed that pollen tube penetration into the styles occurred within four hours after pollination in all three clones. Pollination treatments included selfing,

PLATE XIV

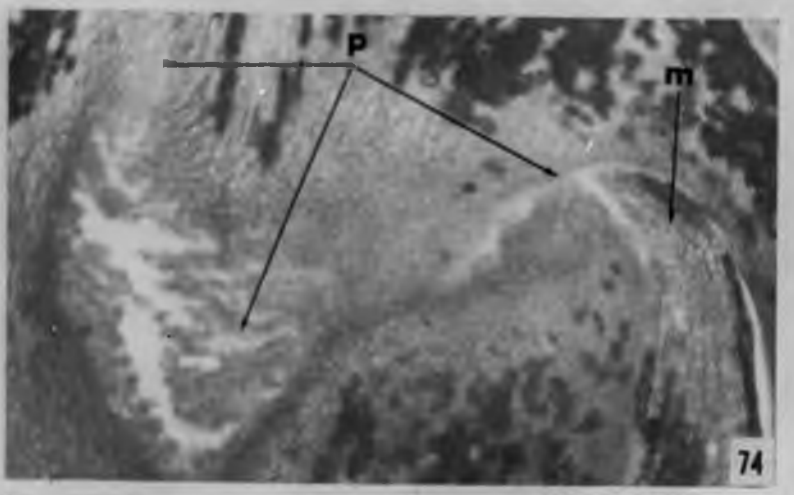
FIGURE 74: Normal pathway of pollen tube growth (p) into the ovary and locule are shown by arrows. The micropyle (m) lies adjacent to the pathway. 136 X.

FIGURE 75: Pollen tubes (t) did not enter the ovule (o) and have burst at the receptacle end of the locule. 76 X.

FIGURE 76: Pollen tube (t) penetration into the micropyle (m) three days after hand pollination at anthesis. 600 X.

FIGURE 77: Embryo sac two days after pollination and containing a pollen tube (t) which has burst at the chalazal end of the sac. What may be two sperm nuclei (s) are shown among the contents of the pollen tube. The egg (e) is slightly out of focus and is situated at the micropylar end of the embryo sac. What may be a binucleate endosperm mother cell (m) and two other nuclei (d) might be the polar group of nuclei. 451 X.

FIGURE 78: Embryo sac three days after pollination. The embryo sac is pear-shaped and may be at the stage of double fertilization. The egg (e) and endosperm mother cell (m) are shown and what may be two sperm nuclei (s) are represented by the darkly stained "spots". One of the sperm nuclei has reached the egg. 311 X.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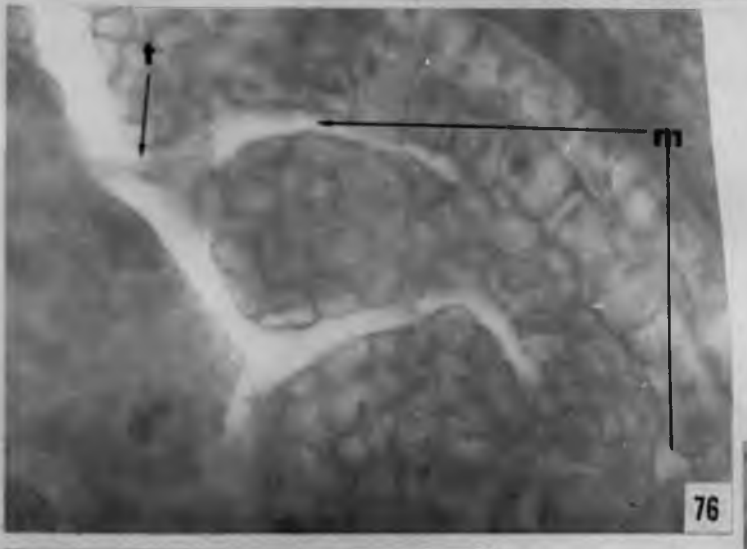
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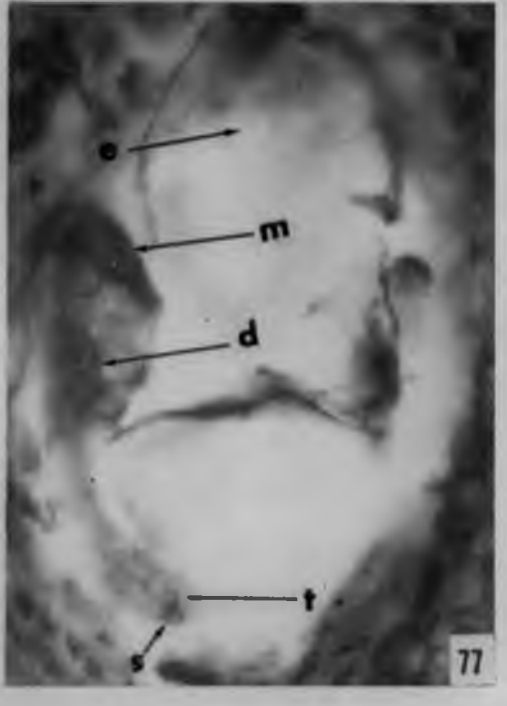
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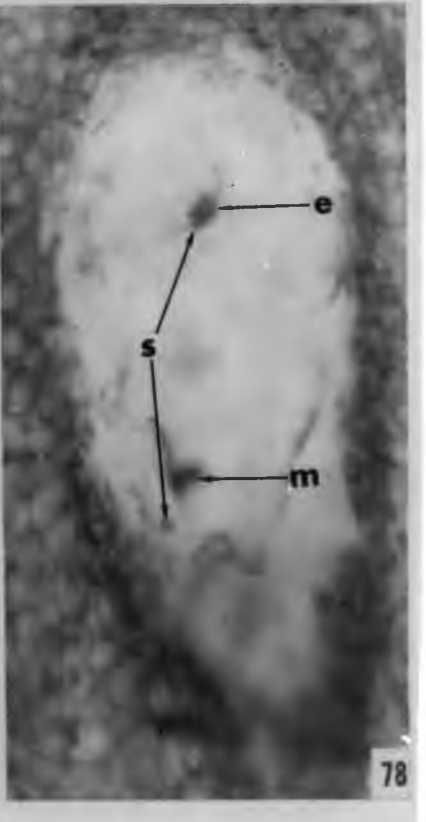
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crossing and the reciprocal combinations. Pollen grains were absent on the stigmas of open pollinated flowers of clones A and B, but a few grains and pollen tubes were present in preparations of clone C.

