A STUDY OF THE CHROMOSOME NUMBER, MICROSPOROGENESIS MEGASPOROGENESIS, EMBRYO SAC DEVELOPMENT, AND EMBRYOGENY IN BLACK GRAMAGRASS, BOUTELOUA ERIOPODA (TORR.) TORR.

by

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I hereby recommend that this dissertation prepared under my direction by L. J. STREETMAN

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After inspection of the dissertation, the following members of the Final Examination Committee concur in its approval and recommend its acceptance:*

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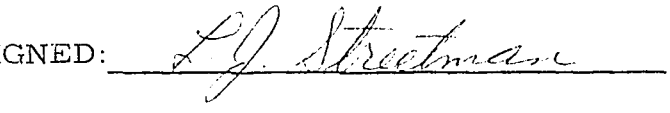
SIGNED: 

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Black gramagrass, Bouteloua eriopoda (Torr.) Torr., is a perennial range grass native to the desert grasslands of the southwestern United States and is a major species in Arizona, New Mexico, and adjacent parts of Mexico. Technical classifications and descriptions of this species have been presented by Griffiths (18)[1] and Hitchcock (24).

Black gramagrass exhibits a high degree of drouth tolerance during seedling establishment and as a mature plant; it is, therefore, especially well-adapted to arid and semiarid range lands. In many areas of low rainfall, this species is the only desirable grass present. Black gramagrass is palatable and nutritious and is readily grazed by animals in both summer and winter.

The many desirable characteristics possessed by black gramagrass make this species valuable for reseeding depleted ranges; however, reseeding has been restricted to experimental plantings because of the

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[1] Numbers in parenthesis refer to Literature Cited.

lack of commercial seed. The seed-setting habits of this grass under range conditions are highly variable and unpredictable. It appears, then, the principal aim of the plant breeder should be the maintenance of the desirable characteristics of this species while improving seed production.

A number of methods for plant improvement have evolved during the past 75 years. Application of a particular breeding procedure is largely dependent upon the cytological behavior of the species. It is the duty of the plant breeder to become thoroughly familiar with the growth and reproductive processes of that species so that the breeding program can progress with maximum efficiency through timely application of proper techniques.

Studies on chromosome number and meiotic behavior provide basic information on chromosome races, nature and degree of ploidy, fertility, and taxonomic relationship of species. In addition, such information aids the breeder in selection of parental plants for intraspecific, interspecific, and intergeneric hybridization. Although interspecific and intergeneric hybridization have not resulted in economically important grass varieties, these divergent crosses often supply a greater source of germ plasm from which future selections can be made.

Apomixis, a reproductive process through which the union of the egg and pollen nuclei is circumvented, imposes unique problems upon

plant breeders. In this process an exchange of germ plasm does not occur, and progeny plants are usually identical with maternal parents. Obviously, then, conventional breeding methods employed on sexual plants are of little value for improving asexually reproducing plants. Studies to determine the mode of reproduction of a species uninvestigated cytologically should be made early in a plant improvement program. Absolute evidence for the presence or absence of apomixis can be obtained only through detailed studies of female gametophyte and embryo development.

Studies on microsporogenesis, megasporogenesis, and other phases of reproduction have not been reported for black gramagrass. The need for this information was the basis for initiation of this study. Specific objectives were (a) to determine the chromosome number of all available accessions, (b) to observe the meiotic behavior of these plants, (c) to follow the development of the female gametophyte, (d) to study the initiation and development of the embryo, and (e) to determine the mode of reproduction.

CHAPTER II

REVIEW OF LITERATURE

The merit of cytological investigations in providing fundamental information as a basis for breeding programs has been conclusively demonstrated. Many forage plants have undergone detailed cytogenetic analysis, and comprehensive reviews of cytological data in relation to forage crop breeding have been presented by Atwood (3) and Myers (34). Hanson and Carnahan (20) state that cytological and genetic studies contribute fundamental information on chromosome numbers, the nature of polyploidy, and the existence of aneuploids and chromosome series useful in breeding. According to Nielsen (36) the ultimate goal of cytogenetic investigations is to serve as an effective tool for developing and maintaining improved forage strains.

Numerous studies and observations concerning management of B. eriopoda have been reported. A review of this information has been presented by Wright and Streetman (47). However, the literature pertaining to black gramagrass reveals little cytological data. Fults (15) reports a somatic chromosome complement of 2N = 21 for three seed

sources of B. eriopoda. The chromosomes ranged in length from 0.25 to 1.50 μ with an average length of 1.00 μ. Two small spherical chromosomes in each complement appeared to be characteristic. After investigating the chromosome complements of 18 biotypes of seven species of Bouteloua, Fults (15) concluded the genus was composed largely of aneuploids. However, Brown (11) states that his data does not confirm this conclusion: he reported the chromosome number of six Bouteloua species; B. breviseta Vasey 2N = 28, B. chondrosioides (H. B. K.) Benth. ex. S. Wats. 2N = 14, B. eriopoda 2N = 28, B. filiformis (Fourn.) Griffiths 2N = 14, B. rigidiseta (Steud.) Hitchc. 2N = 28, and B. trifida Thurb. 2N = 28, and concluded the genus has a basic number of x = 7 and that euploidy is more prevalent than aneuploidy. Recent investigations by Snyder and Harlan (39) and Gould (16) with pollen mother cells indicate a basic number of x = 10 rather than x = 7. Snyder and Harlan made chromosome counts of B. gracilis (H. B. K.) Lag. ex. Steud. plants collected from 108 localities in western Texas, eastern New Mexico, and one locality in western Oklahoma, and reported numbers of 2N = 20, 2N = 40, 2N = 42, 2N = 60, and 2N = 84. The aneuploid numbers of 2N = 42 and 2N = 84 were represented by plants from seven localities. Gould (16) points out that pollen mother cell prophases of 2N = 20 B. hirsuta Lag. plants were regular, whereas in the 2N = 28 material two

to four univalents were observed in many cells. He also reported a diploid chromosome complement of 2N = 20 for B. curtipendula (Michx.) Torr.

Stebbins (42) reported about 70 per cent of the grass species investigated cytologically to be polyploids. Adding to the complexity of the problems which confront grass breeders is the apparent widespread occurrence of apomictic reproduction in the Gramineae. Detailed reviews of the literature pertinent to apomixis have been presented by Stebbins (40) and Gustafsson (19). The classification of the types of apomixis and terminology proposed by Gustafsson is generally accepted by students of cytology and genetics. Stebbins (41) states that positive evidence for the presence or absence of apomixis can be obtained only from laborious and time-consuming studies of megaspore, embryo sac, and embryo development. Atwood (3), recognizing the contribution of cytological work in explaining the process of apomixis, suggests an even greater amount of evidence has been obtained from measuring apomixis indirectly through the progeny test.

On the basis of cytological and breeding studies, Müntzing (33) found certain races of Poa pratensis L. reproduced by an apomictic mechanism. Åkerberg (1), Engleberth (14), Brittingham (10), Tinney (45), and others have confirmed and extended Müntzing's findings. Tinney (45) presents a detailed description of the apomictic mechanism in P.

pratensis. He found the embryo sac develops, without meiosis, from a cell of the nucellus which is located near the chalazal end of the megaspore mother cell. Often the diploid egg begins development into a proembryo before pollination; however, pollination appeared to be necessary for endosperm formation. Nielsen (35) found that five days after anthesis embryos and endosperm of asexual and sexual plants of P. pratensis were essentially parallel and indistinguishable in development. Bashaw and Holt (6), working with Paspalum dilatatum Poir., observed proembryo development in more than ten per cent of the ovules collected before anthesis.

