Cytological studies on the influence of male-sterile mutant ms1 on reproductive function in soybean (Glycine max (L.) Merr.)

Long-Fang Oliver Chen
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CYTOLOGICAL STUDIES ON THE INFLUENCE OF MALE-STERILE MUTANT MS1 ON REPRODUCTIVE FUNCTION IN SOYBEAN (GLYCINE MAX (L.) MERR.)

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Cytological studies on the influence of male-sterile mutant \textit{ms1} on reproductive function in soybean \\
(\textit{Glycine max} (L.) Merr.)

by

Long-Fang Oliver Chen

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

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GENERAL INTRODUCTION

Male-sterile mutants can be manipulated for the study of basic genetic processes, building germplasm pools, and exploiting heterosis (Duvick, 1966). In our laboratory, use of male-sterile mutants in soybean (Glycine max L. Merr.) for cytological and genetic studies is an important research tool. In this introduction, brief reviews on male-sterile systems in angiosperms, the known sterility systems in soybean, studies on the genetic male-sterile, female-fertile ms1 mutant in soybean, mutants in other species similar to that of ms1 mutant in soybean, some rationale of techniques used, and the dissertation objectives in my research are of concern.

Male-sterility systems in angiosperms

As defined by Dorsey (1914), male sterility represents "the condition resulting from defects leading up to the non-formation of pollen, or lack of functional power in it when formed". On the distinction between male sterility and pollen sterility, Jain (1959) suggested that a formal definition of male sterility should involve: 1) deficiency of male individuals in a dioecious strain, 2) absence or atrophy of male organs in a normally bisexual plant, 3) failure to produce normal sporogenous tissues in stamens, 4) inhibition at various stages of pollen development yielding incomplete
or imperfect pollen, or 5) failure to mature, dehisce, or to function, when placed on a compatible stigma. Pollen abortion and pollen failure (4 and 5 above) were considered as pollen sterility by Jain (1959). Heslop-Harrison (1971), on the basis of the developmental stage and cause of male sterility, divided male sterility into three main categories: 1) anther suppression, abortion, phyllody, petallody, and pistillody, 2) abnormal meiosis or gametophyte development, and 3) failure of anthesis or anther dehiscence. Gottschalk (1976) classified genetic male sterility into two types in higher plants. Functional male sterility includes mutants in which archesporial tissue or stamens are not differentiated, anthers do not dehisce or anther and stigma are separated from each other, or stamens are transformed into carpels or other floral parts. The other type of male sterility is the result of breakdown of microsporogenesis or microgametogenesis process. Johns et al. (1981) used the term "structural sterility" to emphasize the fact that the sterility of the mutant under consideration was due to structural abnormalities in the flowers or in the reproductive organs, and "nonstructural sterility" referred to sterility caused by a breakdown of microsporogenesis or microgametogenesis. In my report, I will use the terms defined by Johns et al. (1981).
On the basis of inheritance, male sterility could be categorized as either genetic, cytoplasmic or gene-cytoplasmic (Jain, 1959; Gottschalk and Kaul, 1974; Frankel and Galun, 1977). In flowering plants, these three types of male sterility have been reviewed by Jain (1959). Genetic and gene-cytoplasmic male sterility were reviewed by Gottschalk and Kaul (1974) and Gottschalk (1976); cytoplasmic male-sterility was reviewed by Edwardson (1970) and Laser and Lersten (1972). Johns et al. (1981) also reviewed genetic structural sterility in angiosperms. Nonstructural male-sterile mutants that result in the breakdown of microsporogenesis and microgametogenesis are typically found in higher plants. Although abortion was reported to occur at any stage between microspore mother cells to mature pollen, most of the male-sterility (ms) genes exerted their influence on the final stages of meiosis (Frankel and Galun, 1977; Gottschalk and Kaul, 1974; Laser and Lersten, 1972).

**Sterile mutants in soybean**

In soybean [Glycine max (L.) Merr.], all known male-sterile mutants are of genetic type (Graybosch and Palmer, 1984a). Presently, only two structural sterility mutants are noted (Johns et al., 1981). One, designated flower-transformed (ft) mutant, obtained from a variety irradiated with gamma rays and controlled by single recessive gene, was
described by Singh and Jha (1978). Sterility of this ft ft mutant resulted from nondehiscent anthers. Another structural male sterile mutant controlled by two recessive fs1 fs2 alleles was reported by Johns and Palmer (1982). Because of abnormal filament elongation, self-pollination is inhibited by a spatial separation of the stigma and anthers.