Pollen tubes were not observed within the styles of open pollinated flowers of clones A and B. This was further evidence of the absence of pollen germination. Observations of intact stigmas, which were open pollinated apparently in the morning and examined at noon with a dissecting microscope showed few (about five grains per stigma) or no pollen grains on stigmas of clone A. No pollen grains were observed on the intact and open pollinated stigmas of clone B.

The pathway and subsequent growth of pollen tubes were studied in hand pollinated flowers. The pollen tubes had grown through approximately one-third to one-half the length of styles at 24 hours after pollination. By 50 hours after pollination, the pollen tubes had entered the locules. Figures 75 and 76 show the pollen tube growth in relation to the ovule and Figure 74 shows what I interpret to be the normal pathway of pollen tube growth into the ovule. Figure 74 shows the top of the ovary immediately below a style. The pollen tubes grow down the style into a tissue with large intercellular spaces (p) and grow laterally toward the micropyle (m), which lies adjacent to the pathway of the growth of the pollen tubes. Figure 76 shows a pollen tube (t) penetrating the micropyle (m) in the next section of the ovary from which Figure 74 was made.

Indications of pollen tube penetration into the embryo sacs of clones A and C were seen two to three days after pollination (Fig. 77). The pathway of the pollen tube was indicated by darkly-stained cells and the pollen tube appeared to be disintegrating. Pollen tube penetration into the embryo sac of clone B occurred during the second day after pollination. The actual

time of pollen tube penetration into the embryo sac for this clone was not observed. However, signs of the pollen tube near the embryo sac were not observed from collections made 24 hours after pollination, but a two-nucleate proembryo stage (Fig. 79) was seen 72 hours after pollination.

The differences in time of pollen tube penetration into the embryo sacs may be attributed to the differences in location of the embryo sacs within the nucellus. Measurements of the relative distance between the tip of the outer integument (micropyle) and the tip of the embryo-sac wall perpendicular to its long axis at the micropylar end, of five ovules each at three days after anthesis yielded averages of 0.45 mm., 0.52 mm. and 0.56 mm. for clones B, A and C respectively.

Results of this study seemed to indicate, however, that absence of pollination rather than failures in pollen germination and pollen tube growth is one of the main probable causes of seedlessness in acerola.

Development of Fertilized Ovules

Double Fertilization. Signs of double fertilization within embryo sacs were observed two to three days after pollination of the flowers at anthesis. Embryo sacs at four days after pollination either showed signs of disintegration or contained proembryos with or without primary endosperm cells.

A few embryo sacs collected from flowers three days after anthesis and thereafter contained as many as nine groups of what appeared to be free nuclei (Fig. 82). The number of nuclear groups and the number of nuclei within a group were variable. The groups were connected by densely stained cytoplasm and showed variation in their arrangements in all three clones.

The origin and significance of these groups of nuclei are not clear to me. However, it was noted that disintegration of the groups without further division occurred at later proembryo stages. No endosperm development

PLATE XV

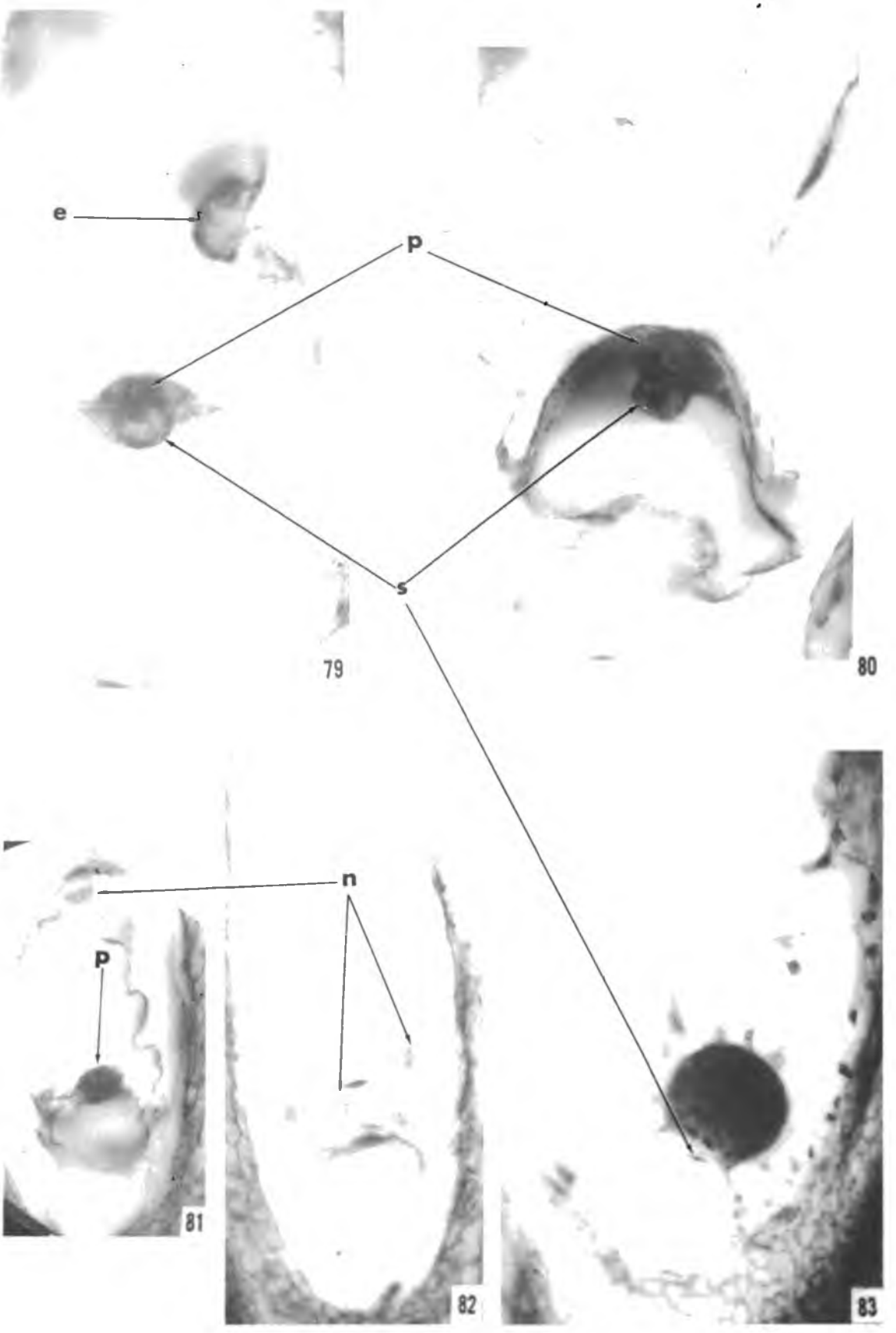
FIGURE 79: Two-celled proembryo stage at three days after pollination. The primary endosperm cell has divided forming two cells (e). The proembryo cell (p) adjacent to the suspensor is flattened and later gives rise to the embryo. The nucleus within the suspensor (s) does not undergo further division and becomes part of the suspensor. 710 X.