Maheshwari (30) has comprehensively reviewed the literature concerned with the development and classification of Angiosperm embryo sacs. Battaglia (7) has further classified the various types of nonhaploid or apomicitic embryo sacs.

Brown and Emery (12) sectioned ovules of five Bouteloua species, B. breviseta, B. curtipendula, B. eriopoda, B. filiformis, and B. gracilis, and did not find evidence of an apomictic mechanism in any of the species studied. Harlan (22), however, has reported apomixis in B. curtipendula. He based the apomictic mechanism upon the asymmetrical division of the megaspore and microspore mother cells, the products of meiosis being two approximately unreduced cells and two cells with only a few chromosomes each. The apomictic plants have chromosome numbers ranging

from 85 to 101. The chromosomes exhibited very little pairing during meiosis. The extreme uniformity of progenies from single plant selections of a normally cross-pollinated species also supports the theory of apomictic reproduction.

Gould (17) made a survey of the incidence and distribution of B. curtipendula plants with high chromosome numbers and apomictic reproduction. The apomicts were found to occur in a broad but well-defined zone from the southwestern United States to northwestern Mexico. In the southwestern United States apomicts were more frequent than sexual plants.

Numerous workers have studied the grass embryo from a morphological point of view. Kennedy (28) presents a brief historical review of early embryo studies. According to Kennedy, Malphigi's description of the embryos of Triticum and Avena in 1687 represents the first work in this field. More recent studies on grass embryo development and morphology have been made by Avery (4), Bennett (9), Harlan (21), Merry (31), Randolph (37), and Reeder (38).

Randolph (37) has described the development of the embryo of Zea. The cells of the proembryo lacked a definite arrangement and sequence of division. Merry (31) found the proembryo of Hordeum sativum Pers. also lacked a definite arrangement of cells and sequence of cell division. Bennett (9) concluded no special significance could be

attached to the sequence of cell division or to the arrangement of cells in the early development of the embryo of P. dilatatum. He also states this indicates factors controlling growth of the proembryo affect the embryo as a whole, rather than affecting definite cells.

Various details of the development of the buffalograss seed from the unfertilized ovary to maturity have been observed and described by Harlan (21). He lists the differences between the buffalograss and wheat caryopsis as thin integuments, the adjacent, basal location of the chalazal and micropyle, and the multicellular suspensor in buffalograss. Harlan (21) also states Buchloë embryo development appears to be similar to that of Bouteloua.

Reeder (38), studying the grass embryo in relation to systematics, placed Bouteloua in the Chloridoid-Eragrostoid group. He concluded the embryos are basically Panicoid in that they are characterized by the Panicoid type of vascularization, and the lower part of the scutellum is free from the coleorhiza. Reeder also states they resemble Festucoids in that they have an epiblast and that when seen in transverse section the margins of the leaves do not overlap.

CHAPTER III

MATERIALS AND METHODS

At initiation of this investigation, only one source of black gramagrass seed was available for study; the collection made by the Soil Conservation Service from a pure stand of black gramagrass north of Flagstaff, Arizona, in the fall of 1948. Being the only seed of any quantity available, this strain has been used exclusively for range reseeding trials by the Soil Conservation Service in Arizona and parts of New Mexico, and for establishment of experimental plots by the Agricultural Research Service at Tucson, Arizona. The "Flagstaff" strain is now considered to be the standard black gramagrass.

Sources of Plants Studied

The lack of divergent plant material made the collection of black gramagrass seed from various locations throughout the area of major distribution necessary. Bulk seed were collected from a number of plants selected at random at each collection site. A minimum distance of five miles separated most collection sites. The erratic seed-producing habits of this species under range conditions made collection of

vegetative material necessary in some instances. The vegetative material was planted in gallon cans containing a soil-sand mixture, and, later, surviving plants were transplanted to the field. Because of the difficulty encountered in transporting and establishing the plants, only seven of the accessions studied were represented by vegetative material. Most of the plants for the chromosome number and microsporogenesis study were obtained on a collection trip through eastern Arizona and western and central New Mexico in the fall of 1957. Highly favorable climatic conditions in this portion of the Southwest made 1957 "one year among many" for seed-set under range conditions. The collection points of this and other trips and the accession numbers assigned to the corresponding plants are shown in Table 1.

In the spring of 1958 the seed which had been collected were planted in flats and later transplanted into an individual plant nursery at the University of Arizona Plant Material Center, Tucson, Arizona, where research facilities for the cytological studies are located. The soil at this station is Gila sandy loam. Water was applied by the flood irrigation method at intervals necessary for maximum establishment and growth. A total of thirty-five accessions, including seven vegetative collections, each represented by one to twenty plants, were successfully established.

Table 1. The accession number and origin of black gramagrass plants studied cytologically.

SW no.*	Origin
240	Flagstaff, Arizona
419**	Santa Rita Expt. Range
422**	30 mi. northeast Tucson, Page Ranch
548	Jornado Range, New Mexico. Pasture No. 9
549	15 mi. north Truth or Consequences, New Mexico, Hwy. 85
550	1 mi. east Nutt, New Mexico, Hwy. 26
551	Trinity Base, New Mexico
552	Jornado Range, New Mexico, Pasture No. 8
553	7 mi. east Hillsboro, New Mexico, Hwy. 180
554	18 mi. south San Antonio, New Mexico, Hwy. 85
555	2 mi. east Hillsboro, New Mexico, Hwy. 180
557	5 mi. east Benson, Arizona, Hwy. 86
558	29 mi. west Hillsboro, New Mexico, Hwy. 180
559	19 mi. west Willcox, Arizona, Hwy. 86
562	9 mi. east Hillsboro, New Mexico, Hwy. 180
563	Steins Pass, New Mexico, Hwy. 86
564	3 mi. east Separ, New Mexico, Hwy. 80
565	10 mi. north Lordsburg, New Mexico, Hwy. to Red Rock
566	Separ, New Mexico, Hwy. 86
567	2 mi. north Stallion Camp, White Sands, New Mexico
568	36 mi. north Truth or Consequences, New Mexico, Hwy. 85
571	5 mi. south Cottonwood, Arizona, Hwy. 279
572	15 mi. east Willcox, Arizona, Hwy. 86
574	36 mi. south Three Points, Arizona, Hwy. 86
576	1 mi. north Sasabe, Arizona
578	Santa Rita Expt. Range, Field 6C
581	Camp Verde, Arizona, Hwy. 79
583	3 mi. east Cornville, Arizona
587	Las Vegas Ranch - north Prescott, Arizona
591	Cornville, Arizona
598**	12 mi. north Rock Springs, Arizona
603**	8 mi. south Kingman, Arizona, Hwy. 93
604**	24 mi. southeast Kingman, Arizona, Hwy. 89
605**	50 mi. north Cameron, Arizona, Hwy. 89
606**	10 mi. east Sells, Arizona, Hwy. 86

* SW refers to accession number used in the USDA-ARS Grass Improvement Program located at Tucson, Arizona.

** Vegetative collection.

Concurrent with collection of divergent plants, the "Flagstaff" strain growing at the University of Arizona Plant Material Center was used for preliminary studies of methods for obtaining suitable plant material for cytological study.

Root Tips

Initially, root tips were to be used for chromosome number determinations. Attempts to root stolons, culms, and whole plants in distilled and in tap water failed in every case. Vermiculite, perlite, and sand also proved to be unsatisfactory as rooting mediums. The most satisfactory method for obtaining root tips was the growing of whole plants in small wire-bottomed flats filled with soil. As the roots emerged through the wire the root tips were collected; by this time, however, the tips were small and often unhealthy. Both 1:3 acetic-alcohol and Carnoy's solution were tried as killing and fixing agents. The 1:3 acetic-alcohol was selected for use in subsequent studies because the cytological material could be stored indefinitely in this solution without deterioration. Staining with acetocarmine, prepared according to Darlington and Lacour (13), resulted in chromosomes so lightly stained that analysis was impossible; however, when iron citrate rather than iron acetate was used as the mordant (5), staining was highly satisfactory.

