REPRODUCTIVE CYTOLOGY OF BLACK CHERRY (PRUNUS SEROTINA EHRH.)

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INTRODUCTION

Black cherry represents the only species generally used for timber production in a genus containing about 175 species of trees and shrubs. Wood from this tree is considered of high-value for quality furniture manufacture, and for paneling or other decorative uses, although its use is probably less than one percent of the hardwood saw-timber cut in the United States. The eastern hardwood growing stock contains only about five percent of the three species complex including black cherry, walnut and ash. Despite the rather small timber harvest associated with black cherry, the present high value of the wood mandates its inclusion in hardwood forest management schemes. With an increasing interest in artificial reforestation of hardwoods a concomitant emphasis on genetic improvement of this and other species has occurred. Recent planning in the New York Conservation Department has included black cherry as a candidate for seed orchard consideration. Regional planning by the U. S. Forest Service includes a large seed orchard project with black cherry (Schreiner, 1966), and work with this species is active in the Tennessee Valley Authority Forest Program (Taft, 1966), as well as in many other tree improvement progress (Gerhold, 1967).

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The growing interest in the genetic improvement of black cherry has created a need for further understanding of its reproductive cytology. In particular, the development of pollen and seed, and the question of self-compatibility are of immediate interest to seed orchard establishment and to breeding work. The study reported herein was designed to investigate microsporogenesis and embryogenesis in detail, and to provide an introductory study of self-compatibility and floral phenology.

LITERATURE REVIEW

Chromosome Number. ----The basic chromosome complement for the genus was reported as x = 8 by Okabe (1928) and Darlington (1928). Kobel (1927) reported a 2n = 32 chromosome number for P. serotina; to the authors' knowledge there has been no dispute of this report. Among the Prunus species grown as fruit trees, considerable chromosome number variation is found. In general, it appears that all of the Sweet cherries are diploid (2n = 16), while the Sour cherries are tetraploid (2n = 32), and may be divided into the Duke and Sour groups (Hruby, 1950). Darlington (1928) suggested that the Sour cherries were derived from an old, independent species, but after cytological studies of meiotic behavior Kobel (1927) and Prywer (1936) reported that they were of hybrid origin. The Duke cherries are generally accepted as being of hybrid origin, resulting from an unreduced gamete of P. avium L. uniting with a reduced gamete of P. cerasus L. (Darlington, 1928, Crane and Lawrence, 1938) .

Microsporogenesis .--Microgametophyte development in the cherry species of Prunus has been discussed by Darlington (1928), Okabe (1928), Hruby (1939, 195 ^{0}), Prywer (1936), and by Tillson (1947); none of these reports included P. serotina . Tillson reported that the sporogenous tissue in the anthers of P. mahaleb L. first appeared by late October and that pollen mother cells were differentiated during November. He also noted that while some other cherry varieties were in a more advanced stage of development, all passed the winter in the pollen mother cell stage. Additional reports on microsporogenesis, in the cherry species have substantiated this developmental timing. In certain cases the pollen mother cell stage was delayed until March, but no precocious development has been reported. Pollen development has followed the normal pattern for all individuals investigated, despite the species variation in chromosome numbers.

Megasporogenesis and Embryo-sac Formation .--Bradbury (1929) reported that two ovules arc formed in the simple pistil of P. cerasus, and that one of these normally degenerates at anthesis. Sterling (1963) conducted a broad survey of carpel morphology in Prunus and observed that all the species studied had biovulate carpels and that generally the simple pistil consists of one carpel. He also reported that bicarpellate flowers do occur in Prunus. Several investigators have reported on megaspore development in cultivated species of Prunus ; however, only a few of these reports pertained to the cherry varieties, and none concern black cherry. Much of this research in both the Sweet cherry (P. avium) and the Sour cherry (P. cerasus) was performed by Pechoutre (1902), Ruehle (1924), Bradbury (1929), Tukey (1933, 1934), Tillson (1947) and Eaton (1959, a,b). Results from these studies have shown good general agreement between both species for all phases of development. The anatomy of development was nearly identical although a difference in the time sequence has been reported. This information provides a particularly interesting comparison since P. cerasus is a tetraploid while P. avium is a diploid species.