FIGURE 80: Four-celled proembryo stage at three days after pollination. This embryo sac did not show signs of endosperm development. Three proembryo cells (p) adjacent to the suspensor nucleus (s) occur in one row and are enclosed in densely stained cytoplasm. 692 X.

FIGURE 81: Proembryo stage at four days after pollination. The proembryo has formed a second row of cells by nuclear divisions and the laying-down of cell walls parallel and perpendicular to the long axis of the suspensor. A nuclear group occurs toward the chalazal end of the embryo sac and is indicated by n. 323 X.

FIGURE 82: Next section of same embryo sac as Figure 81. Free nuclear groups (n) are shown. No endosperm is developing within this embryo sac. 306 X.

FIGURE 83: Proembryo at five days after anthesis showing what may be degeneration of the suspensor nucleus (s). 256 X.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was observed in embryo sacs containing both proembryo and the nuclear groups. Disintegration of chalazal nucellar cells was associated with this type of abnormality. Free-nuclear groups also occurred in embryo sacs without proembryos.

Figure 77 shows an embryo sac two days after pollination. It shows a pollen tube (t), which has burst within the embryo sac, freeing what appear to be two sperm nuclei (s). The egg (e) appears at the micropylar end and what seems to be a binucleate endosperm mother cell (m) occurs at the chalazal end of the embryo sac. Two other polar nuclei appear to have fused and seem to be disintegrating (d).

Figure 78 shows an embryo sac at three days after pollination. Structures interpreted as the egg and the endosperm mother cell are shown at "e" and "m" respectively. The egg nucleus appears to be attached at the chalazal end of a large suspensor-like cell, which in turn seems to be attached to the embryo-sac wall at its micropylar end. Two structures interpreted as sperm nuclei may be represented by the darkly stained "spots" (s).

Embryogenesis and Development of Endosperm. Figure 79 shows an embryo sac three days after pollination. The zygote by this time had divided, forming two cells. Figure 79 shows a large suspensor cell adjacent to the embryo-sac wall at the micropylar end. The suspensor nucleus (s) occurs within the suspensor cell at its chalazal end. The cell comprising the proembryo (p) is adjacent to "s", but occurs outside of the suspensor wall. Both nuclei at this proembryo stage appear to be approximately the same size, but that within the cell at "p" is flattened. The proembryo cell (p) later divides, giving rise to the embryo.

Figure 80 shows a four-celled proembryo stage. The large suspensor cell, containing a nucleus, lies adjacent to the other three proembryo cells

which occur in one row and are enclosed in densely stained cytoplasm. This also represents a proembryo stage which occurred three days after pollination. This arrangement of the four cells may have arisen from nuclear division and the formation of cell walls perpendicular to the suspensor, but this is not certain.

Figure 81 shows a proembryo at four days after pollination. A second row of cells has formed by nuclear divisions and the laying-down of cell walls parallel and perpendicular to the long axis of the suspensor. The proembryo has a rounded top and a flat bottom where it is adjacent to the suspensor cell. (The proembryo from this stage until its maturity stained red in safranin-fast green and was readily identifiable.)

At five days after pollination, the suspensor cell showed definite signs of attachment to the embryo-sac wall (Fig. 83). The body of the proembryo was spherical. The single nucleus which had originated within the suspensor cell appeared collapsed and became part of the suspensor (Fig. 83, s). The row of embryonic cells adjacent to the suspensor remained as a connecting row of cells.

The spherical body of the developing embryo continued to increase in size and cell number at six days after pollination (Figs. 84 to 86). Variations in places of attachment of the suspensor to the embryo-sac wall and the number of developing embryos in an embryo sac are shown by these figures. Figure 84 shows the attachment at the micropylar end and Figure 85 shows the attachment toward the chalazal end of the embryo sac of 923 microns (long axis). Both embryo sacs contained endosperm. Figure 86 shows two developing embryos having a single but enlarged suspensor. No endosperm was observed in this embryo sac.

The twin proembryos may have resulted through fertilization of two free nuclei which functioned as eggs at the time of syngamy with each of two sperm

PLATE XVI

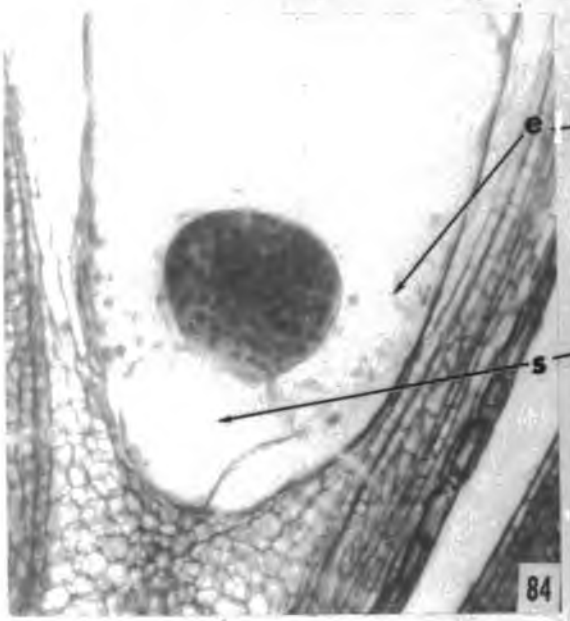
FIGURE 84: Proembryo at six days after anthesis showing the attachment of the suspensor (s) at the micropylar end of the embryo sac. Endosperm (e) has developed. 228 X.

FIGURE 85: Proembryo at six days after anthesis. Suspensor (s) is attached about 923 microns toward the chalazal end of the embryo sac, from the micropyle. Endosperm (e) is developing. 243 X.

FIGURE 86: Twin proembryos (p) having a single but enlarged suspensor. No endosperm has developed in this embryo sac at six days after anthesis. 275 X.

FIGURE 87: Young acerola seedling showing two shoots and a fused hypocotyl-root axis. 3 X.