Other difficulties encountered with the root tip smear technique were a lack of metaphase cells and the tendency for the small chromosomes

to clump. Attempts to accumulate metaphase cells by cold treatment, as suggested by Hill and Myers (23), were unsuccessful. Colchicine treatment of excised root tips was also found to be of little value. The use of para-dichlorobenzene and 8-hydroxyquiniline as employed by Morton (32) and Tjio and Levan (44), respectively, had little effect in reducing chromosome clumping. After numerous attempts to obtain chromosome counts from root tip smears, this method was abandoned in favor of pollen mother cell smears.

Pollen Mother Cells

The daily pollination cycle for black gramagrass was studied to facilitate collection of inflorescences with pollen mother cells suitable for chromosome number determination and meiotic behavior analysis. Visual observations had indicated anthesis begins about 3:30 p. m. Microscope slides covered with a thin film of vaseline were placed at each of 12 locations in an established black gramagrass field. These slides were held in place at a 45° angle by a clamp on a weather vane-like device which was free to rotate with the wind change. The slides were changed at 30-minute intervals from 3:30 to 7:30 p. m. The remainder of the 24-hour period was represented by slides which remained in the field from 7:30 p. m. to 8:00 a. m. and from 8:00 a. m. to 3:00 p.m. This daily cycle was carried out for three successive days. The number

of pollen grains shed during each collection period was determined by counting the pollen grains observed in ten random fields of a 16 mm microscope objective as outlined by Jones and Newell (26).

Subsequent collection and staining of pollen mother cells indicated a maximum number of dividing cells could be obtained by collecting, one to two hours before the onset of pollination, inflorescences which had completely emerged from the boot but had not begun flowering. Inflorescences were collected from all accessions in the fall of 1958. These inflorescences were placed in glass vials containing a 1:3 acetic-alcohol solution and stored in a refrigerator for later analysis.

Acetocarmine prepared as described for use with root tip smears was used for staining the chromosomes of pollen mother cell smears. From one to five plants per accession were analyzed for chromosome number. Diakinesis was the only stage of meiosis in which cells could be accurately analyzed. A minimum of ten such cells were studied for chromosome number and maximum pairing. Anaphase I cells in which chromosome counts could be made with confidence occurred very infrequently. When possible, quartet cells were studied for presence of micronuclei. Pollen viability was checked for each accession. The percentage of pollen grains stained with acetocarmine was the criterion used. A minimum of 500 pollen grains selected at random using a 20X objective and 10X ocular was counted for each entry.

Megasporogenesis and Embryo Sac Development

Cytological material for the study of megasporogenesis and embryo sac development was collected from plants of the "Flagstaff" strain. Progressively older florets, from the time they were distinguishable up to time of anthesis, were harvested to insure obtaining the complete developmental sequence. The florets were placed in formalin-acetic-alcohol (FAA), and immediately subjected to a vacuum to remove air from the tissues. After a killing and fixing period of 48 hours, dehydration was accomplished by the tertiary-butyl-alcohol series as outlined by Johansen (25). The florets were then infiltrated and embedded in paraffin for sectioning with a rotary microtome. Eight microns proved to be an optimum thickness at which to cut the ovaries.

The ovaries were stained with the safranin O-fast green combination, as described by Johansen (25), and counterstained with orange G. Approximately 300 ovaries were analyzed in this phase of the study.

Pollen Tubes

Pollen tube growth in the female tissues was studied using the same plants used for the megasporogenesis and embryo sac study. The florets were killed and fixed in FAA at 1, 2, 3, 4, 8, 12, and 24 hours after pollination. The aqueous lacmoid technique as outlined by Wilson (46) was employed.

Embryogeny

The embryogeny of black gramagrass was studied with the same plants used for the megasporogenesis and embryo sac study. Samples were collected from progressively older material 12, 18, and 24 hours after the time of pollination. After the first 24 hours, florets were collected daily for 14 days. All florets selected were individually tagged at the time each began pollination, thus the exact age of each developing embryo was known. This material was processed by the same microtechniques as those described for the megasporogenesis and embryo sac material. Approximately 700 ovaries were studied to elucidate fully the developmental sequence of the embryo. Measurements, in microns, were made of embryos at various stages of development using a calibrated eye piece micrometer and 16 mm objective. The measurements presented are the average of five embryos.

A 1/3X Micro Ibso photomicrograph attachment and Ig Leica camera back were used for making photomicrographs. Best results were obtained using Kodak Panatomic X film.

CHAPTER IV

RESULTS

Daily Pollination Cycle

Close observation of black gramagrass plants indicated little flowering occurred before 3:30 p. m. This was borne out by the slides exposed between 8:00 a. m. and 3:00 p. m. catching an average of only 91 pollen grains. An average of 56 pollen grains was caught during the 30-minute period between 3:00 and 3:30 p. m. A peak of 278 pollen grains was reached at 5:00 p. m. (Figure 1). The peak shedding period continued until 6:00 p.m., after which there was a steady decline in pollen shed until 7:30 p. m. An average of 65 pollen grains was caught during the period from 7:30 p. m. to 8:00 a. m.

The apparent effect of environmental factors on daily pollen shedding as reported by Jones and Brown (27) was exemplified by variation of the daily peak pollination period of black gramagrass (Table 2). The peak pollination period occurred between 4:00 and 4:30 and 5:30 and 6:00 p. m. on September 10, 5:00 and 5:30 p. m. on September 11, and 4:30 and 5:00 and 6:00 and 6:30 p. m. on September 12. Brief gusty