Tukey (1933) and Tillson (1947), working with P. avium and P. mahaleb respectively reported that two anatropous ovules arose from opposite placentae of the single carpel. Tukey observed that the ovary increased in length only slightly until. two weeks before full bloom, and that both ovules develop equally but slowly, paralleling the development of the ovary. He noted that the nucellus and integuments also develop slowly until fertilization, and then undergo a sudden acceleration in growth. In this same study, he

suggested that the development of the megagametophyte and embryo-sac in P. avium was so similar to the report for P. cerasus (Bradbury, 1929) that any critical comparison would be superfluous.

Eaton (1959 b) noted that most embryo-sacs of P. avium had reached the 8-nucleate stage and had differentiated an egg cell by anthesis. These findings were in good agreement with Bradbury (1929) who reported that embryo-sac development in P. cerasus was simultaneous with blossom development from the bud stage where white petals were just becoming visible to the time of anthesis. Both authors gave similar accounts of the com pleted embryo-sac; it was characterized by an 8-nucleate sac of the classical Polygoman type (see Maheshwari, 1950) . Eaton (1959 b) suggested that in pistils collected at anthesis or later it was possible to differentiate the functional and non-functional ovules by haematoxylin staining; the functional ovule showed a greater affinity for the stain, while the non-functional ovule was constricted, lighter-staining, and contained a degenerating embryo-sac. Reference to a similar degeneration time for the second-order ovules was also reported earlier by Ruehle (1924) and Bradbury (1929).

Fertilization, Endosperm Formation, and anbryogenesis . --Bradbury (1929) noted that double fertilization in P. cerasus occurred three or four days after full bloom and that division of the primary endosperm nucleus, which resulted in a free endosperm, preceded the zygotic divisions. This fertilization timing had been generally confirmed for P. avium by Tukey (1933), Tillson (1947) and Eaton (1959). As the embryo-sac lengthened toward the chalazal end of the ovule, cytokinesis occurred in the free-nuclei endosperm and progressed from the periphery inward toward the chalazal end of the embryo-sac. By continual division the cellular endosperm gradually replaced the nucellar tissue and in turn was replaced by the developing embryo (Bradbury, 1929).

Johansen (1950) stated that no detailed studies were available on the embryony of any species in the Amygdalaceae. He concluded that this subfamily showed little resemblance to the pattern of embryo development so prevalent in the related Rosaceae. However, in a text of embryo development (ed. by Maheshwari, 1963) Cretes stressed the remarkable homogeneity of embryo development in the Rosaceae. Cretes' statement seems the most reasonable in light of earlier work by Tukey 1933 with P. avium and by Bradbury (1929) with P. cerasus. Both authors reported a similar embryogenesis: a slow development until about three weeks after full bloom, followed by a rapid but regular pattern of enlargement. A suspensor cell was reported at the first division of the zygote, and it (cells of suspensor) persisted until maturity. At maturity the embryo for both species had large well-formed cotyledons, surrounded by a thin layer of endosperm and nucellar tissue.

Self- and Cross-Compatibility.--No reports on the self- or cross-compatibility in P. serotina were found in the literature reviewed. Among the cultivated cherries, reports of self-incompatibility prevailed in the Sweet cherry group (Crane and Lawrence, 1938), while results have varied in the Sour or Duke cherries (Crane and Lawrence, 1929, 1931). Cross-compatibility between these groups involves diploid X tetraploid crossing and is usually more successful with the diploid as the female parent. Speculation about the degree of endosperm ploidy has suggested that a higher chromosome number may be undesirable (Crane and Lawrence, 1938). Triploid varieties of cherries are usually sterile and are often vegetatively propagated for ornamental use,

MATERIAL AND METHODS

Microsporogenesis and Chromosome Number.--Microsporogenesis in P. serotina was observed in materials developed in the greenhouse and also from field collections. Ten trees were selected in a natural stand on the east facing slope of Labrador Valley one-tenth mile north of Truxton, New York. The stand was even-aged, approximately fo -rty years old, and occupied an area of approximately 2.5 acres.

Branches were collected at two-week intervals during the spring of 1965, brought into Flu greenhouse, trimmed and placed in water to allow bud development. Flower buds were not discernable on dormant branches; therefore collections were delayed until early anthesis when the raceme became evident. In addition, flower buds were collected in the field at weekly intervals from April through June; all collections were fixed in Newcomer's solution (Newcomer, 1953).