FIGURE 88: Developing embryo and disintegrating endosperm at 10 days after anthesis. 127 X.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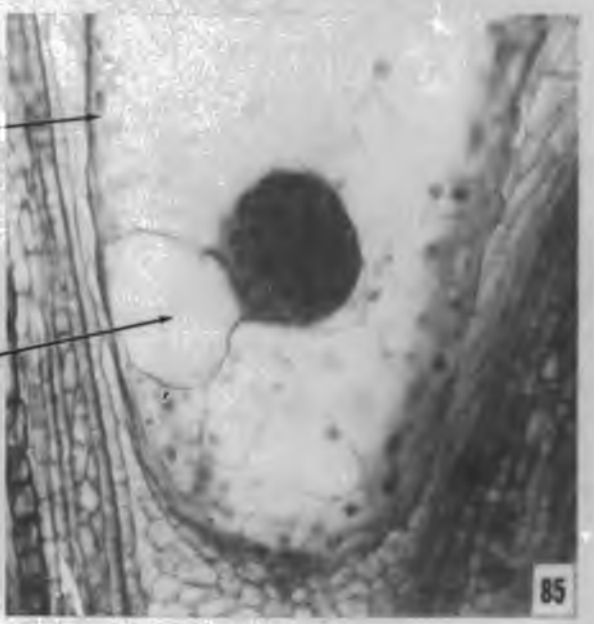
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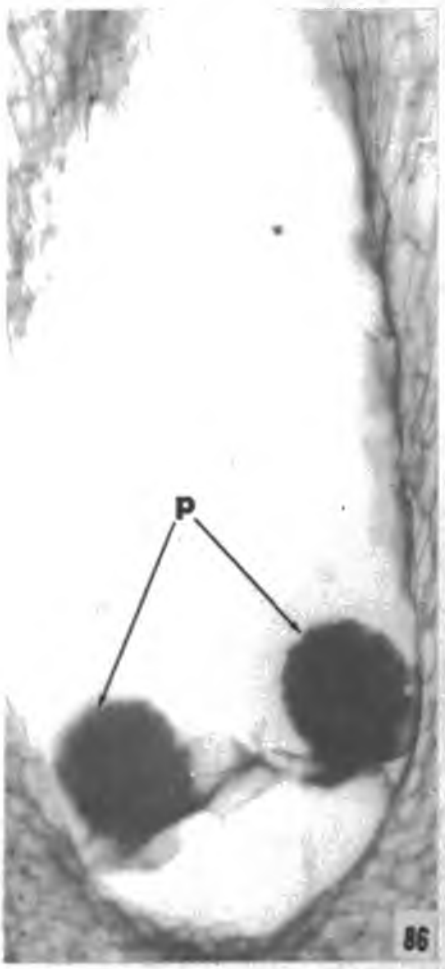
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nuclei which may have been released near the micropylar end of the embryo sac. No nucellar polyembryony was observed throughout this study. The occurrence of a mature twin embryo or two mature embryos from a twin embryo sac (Fig. 40), while exceedingly rare in acerola, might account for the unusual finding of a seedling having twin shoots and a fused hypocotyl-root axis (Fig. 87).

The developing embryo at 10 days after pollination had well-differentiated and developing cotyledons (Figs. 88 and 89). The immature embryo was heart-shaped (Fig. 88) or had a rounded axis (Fig. 89). Figure 88 shows the embryo at the micropylar end and initiating a radicle from cells adjacent to the suspensor which appeared in a succeeding section in the series. Endosperm cells were present in all five ovules examined at this stage. The endosperm cells nearest the young embryo had disintegrated. Nuclei of endosperm cells at the periphery and chalazal end of the embryo sac were in active division while nuclei of endosperm cells adjacent to the disintegrated cells were not in mitosis when the ovules were placed in Craf III solution at approximately 1:00 A.M. The interphase endosperm nuclei contained several nucleoli.

In the case where there was a developing embryo without accompanying development of endosperm cells within the embryo sac of the immature ovule, the embryo and ovule may abort at about 12 days after anthesis as evidenced by Figure 90. This figure shows a collapsed and aborted embryo attached at the micropylar end of the embryo sac. The aborted embryo appears to be slender and long with clearly distinguishable immature cotyledons and radicle.

By 15 days after anthesis the embryo appeared to be morphologically mature (Fig. 91). There was a relatively short radicle and two massive cotyledons in which vascular differentiation had occurred. The cotyledons

PLATE XVII

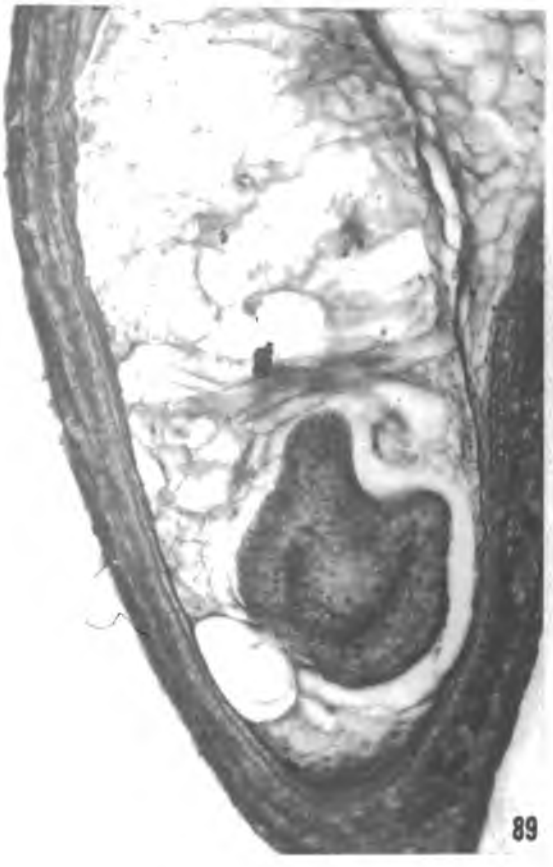
FIGURE 89: Developing embryo at 10 days after anthesis from a section cut perpendicular to the flat plane of the ovule. 72 X.

FIGURE 90: Aborted embryo at 12 days after anthesis. No endosperm was observed in this collapsed embryo sac. The cotyledon (c) and radicle (r) appear slender and long. 207 X.