Several nonstructural sterility mutants, in which sterility results from the breakdown of microsporogenesis or microgametogenesis, are known in soybean. Five synaptic mutants, designated st1, st2, st3, st4, and st5 were reported by Owen (1928a), Hadley and Starnes (1964) (st2 and st3), Palmer (1974) and Palmer and Kaul (1983), respectively. Cytological evidence obtained by the above authors showed that sterility of these synaptic mutants were caused by either desynapsis or asynapsis in prophase I of meiosis. Both male sterility and female sterility were noted in these synaptic mutants. Three additional male-sterile, female-sterile mutants, including one (TGM 103-1) resulting from precocious movement of chromosomes at metaphase I as well as tapetal abnormality (Rubaihaya and Gumisiriza, 1978), one desynaptic mutant, and a mutant with abnormal metaphase I (Jha and Singh, 1978), also were recorded.

At least three partial male-sterile mutants designated msp (Stelly and Palmer, 1980a, b), Arkansas ms mutant (Caviness et al., 1970), and UPSL-18 MS-1 (Jha and Singh
1978), have been reported. All these partial male-sterile mutants are known to be inherited monogenically, with sterility being recessive. Phenotypic expression of msp is variable and abortion of cells may occur at any stage between the late sporogenous and mature pollen stages (Stelly and Palmer, 1982). Meiotic studies revealed that sterility of UPSL-18 MS-1 resulted from abnormal secondary meiotic division (Jha and Singh, 1978). Cytological evidence is not available for the Arkansas male-sterile strain.

Five male-sterile and female-fertile mutants for which gene symbols have been assigned are: ms1 (Brim and Young, 1971; Palmer et al., 1978; Yee and Jian, 1983), ms2 (Graybosch et al., 1984), ms3 (Palmer et al., 1980), ms4 (Delannay and Palmer, 1982), and ms5 (Buss, 1983). Albertsen and Palmer (1979), in their study of microsporogenesis in the ms1 mutant, indicated that male sterility was due to the failure of cytokinesis after telophase II of meiosis. Graybosch et al. (1984) verified that meiosis in the ms2 mutant is normal; however, following cytokinesis the tetrads aborted. No release of microspores or formation of microspore walls were observed in the ms2 mutant. In the ms3 soybean, tapetum malfunction caused the abortion of microspores shortly after the initiation of microspore wall but prior to callose dissolution (Nakashima et al., 1984;
Buntman and Horner, 1983; Palmer et al., 1980). Formation of four-nucleate coenocytic microspores also was observed in ms4 plants (Delannay and Palmer, 1982). However, further cytological studies of this ms4 mutant demonstrated that male sterility was the result of abnormalities in the function of postmeiotic microspore mother cells (Graybosch and Palmer, 1985). They also pointed out that cytokinesis following telophase II of meiosis could be absent, incomplete, or disoriented, resulting in cells with different numbers of nuclei. Cytokinesis is influenced by temperature in the ms4 mutant (Graybosch and Palmer, 1984b). The ms5 mutant was derived from irradiated plants of the cultivar "Essex" (Buss, 1983). However, cytological studies on this mutant are not available.

Several additional nonstructural male-sterile mutants in soybean, such as: Wabash (Chaudhari and Davis, 1977), Semmes MS-1, Semmes MS-2 (Patil and Singh, 1976), UPSM-229 MS-2 (Jha and Singh, 1978), and TGM 242-4 (Rubaihayo and Gumisiriza, 1978) are known but gene symbols have not yet been assigned. Among these, Wabash was recently proved to be an independent mutation at the ms3 locus (Graybosch, 1984). Semmes MS-1 has normal cytokinesis and normal microspore formation but the microspore failed to develop into normal pollen grains (Patil and Singh, 1976). Patil and Singh also observed normal cytokinesis in Semmes MS-2 but the tetrads
degnerated immediately and no pollen grains were noted. Whether it is a similar mutant to that of ms2 is not known. The UPSM-229 MS-1 reported by Jha and Singh (1978) had abnormal meiosis after metaphase I that resulted in unequal distribution of bivalents at anaphase II. Two to four microspores per sporad frequently were observed. Cytokinesis failure, and large pollen grains with four daughter nuclei were investigated in UPSM-229 MS-2 and in TGM 242-4 by Jha and Singh (1978), and Rubaihayo and Gumisiriza (1978), respectively. However, the relationships between these two mutants (UPSM-229 MS-2 and TGM 242-4) and ms1 or ms4 mutant described previously are not yet clear.