Meiotic development was observed in anthers removed from the flowers and placed in a drop of 0.05N HCl for about one minute. The anthers were then -transferred to a .drop of propionic-carmine stain and prepared for cytological evaluation by the smear technique. Photomicrographs of the meiotic stages were taken at 1000X. Chromosome counts were made at late diakinesis using material previously prepared by the smear technique.

Megagametophyte and Embryo Development .--Field collection of flowers for examination of embryo-sac and embryo development was begun May 6, 1965, and continued at 5-day intervals until seed ripening on September 15, 1965. Whole racemes were collected and fixed in Formalin-Acetic Acid-Ethyl Alcohol (FAAI, Johansen 1940), and stored in a refrigerator at 4^0C. The material was dehydrated in a t-butyl alcohol series and embedded in paraffin. Serial sections were cut at 8-10 microns on a rotary microtome, stained in a schedule of safranin 0 and fast green, and permanent slides prepared. Photomicrographs were made depicting the various stages of embryo-sac and embryo development.

Floral Morphology and Phenology. --Phenological observations were made at each 5-day interval utilized in the megagametophyte collection schedule. In addition, bud collections were made at two-week intervals to determine the time of floral primordia differentiation. The bud collections were prepared for microscopic evaluation by free-hand sectioning.

Self -Compatibility Study .---A preliminary evaluation of self-compatibility in P. serotina was initiated by isolating flowers prior to anthesis on ten trees in each of two flowering seasons. On every tree ten pollination bags, each containing at least three racemes, were utilized. The isolation bags were allowed to remain for 30 days, their non-woven cloth construction allowed free air movement and helped to prevent heat build-up from becoming a factor. Controlled pollinations were not conducted, thus the study evaluated only the ability of the flowers to self-pollinate under the prescribed isolation conditions.

RESULTS AND DISCUSSION

Floral Morphology and Phenology. --The dormant vegetative and floral buds of P. serotina were approximately 5-7 mm. long and ovate in shape with several visible imbricate scales. They were differentiated in late summer and by mid-November the floral raceme was evident in sectioned material. The floral buds were in the lateral axial position and the vegetative buds were terminal and somewhat larger. Approximately thirty-five perfect flowers were borne on a cylindrical, glabrous raceme that was 8-12 cm. long when mature. The individual flowers were supported on pedicels 4-8 mm. long and had five green sepals and five white petals (see Figure 1B). The simple pistil was light green; when receptive the stigma was pale green in the center with a cream colored periphery.

The flowers appeared to be predominantly insect pollinated and began opening during the first week of June in the experimental area. Very little variation in flowering within the tree or throughout the stand was observed.