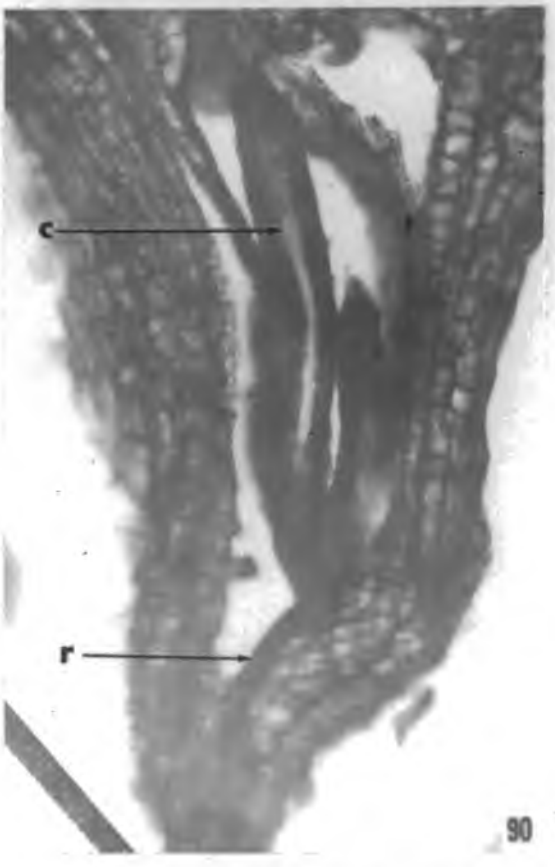
FIGURE 91: Morphologically mature embryo at 15 days after anthesis. The radicle (r) is short and the apical meristem (m) is situated at the base of two massive cotyledons (a and b). One cotyledon (b) shows vascular differentiation and is bent at its center adaxial to the apical meristem. The other cotyledon (a) forms a hood over the first cotyledon and the tip of this hood is adjacent to the point of curvature of the first cotyledon. 18 X.

FIGURE 92: Nuclear division of endosperm cells at 10 days after anthesis. 500 X.

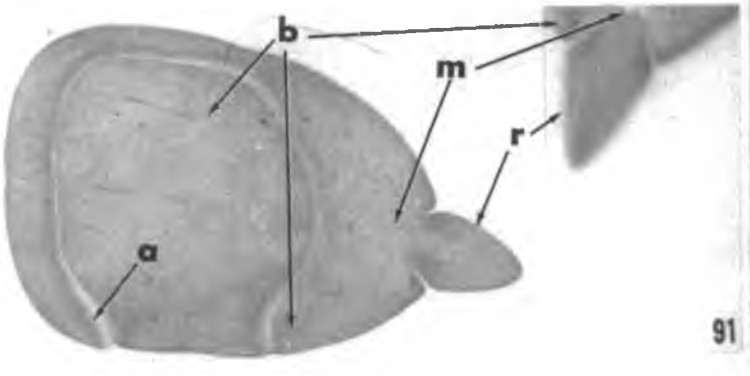
FIGURES 93 TO 95: Root tip chromosomes of clone A. 1438 X.



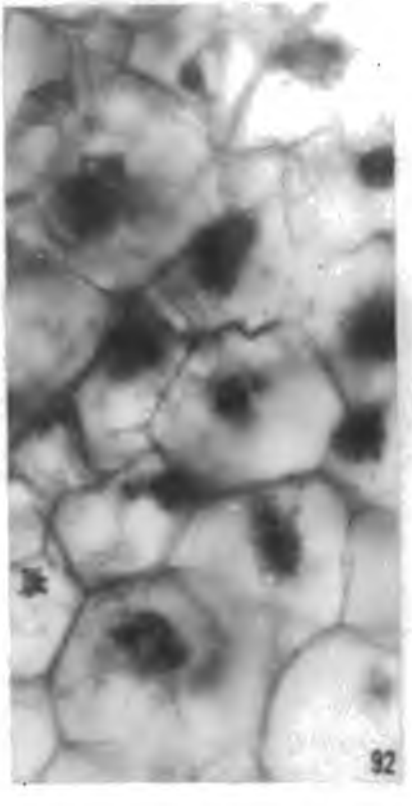
89



90



91



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93

94

95

at maturity were unusually oriented. They were extensions that arose from both sides of the apical meristem. One cotyledon was bent at its center adaxial to the apical meristem so that the tip of this cotyledon rested immediately above the apical meristem. The other cotyledon formed a hood over the first cotyledon and the tip of this hood was adjacent to the point of curvature of the first cotyledon. The endosperm cells were completely digested by this time.

Chromosome Counts

The number of chromosomes within the endosperm cells were not determined in this study. Examination of approximately 50 squashes of embryo sacs after removing their immature embryos showed; (a) endosperm had not developed within the embryo sacs, (b) the endosperm cells were not in mitosis, or (c) those that were dividing were not in prometaphase when they could be counted. Figure 92 shows a paraffin section of endosperm cells in mitosis.

In order to determine the ploidy of the somatic cells, squashes of root tips of the three clones used in the study on gametogenesis were made. Figures 93 to 95 show the diploid number of 20 chromosomes for clone A. This same number of chromosomes for clones B and C were also counted from root tip squashes.

Fruit Set As Related to Seed Set

Fruit set of acerola in Hawaii has been shown by Yamane and Nakasone (103, 104) to be greatly increased by the application of growth regulators and by hand pollinations, in general. In hand pollinations, however, they reported that various degrees of incompatibility might exist as evidenced by lower fruit set percentages when certain self and cross pollinations were made.

This problem of incompatibility was investigated further by studying the effect of pollination on fruit and seed set of the three acerola clones used in the study of gametogenesis. Results of the pollination experiment are presented in Table IV. They indicate that both self and cross incompatibilities might be present in these clones. However, results of Table IV also indicate that poor pollen or the lack of pollination may be another reason for the interacting factors in the plants as evidenced by their inconsistent trends in data obtained.

The inconsistencies in data (interacting percentage values) in Table IV occur in fruit and seed set when clone B was used as the pollen source in self and cross pollinations with the other two clones. When clone A (good pollen source) was self pollinated, an unusually low percentage of seed set was obtained and this may be due to self incompatibility. Cross incompatibility may exist in the cross C X A as evidenced by its lowered trend in percentages of fruit and seed set.

Percentages of fruit set showed an increase from self pollination over open pollination, in general, and from cross pollination over self pollination, in general. However, some kind of undetermined interaction affecting fruit set seems to be present. Preliminary *in vitro* and *in vivo* studies of pollen germination and pollen tube growth and the effects of stylar extracts on *in vitro* pollen tube growth did not definitely indicate what the cause or causes of the interaction were. This question will be treated further in the discussion.