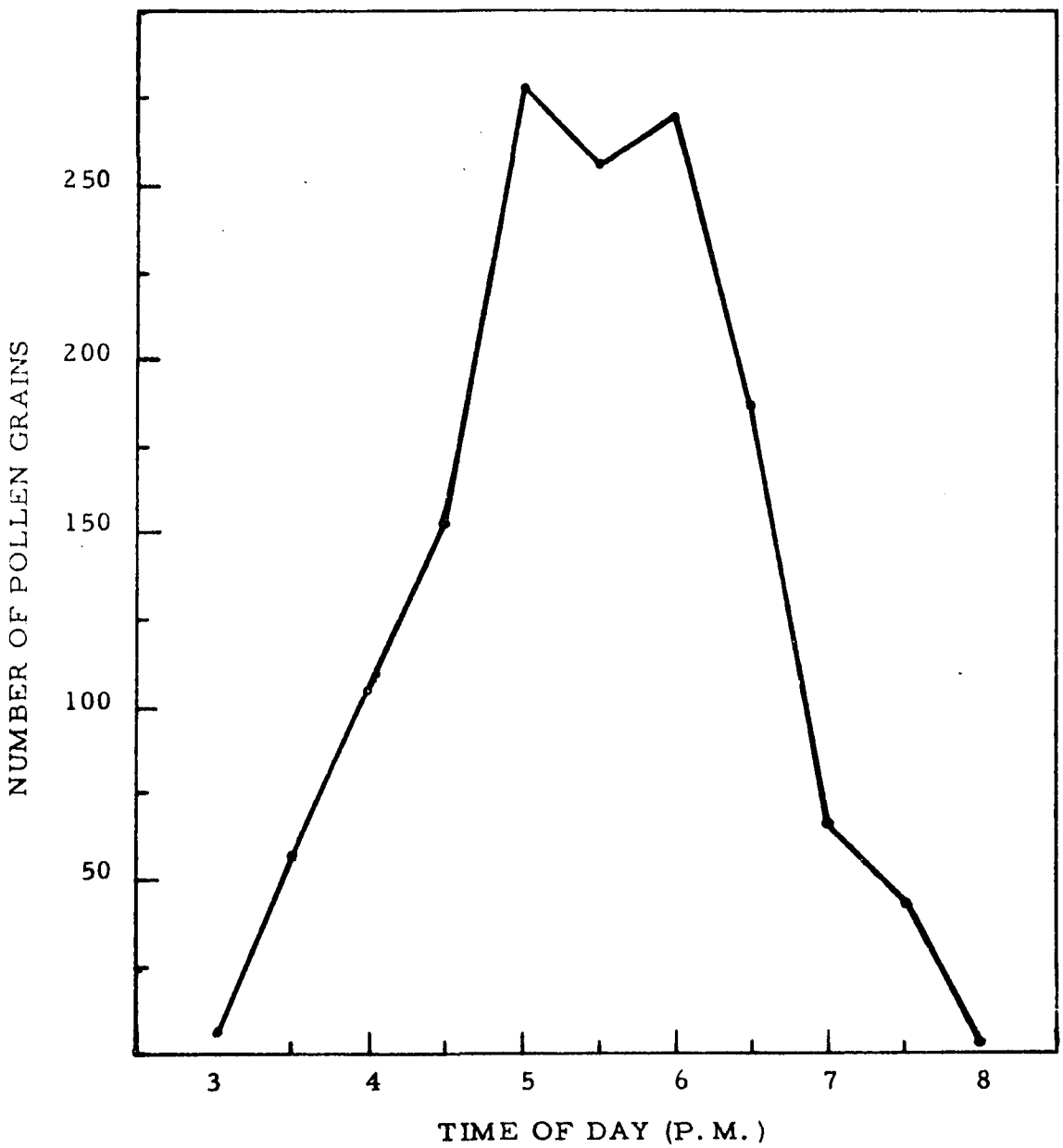


Figure 1. Average number of black gramagrass pollen grains in the air for three days.

Table 2. Total number of black gramagrass pollen grains in the air for three days in 1957.

Time	Date			Average per 30 minutes
	9/10	9/11	9/12	
8:00 a. m. - 3:00 p. m.	79	68	126	6.5
3:00 - 3:30	140	20	8	56.0
3:30 - 4:00	185	57	70	104.0
4:00 - 4:30	204	163	89	152.0
4:30 - 5:00	172	312	350	278.0
5:00 - 5:30	159	411	192	254.0
5:30 - 6:00	417	334	56	269.0
6:00 - 6:30	141	123	294	186.0
6:30 - 7:00	111	46	35	64.0
7:00 - 7:30	64	24	29	39.0
7:30 p. m. - 8:00 a. m.	81	63	51	2.6

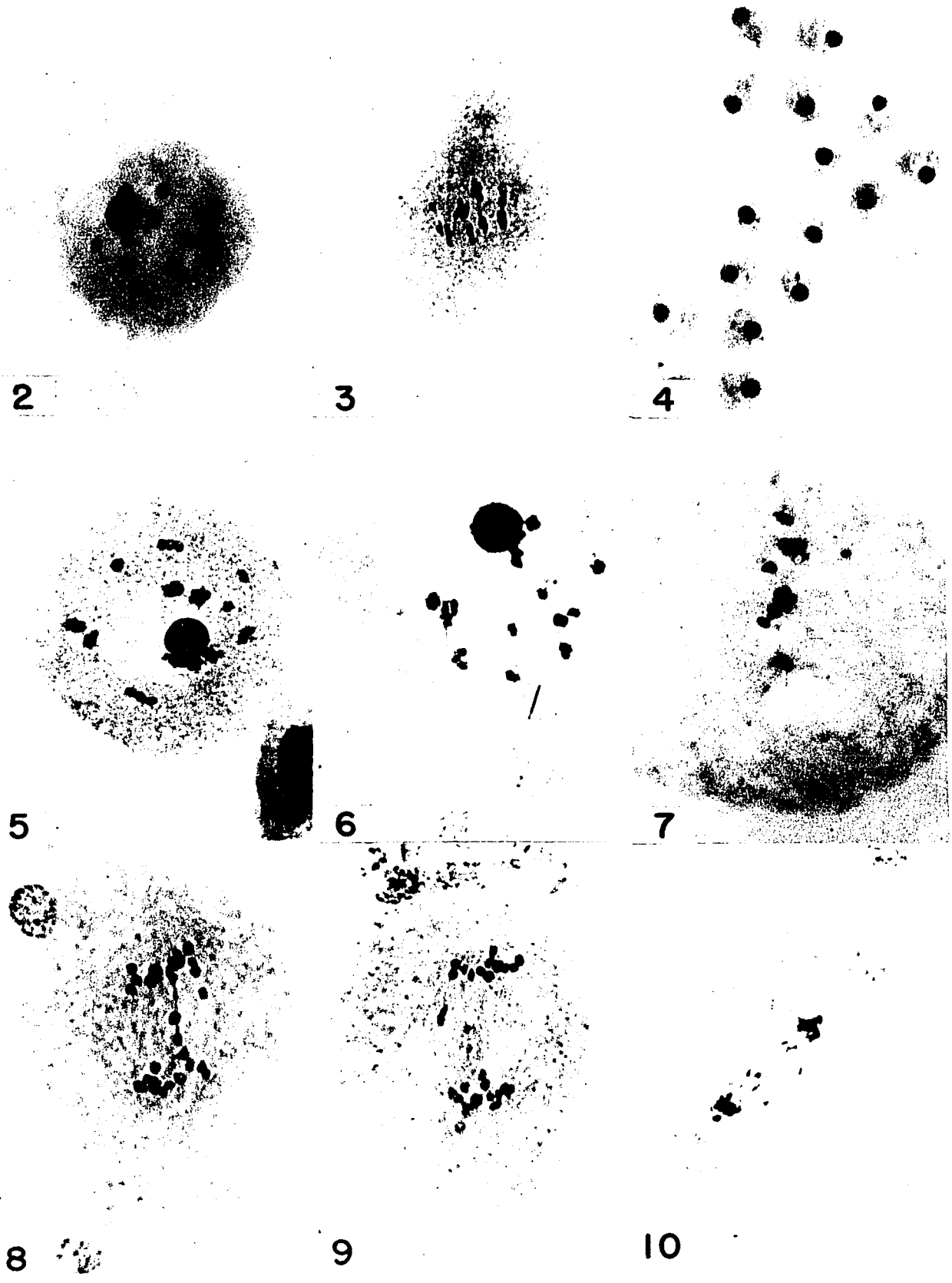
periods were largely responsible for these differences rather than such other influences as temperature and humidity.

Chromosome Number and Meiotic Behavior

Thirty-five different sources of black gramagrass were studied for chromosome number and meiotic behavior. Thirty-four of these accessions had a chromosome complement of 2N = 20. Pollen mother cells at diakinesis and metaphase I usually had ten bivalents (Figures 2 and 3). A small number of diakinesis cells of five accessions were observed to have nine bivalents and two univalents. Meiotic behavior of these and the remaining 28 accessions was otherwise very regular. Micronuclei were not detected in quartet cells (Figure 4) and pollen quality approached 100 per cent.