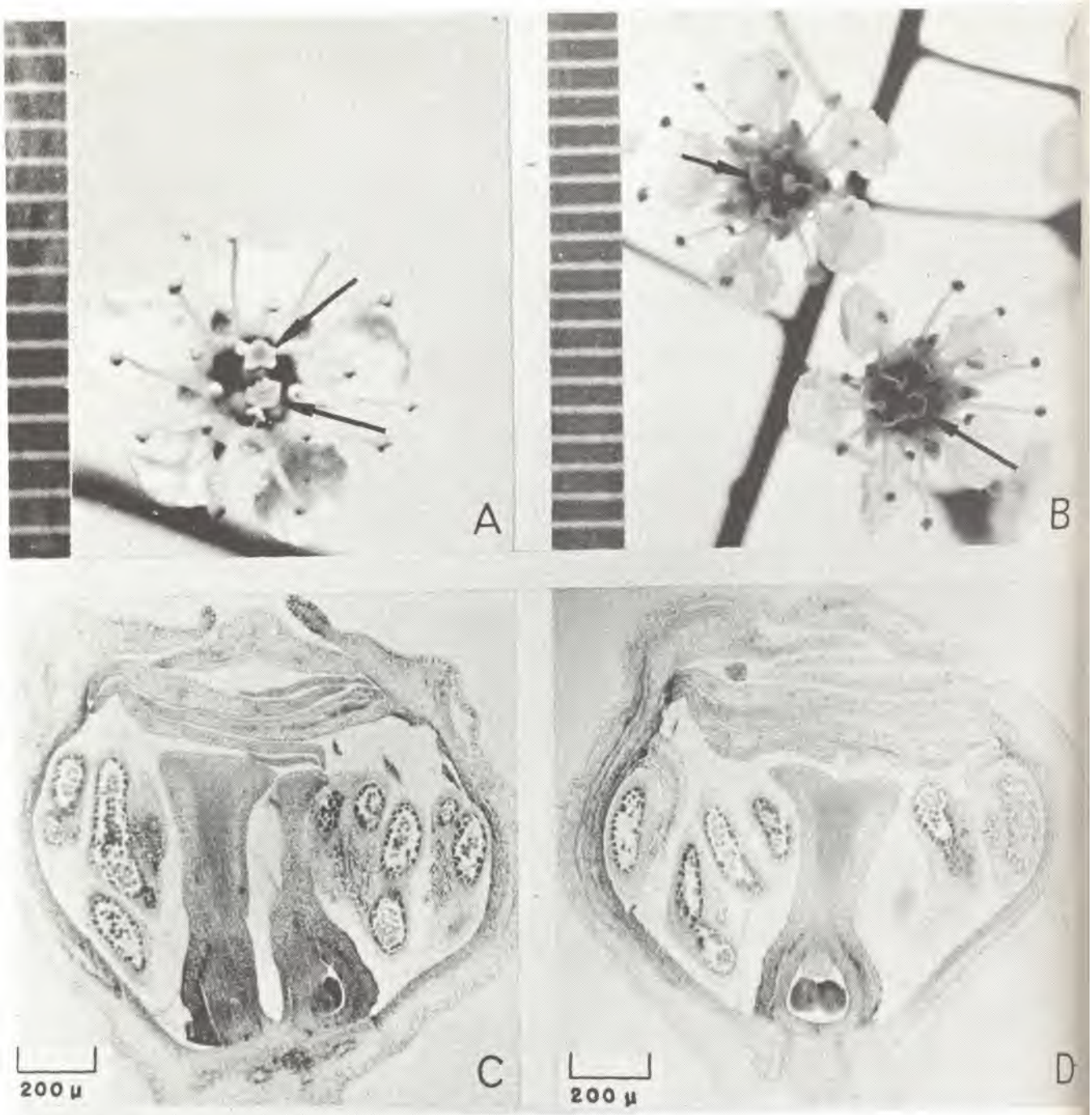

Figure 1.--Normal and abnormal floral morphology. An abnormal 2-pistil flower is shown in A, a section of the same is shown in C, while the normal development is indicated in B and D. The scale in A and B is in mm.

Anthesis began at the base of a raceme and proceeded acropetally. The stamens, 15-20 per flower, were curled under the stigma; the pure white anthers gradually changed to a pale yellow as the filaments became exerted. The stamens were completely extended in two days and then the dorsal surface of the anther began to rupture and dehisce synchronously with stigma receptivity. The period of receptivity for an individual flower was about 2-3 days at maximum. The flowers at the base and middle sections of the raceme were past receptivity when those at the apex were just beginning to open, thus the duration of receptivity for a raceme was estimated to be 5-7 days.

In fertilized flowers the calyx was persistant, while the styles withered and were abscised within three weeks. Unfertilized flowers dropped off at the base of Their pedicels. The fruit, a one seeded drupe, was globose, smooth skinned and 8-10 mm. in diameter when mature. Fruit fall occurred by mid-September and at this time the drupe was purple-black in color. From observations of open-pollinated material in the field, it appeared that a low proportion of fruit set was characteristic of this species under natural conditions.

One of the trees observed had an abnormal flower development at the base of many of its racemes. In these flowers two independent pistils were present, generally one was much smaller than the other (see Figure 1A, C), and only occasionally did embryo-sac development take place in both. The flowers which contained two pistils appeared normal in all other respects and none were observed in which both pistils were fertilized.

Microsporogenesis and Chromosome Number. --Microsporogenesis was observed in buds from branches developed in the greenhouse and also from field collections. The branches used successfully in the greenhouse experiment were collected during late winter and spring, while in collections made prior to the end of February a few racemes appeared but these always aborted before meiosis. No anomolous behavior was observed in either technique and the cut-branch procedure did not affect microsporogenesis or the formation of pollen when collections were made after March 1.