Development of Natural and Induced Fruits

The ovaries at anthesis had morphologically distinct pericarp layers of exocarp, mesocarp, and endocarp which grew by cellular enlargement only and matured 21 to 25 days after anthesis. Growth and development of pericarps of seeded and seedless fruits were morphologically similar. The only

morphological difference of fruits at maturity was that at least one of the three stony endocarps (pyrenes) of each seeded fruit contained a seed while those of the seedless types did not.

Vegetative Parthenocarpy. This type of parthenocarpy occurs independent of any external stimuli which would then be referred to as stimulative parthenocarpy. Percentage of fruit set of 250 flowers emasculated and bagged without pollination was 2.8 percent in clone A. No seeds were present in the seven ripe fruits obtained at maturity. This indicates that pollination is not necessary for fruit set in acerola and that vegetative parthenocarpy is one of the factors involved in its low percentage of natural seed set.

Effects of Crude Extracts. Aqueous extracts of anthers, styles and ovaries were used to induce fruit set on emasculated and bagged flowers of the three clones as a means of studying further the interaction obtained when pollination treatments among the three clones were made. The possibility that self and cross incompatibilities due to some chemical substance existed and that the interaction was not due to lack of pollination alone was investigated.

Data in Table V show that various inconsistent trends (interacting values) in fruit set are present within treatments of the same extract and also among the treatments of the different extracts. The extracts of each clone appear to have independent effects with different combinations. Independent effects with different pollination combinations were also obtained (Table IV). However, the trends of the treatment combinations differed in both cases in the two tables.

Table V shows that treatment with floral extracts, in general, resulted in higher fruit set than was obtained from non-treated flowers. The extracts of styles, in general, seemed to have a lesser effect on fruit set than

TABLE IV. EFFECT OF POLLINATION ON PERCENTAGE OF FRUIT[a] AND SEED[b] SET OF THREE ACEROLA CLONES.

Clones	Open Pollination		Self Pollination		Cross Pollination Pollen Sources						Mean Percentage Set of Clones		Confidence Limits (95% Level)	
					Clone A		Clone B		Clone C					
	Fruit	Seed	Fruit	Seed	Fruit	Seed	Fruit	Seed	Fruit	Seed	Fruit	Seed	Fruit	Seed
A	8.1*	3.6+	39.0+	3.9+	---	---	41.9*+	6.7	65.1*	27.3*	35.4	5.1	30.8-40.0	3.5- 8.2
B	17.5*+	0.9*+	12.0*+	4.2*+	69.0*+	23.8*	---	---	60.0*	36.4*	36.7	7.1	31.5-41.9	5.4- 9.7
C	9.0*	16.6*+	50.0*+	51.1*+	40.8*+	42.9+	28.6+	10.0*	---	---	30.4	20.6*	25.1-35.7	17.0-25.2
Mean Percentage Set of Pollinations	11.5	6.8	31.9	21.7	55.0	29.3	35.3	8.9	63.1	33.3				
Confidence Limits (95% Level)	8.1- 15.6	5.8- 8.8	26.7- 37.3	14.4- 30.3	47.6- 62.4	21.2- 38.0	29.2- 40.8	4.5- 22.0	57.0- 69.0	24.6- 43.7				

a The number of fruit set as percentage of the number of flowers treated.
b The number of seed set as percentage of the number of locules examined.
* Significantly different percentage values for clones.
+ Significantly different percentage values for pollination treatments.

extracts of anthers and ovaries. Further, in general, treatment with extracts of anthers resulted in higher fruit set than treatment with ovary extracts.

Crude extracts of flower buds, whole flowers and fruits of various ages had very variable effects on fruit set of the three clones, and the effects of these extracts were not studied critically.

Effects of Growth Regulators. The efficacy of auxins to induce fruit set by single spray applications on non-emasculated flowers in acerola has been reported by Yamane and Nakasone (103). Induction of parthenocarpic fruit set using the same spray technique (103) with 50 ppm of PCA (parachlorophenoxyacetic acid) on 60 emasculated and bagged flowers of clone A resulted in 78.3 percent fruit set. There were no seeds present in the parthenocarpic fruits at maturity. This percentage value of 78.3 percent fruit set was not statistically different from results obtained when non-emasculated flowers of three clones were used (clone A, 83.3 percent; clone B, 80.0 percent; clone C, 70.0 percent). Unfortunately, the seed content of these fruits were not determined.

A previous study made by the author showed that seed content of 78 fruits in clone A, obtained by similar fashion from non-emasculated flowers, was 1.7 percent per locule or 0.05 per fruit. The seeds, whenever they occurred, were in one locule only of the trilocular fruit.

Seed content of 165 naturally set fruits of clone A determined in this study was 3.6 percent per locule or 0.11 per fruit. There were 148 seedless fruits, 16 seeded ones with seeds in one locule only and one fruit with seeds in two of its three locules.

Results of the combined effects of self pollination followed by growth regulator applications one hour later are presented in Table VI and Figures 7 and 8. This table indicates that a larger percentage of clone C fruits

contained seeds when pollination was not limiting (natural pollination, 9.0 percent fruit set and 15.6 percent seed set). This table also shows that fruit set was increased by chemical stimuli, but the total number of seeds obtained was less when aqueous sprays of growth regulators and of distilled water were used.

Observations of microslides of pollinated stigmas showed that a large number of pollen grains floated when a few drops of water were applied while a small number of grains were not affected. Thus, the applications of aqueous sprays may have washed a large number of pollen grains from the stigmas resulting in a lower incidence of seed set when sprays were used. Thus, the increase in the number of seedless fruits obtained by chemical stimulation may not be due to parthenocarpic fruit development alone.

Figures 7 and 8 show the relative sizes and shapes of mature fruits obtained by self pollinating flowers of clone C and spraying them with growth regulators one hour later. The fruits ripened after 25 days.

The mean weight of fruits that were sprayed with distilled water (checks) was 10.2 grams and fruit shape was more or less spherical. Fruits obtained by induction with gibberellin (GA at 25 ppm) also had a spherical shape, but the mean size was slightly larger (11.4 grams) than the checks. Auxin-induced (PCA at 50 ppm) fruits weighed less (6.8 grams) and had an abnormal shape (Fig. 7). Retarded mesocarp growth toward the styler end of fruits during their development produced constrictions and resulted in triangular-shaped fruits, both seeded and seedless. The flattened flesh was adjacent to each pyrene, while the pyrene itself appeared normal.