The only deviation from the 2N = 20 condition was exhibited by plants collected from the Page Ranch, north of Tucson, Arizona. These plants had an aneuploid chromosome complement of 2N = 28 (Figure 7). Maximum chromosome association observed was seven trivalents, three bivalents, and one univalent (Figure 7). Analysis of diakinesis cells for chromosome number and association was very difficult, a fact which is readily apparent in examination of Figures 5 and 6. The diakinesis cell in Figure 5 has three trivalents directly above, two directly below, and one below and to the left of the nucleolus. One univalent is

Figures 2-10. Photomicrographs of pollen mother cells of black gramagrass. Figure 2. Diakinesis of diploid plants showing 10 bivalents (2N = 20). Figure 3. Metaphase I with 10 bivalents. Figure 4. Typical quartet cells of diploid plants. Figures 5 and 6. Diakinesis of 2N = 28 plants showing varying degrees of chromosome association. Figure 7. Metaphase I cell of 2N = 28 plants showing seven trivalents, three bivalents, and one univalent. Figures 8-10. Anaphase I cells of 2N = 28 plants showing lagging chromosomes. All figures approximately 900X.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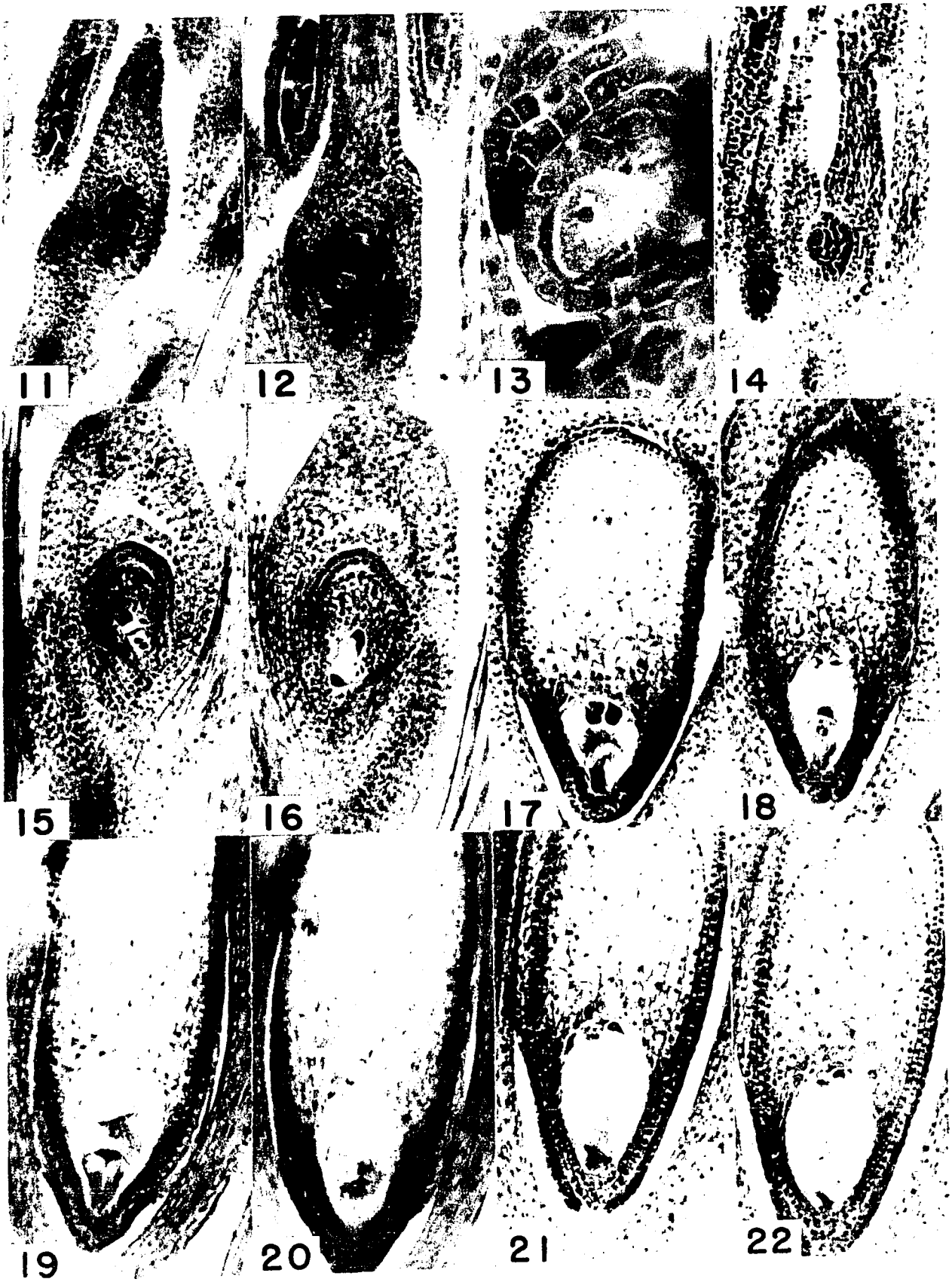
located at eleven o'clock in the cell, the other being situated slightly above and to the right of the nucleolus. The remaining chromosomes are associated as bivalents. Over 50 per cent of the diakinesis cells analyzed had this association of six trivalents, four bivalents, and two univalents. Minimum chromosome association was found to be four trivalents, six bivalents, and four univalents (Figure 6). The number of lagging chromosomes at anaphase I ranged from zero to eight (Figures 8-10). More than 50 per cent of the cells observed had four laggards at this stage of meiosis. No micronuclei were observed in quartet cells; however, relatively few of the cells were obtained for analysis. The pollen grains observed were of equal size and had a viability of approximately 67 per cent, yet sterility in these plants was found to be very high.

The differences in cell size of the 20 and 28 chromosome plants (Figures 2 and 3 and 5-7, respectively) were real and appeared to be directly correlated with chromosome number.

Megasporogenesis and Embryo Sac Development

The development of the female gametophyte began with the differentiation of an archesporial cell from a hypodermal cell of the nucellus (Figure 11). The archesporial cell enlarged and the nucleus became prominent (Figure 12). Although no division figures from which

Figures 11-22. Photomicrographs of ovaries (longitudinal sections) showing megagametophyte development in black gramagrass. Figures 11 and 12. Differentiation of archesporial cell. Figure 13. Megaspore mother cell in prophase I of meiosis. Figure 14. Dyad cells. Figure 15. Linear tetrad of megaspores, the three nearest the micropyle disintegrating. Figure 16. Four-nucleate embryo sac. Figure 17. Typical mature embryo sac. Figure 18. Mature embryo sac; antipodals and synergids partially disintegrated. Figures 19 and 20. Serial sections of mature embryo sac with prominent polar nucleus and synergids. Figures 21 and 22. Serial sections of mature embryo sac showing two polar nuclei and an egg nucleus; antipodals and synergids disintegrating. Figures 11 and 12 and 14-22 approximately 200X. Figure 13 approximately 900X.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chromosome counts could be made were observed, megaspore mother cells which appeared to be in prophase I were readily detected (Figure 13). The megaspore mother cell then underwent two transverse divisions, the first producing two dyad cells (Figure 14), the second producing a linear tetrad of four megaspores. Soon after the second division the three megaspores nearest the micropyle disintegrated, leaving the chalazal member as the functional megaspore (Figure 15).

Three successive divisions of the remaining megaspore resulted in a 2-nucleate, 4-nucleate (Figure 16), and finally an 8-nucleate embryo sac. The 8-nucleate embryo sac then gave rise to the mature female gametyphyte with an egg, two polar nuclei, two synergids, and three antipodals (Figure 17). Typically, the synergids and antipodals began to disintegrate before fertilization, leaving only the egg nucleus and the polar nuclei (Figure 18). Over 90 per cent of the embryo sacs observed at the time of pollination had the two polar nuclei fused. The polar nuclei observed before fusion appeared to be somewhat smaller than the typical egg nucleus. This is illustrated by comparison of the polar nuclei in Figure 22 with the prominent egg nucleus in Figure 18. On the other hand, once fusion had occurred, the 2N polar nucleus was slightly larger than the egg nucleus and had a much greater stainability (Figures 19 and 20).