Field collections of naturally developed material began in April and continued until June. Meiotic activity was initiated by May 1 when the raceme emerged from the bud scales and measured about one centimeter long. Meiotic development progressed acropetally, and during the second week of development in the field all stages of meiosis could be found within the flowers of one raceme.

Prior to the beginning of interphase the microsporangia contained a mass of homogenous sporogenous cells, much compressed and angular in outline. During these early stages it was difficult to separate individual cells; as microsporogenesis progressed the meiotic material separated more easily. A pollen mother cell in late interphase is shown in Figure 2A. Before this stage of development the pollen mother cells are smaller and the nucleus less basophilic. As development continued, cell size and nuclear volume increased and the nucleoli and chromatin material became organized.

When interphase passed into meiotic prophase, the chromosomes became randomly distributed, and usually only one nucleolus was present. Pairing of the homologous chromosomes followed rapidly; in most of the material observed two of the bivalents seemed to be associated with the nucleolus (Figure 2B).

Following synapsis and a relatively long pachytene stage, the bivalents contracted and the characteristic pairing relationship abated as the material entered the diplotene stage (2C). During late diakinesis (2D) the nucleolus disappeared, the bivalents became distributed around the periphery of the nucleus, and the nuclear membrane disappeared. Bivalent formation was regular throughout prophase I with no conspicuous sign of