When 25 ppm of PCA was sprayed on non-emasculated flowers of clone A at anthesis less inhibition in mesocarp growth was observed at the lower concentration. The mean weight of these fruits (4.8 grams) was slightly less than that of naturally set fruits (5.5 grams).

TABLE V. EFFECT OF FLORAL EXTRACTS ON PERCENTAGE FRUIT SET[a] OF THREE ACEROLA CLONES.

Extract of Clones	Fruit Set Percentages of Clones A	B	C	Mean Percentage Fruit Set by Extracts	Confidence Limits (95% Level)
Anther Extract	---	---	---	44.3*	39.1-49.5
A	62.0*+	52.9+	37.5*+	51.7	43.0-60.4
B	34.7+	17.6*+	37.5+	29.7	21.7-38.4
C	42.0*+	66.0*+	45.8+	51.4	42.7-60.4
Stylar Extract	---	---	---	8.1	5.5-11.2
A	4.0*+	24.0*	1.1*+	7.9	4.5-16.7
B	8.0+	17.6*+	0.0*+	6.8	3.4-11.6
C	3.5*+	29.4*	2.2*+	9.6	5.9-14.6
Ovary Extract	---	---	---	25.2*	21.1-29.9
A	17.4*+	34.0*+	23.3+	24.7	18.4-32.0
B	29.8*+	12.7+	10.0*+	15.6	10.2-22.1
C	35.0+	38.5+	32.2+	34.7	27.7-42.0
None	2.1+	5.6*+	0.0*+	2.6	1.6-5.5
Mean Percentage Fruit Set of Clones	22.4	27.9+	15.5		
Confidence Limits (95% Level)	18.2-27.2	23.1-32.7	12.6-18.4		

a Number of fruits set as percentage of the number of flowers treated.

* Significantly different percentage values for extracts.

\+ Significantly different percentage values for clones.

TABLE VI. EFFECT OF SELF POLLINATION FOLLOWED BY AQUEOUS SPRAYS ON FRUIT AND SEED SET OF CLONE C.*

Spray Treatments	Total Fruits Set	Number Fruits with the Following Number of Seeds				Total Seeds Set	Mean Number of Seeds Per Fruit	Percentage of Seeds Per Locule
		0	1	2	3			
None	15	3	4	5	3	23	1.53	51.1
Distilled Water	11	3	6	2	0	10	0.91	30.3
PCA	20	11	6	2	1	13	0.65	21.7
GA	20	9	8	2	1	15	0.75	25.0

*20 flowers per treatment.

GA solutions at 10 and 25 ppm were later sprayed on the 25 ppm PCA treatment as single spray applications at three, five, ten and fifteen days later and as repeated spray applications of GA on these different days to see if fruit weight increased. In general, a total increase in fruit weight of 21.3 percent resulted when 10 ppm of GA was applied and 37.7 percent when 25 ppm of GA was applied. However, results within replications were inconsistent and this phase of fruit development appears to deserve further study before any conclusions can be made.

DISCUSSION

The tetrasporic, 16-nucleate embryo sacs in some Malpighiaceae appear to be a specialized type known to only a few genera of scattered families, Compositae, Gunneraceae, Liliaceae, Malpighiaceae, Pandanaceae, Penaeaceae, Piperaceae and Umbelliferae (56, 77). In this specialized type of embryo-sac development, all four megaspores seem to take part. This is unlike the normal eight-nucleate types of embryo sacs in which development is from a single functional megaspore and in which the three remaining megaspores degenerate.

Each of the four functional megaspores of the specialized types of embryo sacs may give rise to four nuclei, and therefore, 16 nuclei are present in each mature embryo sac of acerola.

If the monosporic, eight-nucleate embryo sac resulting from the three nuclear divisions of the functional megaspore is the fundamental type in angiosperms, then the tetrasporic, 16-nucleate embryo sac, resulting from two nuclear divisions of each megaspore in the specialized type of development appears to be a modification of the normal type. Thus, the specialized type of embryo-sac development has an increase in the number of megaspores taking part in the formation of the embryo sac and a decrease in the number

of nuclear divisions taking place between the megaspore mother cell and the mature embryo sac.

Results of this study have shown that numerous phenomena, which may cause seedlessness in acerola, exist. Seedlessness in acerola may be a result of failure of fertilization and success of parthenocarpic fruit development or it may be a result of fertilization of the embryo sac and abortion of the developing embryo.

Embryo abortion in acerola is not caused by auxin application at anthesis. Auxin-induced embryo abortion has been reported in some plants (102, 103, 104, 108) while the embryos are not affected in others (105). This is evidenced by the seed set study in which auxin was applied to hand pollinated flowers at anthesis and normal seeds were obtained at fruit maturity.

Instead, embryo abortion in acerola appears to be caused by a failure in endosperm formation. The study on embryogeny in acerola indicated that free nuclear groups were present in embryo sacs containing proembryos but no endosperm cells.

In an embryo sac containing neither endosperm nor free nuclear groups, an aborted embryo with well-differentiated cotyledons was observed 12 days after anthesis (the actual time of embryo abortion, however, was not determined). Further evidences that endosperm is utilized by the developing acerola embryo, in cases when endosperm was present, were shown by the digestion of endosperm cells adjacent to the young embryo and the absence of endosperm in the seed. Like most angiosperms, endosperm appears to be necessary as a source of nutrition for the developing acerola embryo, although exceptions where endosperm is suppressed and embryo development is often completed without endosperm are known in Orchidaceae and Podostemonaceae (106).

Seedlessness related to parthenocarpy in acerola seems to be influenced by two main factors, lack of pollination caused by inadequate pollinating

agents and the inherent parthenocarpic nature of the plant even when pollination is accomplished. Lack of pollination was also found to be a causal factor in low fruit and seed set of acerola in Hawaii by Yamane and Nakasone (104).

The inherent parthenocarpic nature of the plant is evidenced by a number of abnormalities in development of the ovules and of the embryo sacs. Results of this study showed that ovules were normally anatropous at maturity, but abnormal ones were upside down with their micropyles pointing toward the receptacles or were semi-anatropous with their micropyles pointing toward the attachment of funiculus and placenta. The chances of pollen tube penetration into the ovules and fertilization of the embryo sacs in acerola seem to be limited by the abnormal ovule orientations. Pollen tubes penetrating the chalaza was not observed in this study, although in Casuarina suberosa, this is reported as the normal situation (106).