Gottschalk and Kaul (1974) pointed out that most of the ms genes influence the final stage of meiosis between interphase II and pollen formation, with a few ms genes acting during the early and middle stages of the first meiotic prophase and only a very small number of ms genes becoming effective between diakinesis and anaphase II. In soybean, most nonstructural sterility mutants with abnormal meiosis in early meiotic stage (prophase I), which include most of synaptic mutants, proved to be both male sterile and female sterile. The partially male-sterile msp mutant is known to be variable in phenotypic expression (Stelly and Palmer, 1982). Three ms mutants (UPSM-229 MS-1, UPSM-18 MS-1 and TGM 103-1)
have effects between diakinesis and telophase II, and the other nine mutants (ms1, ms2, ms3, ms4, Semmes MS-1, Semmes MS-2, UPSM-229 MS-2, TGM 242-4, and Wabash) on which cytological studies have been conducted all show cell abortion between the stages of late telophase II and pollen formation.

Mutants in other species similar to ms1 in soybean

In soybean, failure of cytokinesis following telophase II of meiosis was observed in four male-sterile mutants: ms1, ms4, UPSM-229 MS-2, and TGM 242-4. In the early literature of male-sterile mutants in higher plants, only a few examples were reported of incomplete cytokinesis or failure of cytokinesis as a cause of male-sterility. Beadle (1932) reported a variable sterile mutant in maize in which there was failure of cytokinesis during the first meiotic division or during the second division of meiosis. Cole (1959) described a male-sterile line in green sprouting broccoli in which the microspores in the sterile anther do not separate. They remain bound together in a sticky matrix and gradually degenerate. Whether the nonseparate tetrads found in their study were due to failure of cytokinesis is difficult to judge because no photograph was provided in their report. Klein (1969) observed mutant 395 in Pisum with incomplete cytokinesis followed by degeneration. Three additional
mutants similar to mutant 395 in *Pisum* also were noted by Gottschalk and Kaul (1974). Abdalla and Hermsen (1972) reported an undivided microsporocyte sterility mutant in *Solanum verrucosum* with four nuclei enclosed within a common cell wall. Kitada et al. (1983) obtained one meiotic mutant with incomplete cytokinesis from a MNU (N-methy-N-nitroso-sourea)-induced population in rice. Recently, an alfalfa (*Medicago sativa*) mutant with jumbo pollen (*jp*) resulting from failure of postmeiotic cytokinesis was noted by McCoy and Smith (1983). Wilson (1928) indicated that lack of oxygen, narcotics, low temperature, change in the concentration of the surrounding medium, and mechanical shock tend to cause failure of cytokinesis in plants. However, the exact cause of failure of cytokinesis in these male-sterile mutants needs further study.

Studies on somatic mitotic division have shown that cytokinesis could be modified or inhibited by application of sucrose or by drugs such as caffeine, nicotine, chloral hydrate, etc. (Shigenaga, 1937; Pickett-Heaps, 1969) and by colchicine (Whaley et al., 1966). Mahlberg et al. (1975) also observed incomplete cytokinesis in callus cells of *Nicotiana, Oryza*, and *Pinus*. 
Male-sterile (ms1) mutant in soybean

Regardless of finding mutants similar to that of ms1 in soybean from other species, there are at least five independent mutations at the ms1 locus in soybean. Four mutants, designated the North Carolina, Urbana, Tonica, and Ames male steriles, arose spontaneously and independently of each other in the United States (Palmer et al., 1978). Another mutation at the ms1 locus is the Shennong male sterile recently reported in China (Yee and Jian, 1983). Therefore, seven independent male-sterile lines in soybean were proved to have the same character of failure of cytokinesis after telophase II of meiosis, and five were known to occur at the same locus. Homozygous recessive ms1 ms1 plant also are known to be associated with formation of polyembryonic seedlings, haploids and polyploids among their progeny (Kenworthy et al., 1973; Cutter and Bingham, 1977; Beversdorf and Bingham, 1977; Crane et al., 1982). This inspired us to study the relationship among the characters of male-sterility, polyembryony, haploidy, and polyploidy among different ms1 source populations.