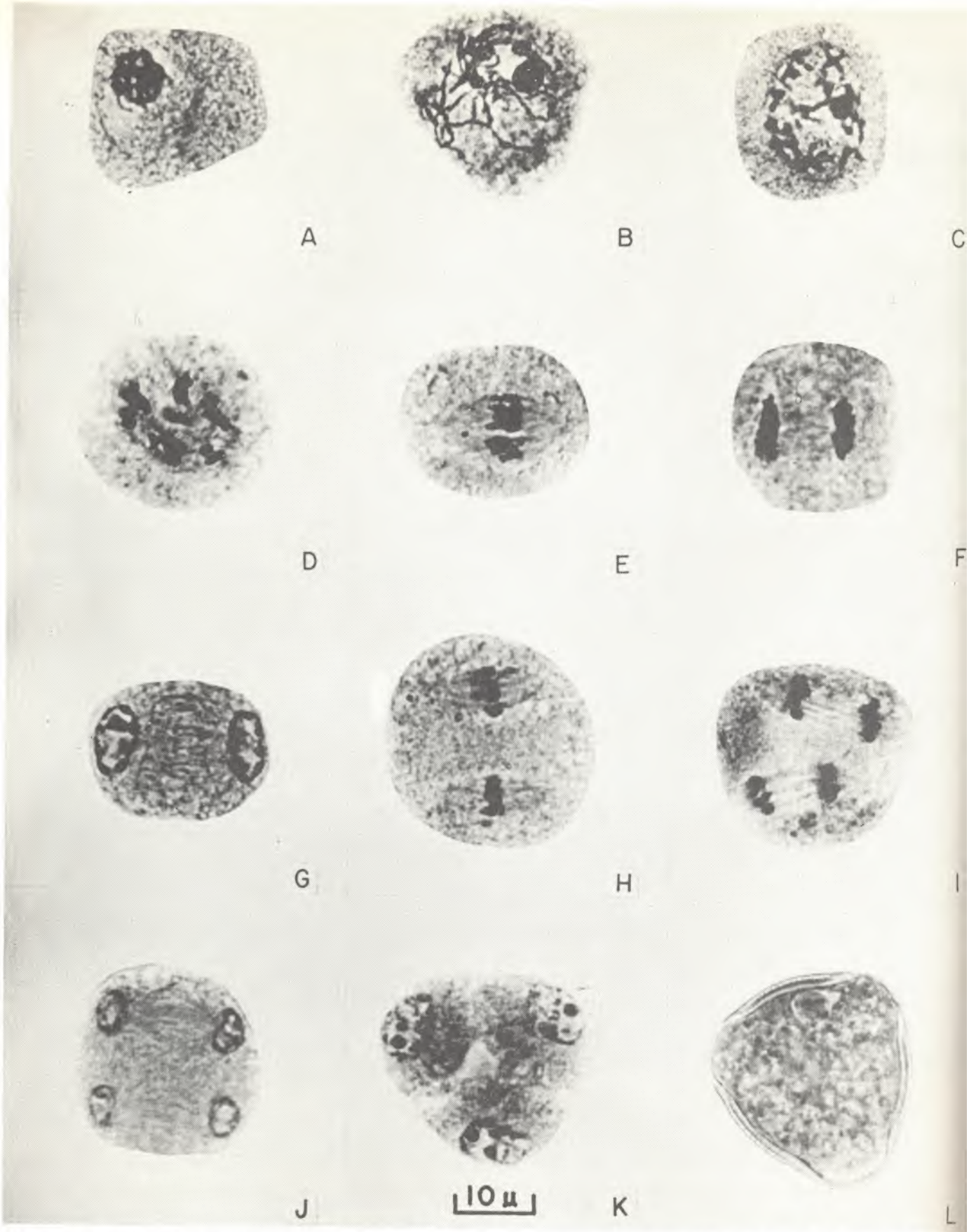

Figure 2.--Microsporogenesis in black cherry. Prophase I is shown in A through D, Metaphase I in E, Anaphase I in F and Telophase I in G. The second meiotic division is illustrated in H through K, with the mature pollen depicted in L. (See text for discussion; scale for all figures is shown in K).

secondary association. The chromosomes of black cherry are small and during late diakinesis the length of the bivalents Varied from approximately 1 to 1.5 microns. This stage is also the best time to ascertain the chromosome number, which was confirmed to be 2n = 32.

Metaphase I was observed with no apparent irregularities. A prominent spindle was formed, the bivalents oriented on the metaphase plate (2E), and anaphase I was signaled when the homologous pairs separated and moved toward their respective poles (2F) . Quite frequently the smaller chromosomes were precocious in leaving the metaphase plate. Upon reaching the poles, a nuclear membrane formed at telophase I (2G) . Following inter kinesis, during which the chromosomes became diffuse and the nucleoli reappeared, the second meiotic division began.

By late prophase II the nucleoli disappeared and the dyads were compact spheres approximately one micron in diameter. The beginning of metaphase II was marked by the disappearance of the nuclear membrane and formation of the spindles, upon which the dyads oriented (211). After anaphase II (21), the four groups of monads reached their respective poles, a nuclear membrane developed around each. group and these telophase II nuc1ei passed into an interphase condition (2J) with the reappearance of the nucleoli.

The nuclei were in this condition when the cell underwent simultaneous cytokinesis, resulting in the quadripartitioning of the mother cell to give four tetrahedral microspores (2K) . Maturation and enlargement of the pollen grain occurred as the raceme continued to elongate. Although division of the microspore nucleus was not observed, binucleate pollen was noted (2L).

Megametophyte and Embryo Development . --Microscopic evaluation of sections made from flower bud samples collected from May 7 through June 4, 1965, revealed that the female reproductive development in black cherry is similar to that of related domesticated species. The simple pistil was already formed at the time collections started, but. the two ovules were only primordial protuberances in May 12 collections. There was considerable variation in the stage of development on a raceme, making it difficult to precisely correlate specific developmental stages.

Approximately three weeks before anthesis the ovules appeared as two separate prorances from the contiguous carpellary margins of the ovary wall. Initially their apices were toward the base of the pistil but as growth proceeded the ovular tips were directed upward, toward the style resulting in anatropous orientation. As floral development entered the second week before anthesis the integuments were visible as a ring of tissue around the ovule base, almost completely enclosing the nucellus. Subsequently, the inner integument formed the micropyle, the dual nature of the integuments was apparent only in this region. With growth of the ovary wall, the ovules were elevated toward the top of the locule, making their parietal placentation evident. Growth of the ovary was somewhat slower than the ovules and they soon filled the locule, as seen in Figure 3A.

Growth and development of the two ovules was approximately equal; both usually enlarged rapidly and doubled in length before anthesis, occasionally the secondary ovules did not and were much smaller. During the final week of development the megaspore mother cell differentiated from the nucellar tissue at the micropylar end of the ovules. This occurred beneath 6-8 rows of parietal tissue resulting in the ovules being classified as crassinucellate (3B) . Division of the megaspore mother cell was not observed but in (3B) the restating linear tetrad with the three distal megaspores is shown in an advanced stage of deterioration. The chalazal megaspore pictured in 3B had already undergone two meiotic divisions and was at the four nucleate stage. The mature 8-nucleate Polygonum typeembryo--sac resulted after division, differentiation and subsequent migration of nuclei(3C) . The polar nucleifused just prior to anthesis resulting in a seven-celled