In sharp contrast to the antipodals and synergids which generally became cellular in form (Figures 17 and 19, respectively) the egg and polar nuclei remained without cell walls: rather, they were observed to lie in a mass of cytoplasm which apparently served to maintain the relationship of these structures (Figures 21 and 22). Once the antipodals disintegrated, cytoplasmic strands to this area of the embryo sac usually diminished, thus severing the continuity between the chalazal and micropylar regions of the gametophyte.

Such abnormalities as enlargement of nucellar cells to form embryo sacs and the occurrence of proembryos were not observed during the development of the female gametophyte.

The time of development of the female gametophyte was found to correspond closely with the male gametophyte by observing both within the same flower at the same time.

Pollen Tube Growth

Although Wilson's (46) method for staining pollen tubes was superior to techniques outlined by Darlington and Lacour (13), the results from this phase of the study were unsatisfactory and inconclusive. Ovaries collected one hour after pollination were found to have pollen tubes growing through the stigma branches and into the style. Preparation of older ovaries were of little value since pollen tubes could not be traced through

the style and into the ovary tissues. Stigmas were generally absent from ovaries collected 24 hours after pollination, indicating that fertilization had already occurred. Over 150 ovaries were observed for pollen tube growth. The maximum number of germinated pollen grains observed on the stigma of an ovary was five, while most stigmas had only one or two. This is in sharp contrast to such grasses as Panicum antidotale Retz., Eragrostis chloromelas Steud., and E. lehmanniana Nees. where stigmas with 25 to 50 [1] germinating pollen tubes were of common occurrence.

Embryogeny

Fertilization was not observed by the writer, but the development of the male and female gametophyte warrants the assumption that this process occurred in the usual manner. Fertilization of the egg nucleus and the 2N polar nucleus appeared to occur simultaneously; however, the zygote lagged somewhat behind the endosperm in initiation of cell division (Figure 23). The time lapse between pollination and initiation of the proembryo and endosperm was usually between 12 and 18 hours. Once fertilization had occurred the cells of the integument and other ovary tissue divided and elongated rapidly.

Forty-eight hours after pollination, the proembryo consisted of four to eight cells located in the micropylar region of the nucellar cavity.

[1] Unpublished observations by author.

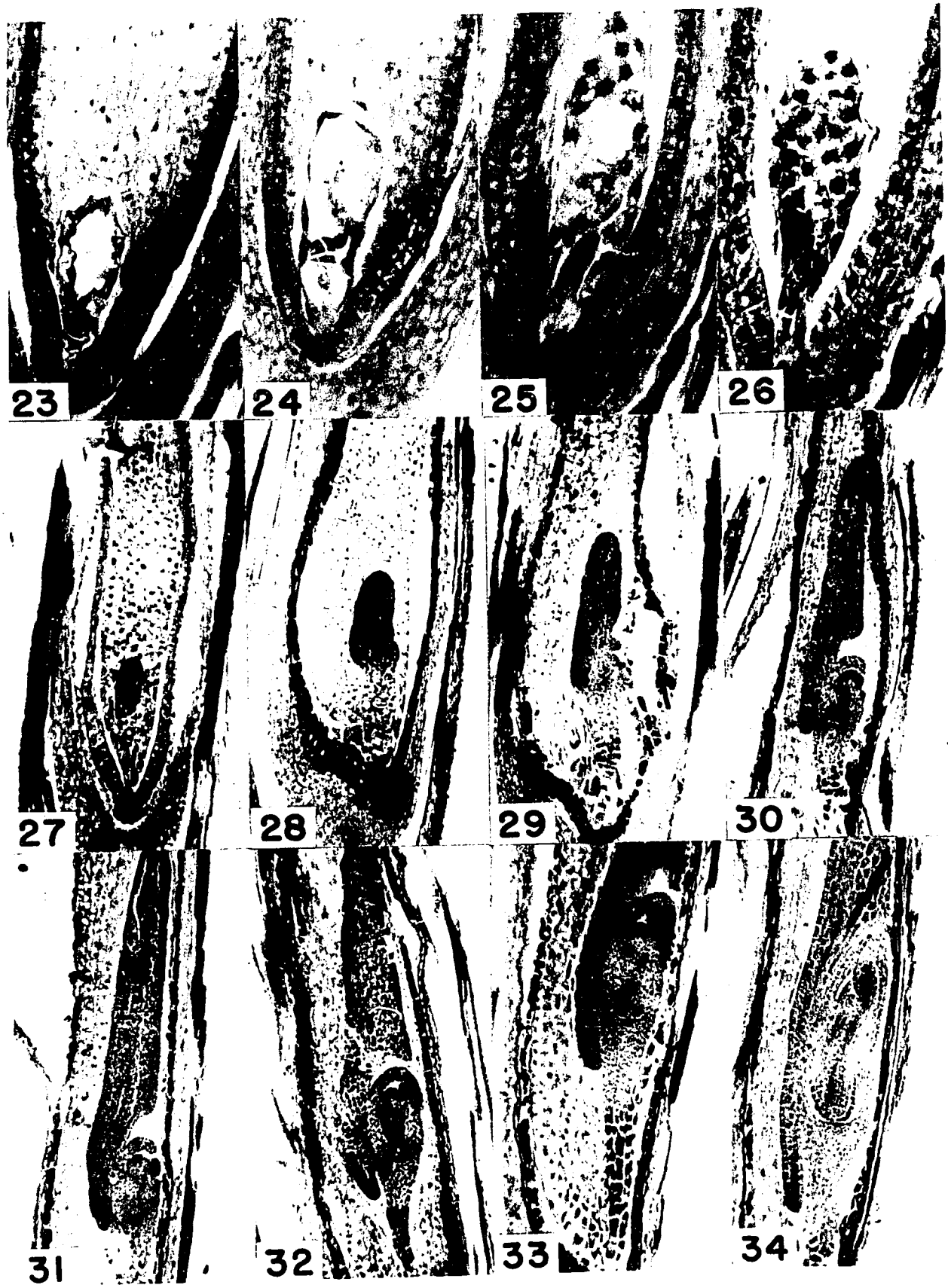
Free nuclear endosperm lined the periphery of this cavity (Figure 24). There appeared to be little regularity in arrangement and sequence of division in cells of the proembryo. Three days after pollination the 10 to 15 celled proembryo assumed a club-shaped appearance and was approximately 73 μ in length while the endosperm remained free nuclear (Figure 25). By the fourth day the endosperm began to change from free nuclear to cellular (Figure 26).

Differentiation of the proembryo into various embryo structures began between the fourth and fifth day. The epidermis was readily detected and there was some evidence of a suspensor being formed. By this time the endosperm had completely changed to the cellular form (Figure 27). The embryo was at this time approximately 131 μ in length.

During the next 24 hours growth of the embryo was rapid. An increase in the rate of cell division in an anterior subapical region resulted in a protuberance which was the initiation of the coleoptile (Figure 28). By the end of this 24-hour period the coleoptile initial was discernible, and the scutellum had increased greatly in length by division of cells of the apex. The suspensor, which extended to the region of the micropyle, was clearly delimited from the body of the embryo (Figure 29). The over-all length of the embryo at this time was approximately 438 μ.