Ovule sterility caused by an absence of division or delayed divisions of the megaspore mother cells, disintegration of nucellar cells and abortion of embryo sacs before anthesis were also observed. Fertilization cannot possibly occur in these abnormal types and they also indicate the inherent parthenocarpic nature of the plant.

Pollen grains of acerola are generally germinable on the day of anthesis only and normal germinability is related to anther dehiscence as in most plants. However, pollen germination within the larger lobes or microsporangia of an undehisced anther on the day of anthesis was also observed. Germination of pollen grains in undehisced anther sacs has been reported in cleistogamous flowers (106). However, reports in the literature of this abnormality in plants whose flowers are produced well above ground and open normally at anthesis are not known to the author. Non-dehiscence of this particular anther of clone A at a time when dehiscence of the other anthers

of the same flower occurred seem to indicate an abnormal development of the flower. The few aborted pollen grains in clone B may also be attributed to abnormal development since only one anther sac of this particular anther showed the abnormality.

Various and inconsistent trends of comparisons of percentages in pollen germination and fruit set were obtained in different combination treatments when the effects of treatments using crude aqueous floral extracts were studied. Inconsistent trends in percentages of seed set of different treatment combinations were also observed in the study on the effects of pollination on fruit and seed set in acerola.

The inconsistent trends in percentages obtained when treatment combinations were compared were interpreted as interactions of the effects of two or more factors within the treatment combinations.

The effects of clone B stylar extract on fruit set (Table V) show a significantly higher percentage value for clone B. The effects of the same stylar extract on pollen germination (Table III) of clone B also yielded a significantly higher percentage. However, the trend of the stylar extract treatments of the three clones on fruit set (Table V) was exactly opposite to that of pollen germination (Table III). Fruit set was significantly increased in clone B upon addition of its own stylar extract but pollen germination in this treatment combination showed a decreased trend. This is expected if auxin is involved since it may be promotional to fruit set but normally is inhibitory to pollen germination and pollen tube growth (53, p. 233).

However, the reasons for the increases in fruit set and pollen germination of clone B when its own stylar extract is added are not known to the author at present since interactions exist with the different extracts and treatment combinations.

The relatively low overall fruit set of the three clones, obtained by use of floral extracts of clone B, may be due to inhibiting substances, either present in the extracts or synthesized within the flowers after treatment. Another possibility is that the endogenous supply of at least one of the growth factors which influence fruit set is normally present in sufficient amounts and the further addition of similar growth factors by extracts causes an inhibition. However, the identification of naturally-occurring growth factors and their effects on inducing parthenocarpy were not determined in this study.

Various interacting factors in the treatment combinations of the study on the effects of pollination on percentage of fruit and seed set (Table IV) were also indicated by inconsistent trends in data obtained. When clone B was used as the pollen source in self and cross pollinations with the other two clones, fruit and seed set were generally reduced. Studies on anther dehiscence and pollen germination showed that clone B was a poor pollen source. Thus, lack of pollination or a low number of pollen grains deposited on the stigmas may account for one of the interacting factors in low fruit and seed set in acerola. On the other hand, self incompatibility may be another factor causing interaction.

Cross incompatibility may be the cause of lower fruit set in the cross C X A as compared to the reciprocal combination, since both clones are good pollen sources. Natural seed set is low in clone A (3.6 percent) and relatively high in clone C (16.6 percent). The fact that seeds within fruits are mobilizing centers and can influence floral abscission when both fruits and flowers occur on the same branch has been reported by several workers (21, 54).

Preliminary <u>in vivo</u> studies on pollen germination and pollen tube growth did not definitely indicate whether or not there was some physiological

hinderance to fertilization (incompatibility) such as slow pollen-tube growth when clone B was used as the pollen source or when the cross C X A was made. However, the number of pistils studied were of small magnitude and the possibility of self and cross incompatibility should not be refuted without further study.

Induction of parthenocarpy on emasculated and bagged flowers and of fruit set on hand pollinated flowers using growth regulators showed increased fruit set over vegetative parthenocarpy and hand pollination in acerola. Also normal seeds were obtained at fruit maturity from hand pollinated flowers sprayed with growth regulators. This seems to indicate that pollination and fertilization of receptive embryo sacs are not the only factors involved in fruit and seed production, and although the former may occur, floral abscission may take place unless a more favorable condition contributing to fruit development is experienced. PCA and GA appeared to contribute to such a condition. However, the interactions of pollination, syngamy, fruit set, ovule development and maturity, application of growth regulators and the inherent parthenocarpic nature should be determined by further study.

SUMMARY

Studies on floral bud differentiation and development, gametogenesis and fruit morphogenesis in relation to fruit and seed production in acerola are presented in this thesis.

Floral bud differentiation from initiation until emergence occurred in eight to ten days on primary branches which were in active vegetative growth. Approximately seven days after the flower buds emerged, their anthesis was observed. Thus, 15 to 17 days after floral initiation, the open flowers were produced. 21 to 25 days elapsed between anthesis and ripe fruits.

Production of flowers and fruits simultaneously on the same plant was a common phenomenon.

There was much variation and abnormality in ovule development, megagametogenesis and embryo-sac development and what I have interpreted to be the normal and abnormal types are described and discussed. Normal embryo-sac development was like the Fonnea-type. No nucellar polyembryony was observed in acerola. Parthenocarpy in acerola seemed to be an inherent character and was evidenced by the abnormal types of development.

In general, pollen grains of acerola showed normal development. Two exceptions are described. Pollen grains of acerola are generally germinable only on the day of anthesis and germinability seemed to be influenced by anther dehiscence.

Signs of double fertilization within embryo sacs were observed two to three days after anthesis. The embryos appeared to be morphologically mature by 15 days after anthesis.

Seedlessness in acerola was found to be the result of both failure of fertilization (parthenocarpy) and embryo abortion. Auxin-induced embryo abortion was not shown to occur in acerola. Embryo abortion appeared to be caused by failure of endosperm development in some fertilized embryo sacs. Endosperm developed in normal types, but was completely digested just prior to embryo maturity.

The diploid number of chromosomes of acerola is 20.

No consistent responses were obtained in fruit and seed set experiments using hand pollination and crude aqueous extract treatments. The probable causes are presented and discussed. Hand pollination of acerola flowers at anthesis resulted in general increases in fruit and seed set, but seed set did not exceed 51.1 percent.

Use of aqueous solutions of auxin and gibberellin at anthesis was found to yield beneficial effects in fruit set of emasculated and non-emasculated flowers. Fruit shape and weight, however, differed between treatments with auxin and gibberellin.

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