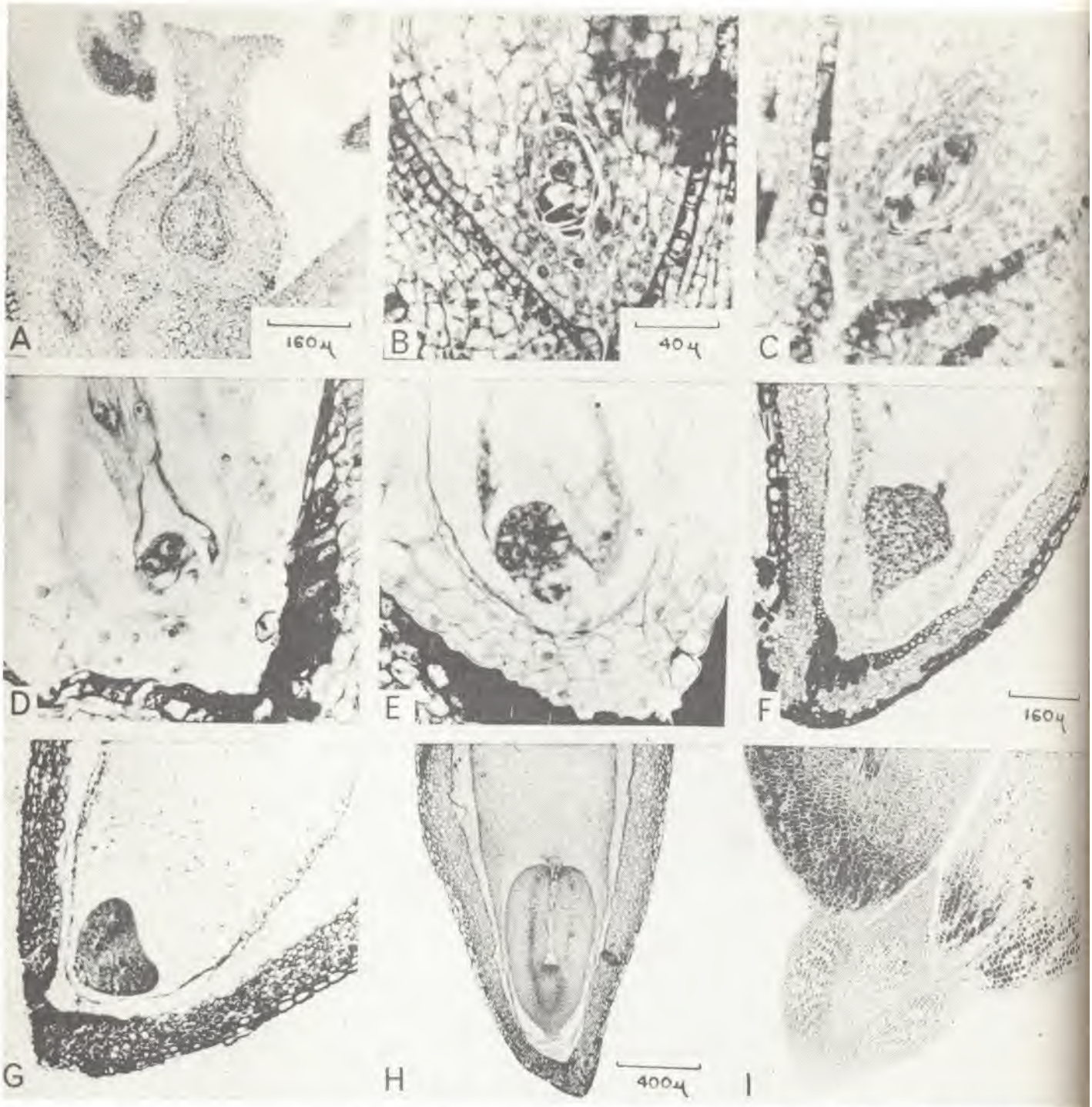

Figure 3.--Embryogenesis in black cherry. A section of the pistil (A) is shown prior to the reduction division illustrated in B. The mature embryo-sac was observed on 3 June (C) while early proembryo development occurred from 17 June (D) to 23 June (E). By 1 July the embryo began rapid development (F) and the heart-shaped stage was reached in a few days (G). Differentiation of cotyledons and vascular tissue is illustrated in H (July 12 collections) and the mature embryo in I. (Scale for A is indicated, for B through E scale is given in B, F and G scale in F, and H to I scale is in H).