On the seventh day the coleoptile, by rapid growth of its upper ridge, began to curve over and envelop the plumule initial. Growth of the

Figures 23-34. Photomicrographs of ovaries (longitudinal sections) showing the embryogeny of black gramagrass. Figure 23. Proembryo and endosperm development--12 hours after pollination. Figure 24. Four-celled proembryo--two days after pollination. Figure 25. Ten to 15-celled club-shaped proembryo; free nuclear endosperm--three days after pollination. Figure 26. Endosperm becoming cellular--four days after pollination. Figure 27. Embryo with epidermis forming; endosperm cellular--five days after pollination. Figure 28. Embryo with slight protuberance in the anterior subapical region which is the coleoptile initial--six days after pollination. Figures 29 and 30. Embryos showing progressive development of the coleoptile, scutellum, and suspensor--seven days after pollination. Figure 31. Embryo showing iniation of foliage leaf below the crescent-shaped coleoptile--eight days after pollination. Figure 32. Embryo showing coleoptile completely surrounding the plumule, downward growth of scutellum, and evidence of root cap development--nine days after pollination. Figure 33. Embryo showing multicellular suspensor--ten days after pollination. Figure 34. Mature embryo--12 days after pollination. Figures 23-26 approximately 200X. Figures 27-34 approximately 100X.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scutellum, in addition to the rapid upward extension, had begun in a downward direction. A slight cleavage of lower tissue of the embryo almost parallel to the lower tip of the scutellum marked the beginning of the coleorhiza (Figure 30). The coleoptile-coleorhiza axis, at a right angle to the longitudinal axis of the embryo, was approximately 340 μ in length, and the over-all length of the embryo was approximately 730 μ.

Between the seventh and eighth days the first foliage leaf primordium was initiated as a group of cells around the plumule (Figure 31). The crescent-shaped coleoptile continued to curve over the plumule and by the ninth day had reached the anterior side of the embryo, completely enclosing the plumule and foliage leaf (Figure 32). At this stage the lower tip of the scutellum had grown downward almost parallel to the upper portion of the very prominent multicellular suspensor. Differentiation of the root cap had also begun, and the coleorhiza region was clearly organized in the lower portion of the embryo.

By the tenth day the embryo was anatomically complete (Figure 33) with the exception of the epiblast, which did not become visible until the twelfth day. The epiblast developed on the abaxial side of the embryo just above the coleorhiza region. By this time the foliage leaf had curved over the plumule, and the coleorhizal region was strongly developed (Figure 34). The scutellum remained free of the coleorhizal region of the

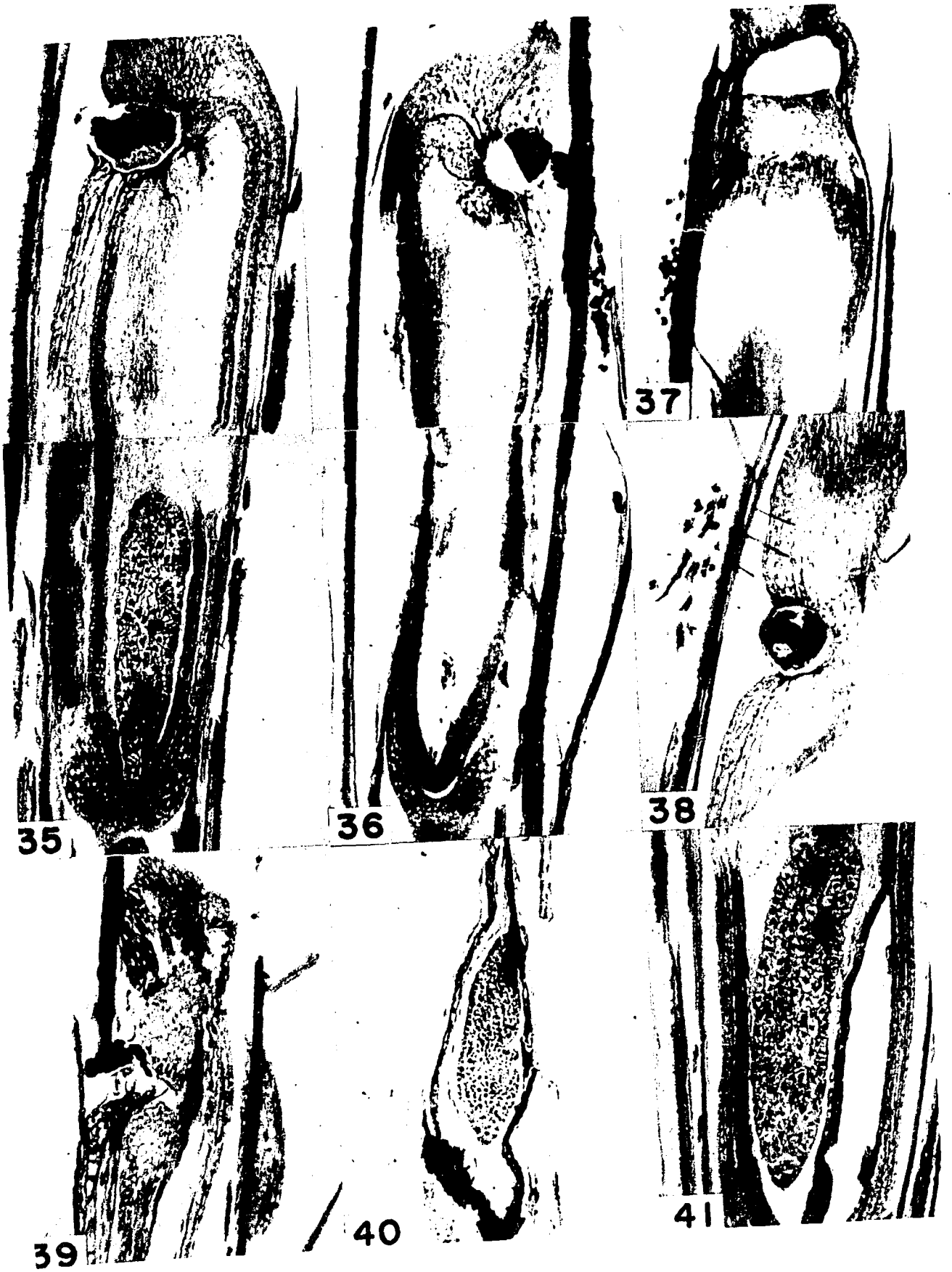
embryo. An epithelial layer was formed about the surface of the scutellum adjacent to the endosperm. The cells of this layer were only slightly cubical in shape, thus the palisade type cell formation of the epithelial layer common to the cereal grains does occur in black grama-grass. At maturity the endosperm, which extended the complete length of the caryopsis, was composed of large thin-walled but tightly compacted cells filled with starch grains. The mature embryo had a scutellum length of approximately 1160 μ, a plumule-radical axis of 580 μ, a total length of 1990 μ, and a thickness of 380 μ. The total length of the mature caryopsis was approximately 2400 μ.

Ovaries Damaged by Insects

While studying the development of the embryo, insect-like eggs or embryos were found embedded in the ovary tissue just below the base of the style (Figures 35, 36, 38, and 39). Slides were submitted to the Department of Entomology, University of Arizona, for identification. Members of the department concluded the objects were insect embryos; however, the identity of the insect could not be established from the histological material. Studies are being conducted to isolate and identify the organism causing damage.

The first notice of insect-damaged ovaries occurred during the study of ovaries collected three days after pollination. Once the insect

Figures 35-41. Photomicrographs of ovaries (longitudinal sections) of black gramagrass showing insect damage. Figure 35. Insect embryo embedded in tissue of ovary with normally developing embryo and endosperm. Figure 36. Aborted ovary showing insect damage. Figure 37. Ovary with tissue below style eaten away by insect. Figures 38 and 39. Later stages of insect development. Figure 40. Insect-damaged ovary with developing embryo but lacking endosperm. Figure 41. Insect-damaged ovary with developing endosperm but lacking an embryo. All figures approximately 100X.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problem was realized all slides of younger ovaries, including those of megasporogenesis, were re-examined for insect damage. No damage was detected, suggesting that insect infestation apparently did not occur until three days after pollination. It is possible, however, that such infestation occurred earlier in ovary development and could not be detected histologically. Identification of the insect and its habits should aid in determining the time of infection. Twenty-eight per cent of the ovaries examined three days after pollination had been infected, whereas fifty per cent of the ovaries collected ten days after pollination showed damage. This increase in number of damaged ovaries with time may have been real, or merely because damage was more easily detected in later stages of development.