Occurrence of twin seedlings from nonmale-sterile soybeans was reported by Owen (1928b), and Shorter and Byth (1975). Owen (1928b) observed an average of 0.44% of double-embryo seeds in a variety from China. Shorter and Byth (1975) reported a relatively high frequency (6.3% to 13%) of
twin seedlings in three Australian cultivars. Chromosome counts from their limited number of twins demonstrated all twin seedlings were diploid-diploid, except for one diploid-haploid. They also pointed out that the character of producing twins tended to be inherited and the frequency of twin seedlings varied when seeds were produced in different environments. Thus, the occurrence of polyembryony should be due to genotype specificity and tends to be modified by the environment. Previous studies on the occurrence of polyembryony among progeny of homozygous recessive ms1 ms1 plants reported averages of 4.0%, 2.3%, and 2.2%, respectively, by Kenworthy et al. (1973), Beversdorf and Bingham (1977), and Sorrells and Bingham (1979). Nevertheless, the seed from these three studies all came from North Carolina ms1 (Brim and Young, 1971). Haploids and polyploids were found in monoembryonic seedlings as well as polyembryonic seedlings in the previously mentioned three studies. Ploidy levels up to hexaploids (2n=120) were reported by Beversdorf and Bingham (1977). One of our primary interests was to determine if there is any difference in frequency of polyembryony and polyploidy among progeny of homozygous recessive ms1 ms1 plants of different source populations.

Studies of megasporogenesis and megagametogenesis
(Cutter and Bingham, 1977; Kennell and Horner, 1985a) indicated that the occurrence of polyembryony and polyploidy might result from abnormal embryo-sac development, producing multiple nuclei and fusion of these extra nuclei before fertilization. Kennell and Horner (1985a) also noted that one to four functional megaspores were observed in the embryo sac. Genetic studies of phenotypic expression of the twin seedlings and haploid plants showed that parthenogenesis, androgenesis, or multiple fertilization are the possible explanations of some of the twins (Kenworthy et al., 1973; Beversdorf and Bingham, 1977). Kenworthy et al. (1973) found a diploid-haploid twin with purple and white flowers, respectively. Both plants were sterile. However, the haploid white-flowered plant was believed to have arisen from gametophytic maternal tissue. Beversdorf and Bingham (1977) reported a haploid-haploid twin set bearing a fertile sector. They believed either that androgenesis from pollen carrying Ms1, or the reversion of the ms1 allele to Ms1, might have occurred.

The action of the ms1 ms1 egg sacs is very similar to that of the indeterminate gametophyte mutant in maize (Kermicle, 1971). The indeterminate gametophyte (ig) mutant, when crossed as female, is known to enhance the frequency of androgenesis (Kermicle, 1969), polyembryony, hetero-fertilization, and seed defectiveness (Kermicle, 1971).
Studies on the structure of the mature embryo sac of \textit{ig ig} plants and that of \textit{Ig Ig} plants manifested multiple egg cells, central cells, and polar nuclei in the \textit{ig ig} embryo sac (Lin, 1978). This is very similar to the finding of multiple eggs and nuclei in the \textit{ms1 ms1} embryo sacs by Cutter and Bingham (1977) and Kennell and Horner (1985a, b).