embryo-sac at full bloom. Seldom was a secondary ovule observed that had reached this stage of development. Those that maintained equal development with the primary ovule did so only up to formation of the embryo-sac. After division and enlargement of the chalazal megaspore, normal embryo-sac development began to lag. The nucellus of secondary ovules was rounded at the micropyle and withdrawn from the integuments; less than 0.20 percent of the seed observed contained two fully developed embryos.

Double fertilization was not viewed, but observation of material collected throughout the week following anthesis indicated that it occurred during this period. The ovary, nucellus and integuments of the primary ovule then began a period of rapid growth and enlargement. If fertilization occurred this accelerated development continued for approximately five weeks and the primary ovule filled the locule, compressing and crushing the secondary ovule. When fertilization did not occur, enlargement of the ovary and primary ovule was considerably reduced and ceased within a week. The embryo-sac of unfertilized material did enlarge slightly after anthesis but within four days it was totally degenerated.

In fertilized material three days after anthesis the embryo-sac had lengthened toward the chalaza and the zygote had enlarged with its most distal portion becoming highly vacuolated. The fusion nucleus appeared larger than before anthesis but had not yet begun to divide. The synergid cells were almost totally degenerated and the antipodals were no longer visible. In the subsequent two weeks the zygote underwent only two or three cell divisions producing the proembryo initials and a large suspensor cell.

In contrast to the zygote, the primary endosperm nucleus began to form free nuclear endosperm by five days after anthesis, and the nucellus increased in volume; while the embryo-sac, narrow and elongated with the free nuclei located in its central region, penetrated deeper into the nucellus (3D). However, by two weeks after anthesis, when the zygote entered the proembryo stage and began rapid growth, the embryo-sac became spherical and much enlarged. At the same time, a well-developed haustorium was visible at the chalazal end. The free nuclei of the endosperm were concentrated around the embryo and in the thin layer of cytoplasm along the periphery of the embryo sac (3E). Three weeks after fertilization, wall formation was initiated in the nuclei at the micropylar end of the embryo-sac and gradually extended to the chalazal end (3F). The highly vacuolated haustorium remained coencoytic throughout development.

The embryo attained the heart-shaped stage by four weeks after anthesis (3G) and the characteristic form of the dicotyledon embryo was reached during the subsequent week (3H). At this time the cotyledons were well developed and the ovule had attained ninety percent of its final size. The seed coat hardened rapidly and growth of the nucellus and integuments subsided. Endosperm formation and enlargement of the embryo-sac continued, and embryo development was accelerated, enlarging in July from 0.15 to 4.9 mm. Eight weeks after anthesis the accelerated development ceased, the endosperm still surrounded the embryo, but only a small remnant of nucellus was present at the chalazal end. Throughout August the remaining endosperm was consumed and the embryo increased slightly in length to 5.0 mm. By September the embryo was morphologically mature and the protoderm, procambium, apical meristerns, and root cap were completely formed.

Self-Compatibility Study. --The results of the two-year study involved the isolation of approximately 18,000 flowers. From this material, only four seeds were obtained and none of these germinated. The data suggested strong self-incompatibility for the species; nevertheless, these results must be viewed with caution since control pollinations were not made to determine the effect of isolation on the developing flowers. Additional work in the cross- and self-compatibility of black cherry is strongly recommended; until such work is completed, it would seem wise to withhold final judgment in relation to a seed orchard or breeding program.

SUMMARY

The data obtained for pollen and seed development in black cherry indicated no abnormal development in this tetraploid species. Floral primordia was differentiated in the late summer, the following spring, the predominantly insect-pollinated flowers were fertilized and the seed matured by mid-September. Isolation of flowers prior to pollination prevented seed development, a fact interpreted as evidence but not final proof of a strong self-incompatibility.

Since the study area included only one stand at a single location, the data must be restricted to that frame of reference. The range of black cherry extends throughout the eastern-half of the United States and south to Guatemala (McVaugh, 1951). With such a large range, it is possible that considerable genetic variation exists and additional geographic variation studies are strongly recommended.

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