Fifteen per cent of the infested ovaries had normally developing embryos and endosperm (Figure 35) that may or may not abort before maturity. However, Andersen (2) was of the opinion that ovaries of P. pratensis infected by insects would not complete development of the caryopsis. Eighty per cent of the infested ovaries had aborted without fertilization (Figure 36) having occurred. Many of these had the upper portion of the ovary hollowed out (Figure 37). The remaining five per cent had either the egg or polar nuclei unfertilized. An assumption could be made that an embryo developing without endosperm (Figure 40) and endosperm developing without an embryo (Figure 41) would be nonfunctional.

CHAPTER V

DISCUSSION

The accessions of B. eriopoda studied clearly indicated this to be a diploid species with a chromosome complement of 2N = 20. However, if the originally proposed basic chromosome complement of x = 7 for the genus Bouteloua is accepted, the 2N = 20 plants of this study would be aneuploids (11, 15). Such aneuploid plants would be expected to exhibit a high degree of meiotic irregularities. Since the frequency of irregularities was low, less than one per cent of the cells observed exhibiting any degree of meiotic irregularity, this concept is unsupported. On the other hand, meiotic irregularities were prevalent in the 2N = 28 plants. If the 2N = 28 plants were considered as autotetraploids, some quadrivalent association would be expected, and this type of association was not observed. The results of this chromosome survey thus furnished additional evidence for the basic chromosome number of x = 10 first proposed by Snyder and Harlan (39) and later supported by Gould (16).

The race with a chromosome complement of 2N = 28 possibly arose by the union of an unreduced female gamete and a normal male

gamete resulting in a triploid, 2N = 30. The triploid produced this aneuploid in subsequent generations. A basis was thus theoretically provided not only for a series of aneuploids but also for polyploidy higher than the triploid level. Fults' (15) report of 21 somatic chromosomes for B. eriopoda was made on the basis of results obtained with plants collected near Tucson, Arizona. These plants may have been from this aneuploid population. The 2N = 28 plants were highly sterile; however, they had the ability to maintain and increase themselves vegetatively. These plants also had a very open and decumbent growth which enhanced their ability to reproduce asexually. Since aneuploids reproduce sexually only occasionally, natural selection could have eliminated forms unable to spread and compete under extreme environmental conditions. This could account for the apparent absence of the expected aneuploid series from this population.

Development of the female gametophyte began with the differentiation of a subepidermal cell of the nucellus that eventually gave rise to an 8-nucleate embryo sac of the "Polygonium" type as classified by Maheshwari (30). Meiotic chromosome counts could not be made; however, the killing and fixing solution used had a relatively slow penetration rate and did not yield optimum cytological material. Faster penetrating solutions resulted in plasmolyzed cells unsuitable for analysis.

No irregular nucellar activity was noted, and embryo development was not observed until 12 to 18 hours after pollination. Although fertilization of the egg and polar nuclei by the sperm nuclei was not observed, the normal development of the male and female gametophytes suggested sexual reproduction. A high degree of variation between and among individual plants of all accessions not only indicated reproduction to be sexual but also suggests that the species may be largely cross-pollinated. The results of this phase of the study suggest improvement of black gramagrass could be accomplished by employing breeding methods commonly used for sexually reproducing plants.

Bashaw and Holt (6), Beck and Horton (8), Stover (43), and others have reported grass embryo sacs with a varying number of antipodals from four to ten. Maheshwari (30) states that large numbers of cells result from division of the antipodals in the Gramineae, yet the antipodals of black gramagrass were never observed to exceed three. Soon after the embryo sac reached maturity the antipodals disintegrated and were apparently digested by other cells of the female gametophyte.

The development of black gramagrass embryos was similar to that of other grass embryos. The lack of regularity in arrangement and sequence of division observed in cells of the proembryo is in agreement with the work of Bennett (9) with Dallisgrass, Randolph (37) with corn,

and Merry (31) with barley. These workers interpreted this as an indication that factors controlling the growth of the embryo affect the embryo as a whole rather than individual cells.

The embryo of black gramagrass had a very prominent multicellular suspensor at maturity and the shield-like scutellum, with a striking green pigment, completely enveloped the adaxial surface of the embryo.

The embryo reached maturity anatomically about 12 days after pollination. Maturity was thus reached rapidly in comparison with corn embryos which require 45 days (37), barley 35 days (31), and Dallisgrass 14 to 18 days (9). Harlan (21) has indicated embryo development of Bouteloua is similar to Buchloë; however, the time required for development was not specified.

The occurrence of insect eggs or embryos embedded in developing seed could have a marked effect upon seed production of this grass. The data obtained during this study indicated production could suffer a 50 per cent reduction. Kneebone (29) obtained a significant increase in blue gramagrass caryopsis production from dieldrin applications. He attributed this primarily to the control of thrips. It is interesting to note that the plants from which florets for this study were collected had been sprayed weekly, after the onset of inflorescence production, with a

mixture of malathion and dieldrin. Obviously, other insecticides or treatment schedules will be required to adequately control the insects.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The chromosome number, microsporogenesis, megasporogenesis, embryo sac development and embryogeny of B. eriopoda were investigated. Thirty-five accessions were observed for chromosome number and meiotic behavior. Plants of the "Flagstaff" strain, considered to be the standard black gramagrass, were used in the remaining phases of the study.

The plants were grown at the University of Arizona Plant Material Center, Tucson, Arizona. Laboratory facilities were also located at this installation.

The results of this study warrant the following conclusions:

1. Root tips were found to be unsatisfactory for somatic chromosome counts because of (a) the difficulty of obtaining healthy root tips in sufficient quantity, (b) the low frequency of metaphase cells, and (c) the tendency of the chromosomes to clump.

2. B. eriopoda is a diploid species with a normal complement of 2N = 20 chromosomes.

3. Meiotic behavior of the diploid plants was normal other than the infrequent occurrence of two univalents at diakinesis.

4. Aneuploid plants with 28 chromosomes were found at one location. Meiosis was irregular and the plants were highly sterile.

5. The larger meiotic cells of the 2N = 28 plants were directly related to the increased number of chromosomes.

6. Meiotic chromosome counts could not be made from the megaspore mother cells studied; however, meiosis appeared to proceed normally.

7. This species had an 8-nucleate embryo sac of the "Polygonium" type. This is the most common Angiosperm embryo sac.

8. The number of antipodals never exceeded three as reported in many other grasses.

9. Megasporogenesis and embryo sac development, being normal, indicates sexual reproduction in the plants studied.

10. Individual plant variation within and among accessions furnished additional evidence for sexual reproduction and also suggested that the species was largely cross-pollinated.

11. The daily pollination period of this grass occurred from 4:30 to 6:30 p. m. Slight variations of this period were caused by fluctuating environmental conditions.

12. The time lapse between pollination and fertilization was 12 to 18 hours.

13. The proembryo was a mass of cells which lacked an orderly arrangement and sequence of division.

14. The endosperm was free nuclear until four days after pollination.

15. The embryo had a more strongly developed scutellum and suspensor than many grasses.

16. The embryo was mature anatomically about 12 days after pollination.

17. Unidentified insect embryos were found embedded in ovary tissue three to ten days after pollination. This infestation could substantially reduce seed production of this grass.

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