Cytological studies on the behavior of polyploids and their progeny could answer some of the questions regarding the mechanisms of male sterility in soybean. In this dissertation, studies on triploids, derived from homozygous recessive \textit{ms1 ms1} plants, and their progeny are included. Triploids are one of the primary sources for obtaining aneuploids in most plant species (Khush, 1973). In our laboratory, several trisomies have been established (Palmer, 1976; Palmer and Heer, 1976a; Gwyn et al., 1985). This study reports on attempts to obtain aneuploids from triploid progeny. The only source of triploid now available in soybean is from the screening of homozygous recessive \textit{ms1 ms1} progeny. Previously, attempts to obtain triploids \((2n=3x=60)\) through natural cross-pollination and artificial cross-pollination between autotetraploids and diploids were unsuccessful (Porter and Weiss, 1948; Sadanaga and Grindeland, 1981). Failure to obtain triploids from crosses between diploids and induced tetraploids could be explained
either by the hypothesis that endosperm development depends on a 2:1 ratio of female to male genomes (Nishiyama and Inomata, 1966) or by the endosperm balance number (EBN) hypothesis (Johnston et al., 1980). Under either hypothesis, crosses between diploid and induced autotetraploid soybeans fail to produce triploids because the maternal : paternal genome or EBN ratio is 4:1 when the autotetraploid is the female parent and 1:1 when the diploid is the female parent (Sadanaga and Grindeland, 1981).

Cultivated soybean (Glycine max) (2n=2x=40), which behaves cytogenetically and genetically as a diploid, has been suggested to be a tetraploid (Hadley and Hymowitz, 1973; Palmer, 1976; Bingham et al., 1976; Crane et al., 1982). The existence of duplicate-factor inheritance for several traits (Bernard and Weiss, 1973), high rate of extra chromosome transmission of trisomics (Palmer, 1976), occurrence of bivalents in haploid soybean (2n=x=20) (Crane et al., 1982), and recent evidence from the molecular level (Lee and Verma, 1984), all favored this hypothesis. Cytological studies on the triploids and their progeny might provide some evidence as to whether the obtained triploids are actually hexaploids. In addition, triploids might be a potential source of aneuploids for further trisomic studies.

Another problem addressed in this dissertation is the question "Are coenocytic pollen grains from male-sterile
anthers capable of germination and can they affect fertilization?". Albertsen and Palmer (1979) reported that male sterility in various ms1 mutants was caused by the failure of cytokinesis after telophase II of meiosis. The four nuclei become enclosed in a single-cell structure, followed by either degeneration or the occasional development of extensions resembling pollen-tubes. Skorupska and Nawracala (1980) observed growth of single pollen tube in styles of male-sterile plants in the Urbana ms1 line. Occasionally, sterile ms1 ms1 plants produced seed in the greenhouse where pollinating vectors seemed to be absent (Cutter, 1975; Palmer and Heer, 1976b; Skorupska and Nawracala, 1980). These observations suggested that the coenocytic pollen grains might germinate, grow, and possibly participate in fertilization. Thus, studies on the efficiency of pollen germination and pollen-tube growth in the coenocytic pollen grains of male-sterile plants became our third objective.

**Rationale of aniline blue fluorescence method**

Fluorescence microscopy was used to study pollen germination and pollen-tube growth. The aniline blue fluorescence (ABF) method was used in this study. The ABF method recently was reviewed by Dumas and Knox (1983). Currier (1957) indicated that callose was known to occur in
pollen grains, pollen-tube walls and as plugs in pollen tubes. Staining with aniline blue reveals a yellow fluorescence as evidence of callose. Presently, callose is known as a cell-wall polysaccharide composed generally of 1, 3-β-glucans, and can be localized by the decolorized aniline blue fluorescence (Dumas and Knox, 1983). Aniline blue is a triarylmethane dye containing several minor impurities, among which is fluorochrome which complexes with callose and other cell-wall polysaccharides. Fluorochromes are known to contain two para-substituted aromatic rings with the formula C_{25}H_{18}N_{2}Na_{2}O_{7}.H_{2}O (see review by Dumas and Knox, 1983).

Presently, study of pollen germination and pollen-tube growth by means of fluorescence microscopy follows either the method described by Currier (1957) or Martin (1959). In the method of Currier (1957), 0.005 or 0.01% aniline blue prepared in 0.15 M K_{2}HPO_{4} (pH approx. 8.2) was used, while in that of Martin (1959), 0.1% aniline blue was prepared in 0.1 M K_{3}P_{4}. I slightly modified both of their methods by preparing the 0.1% aniline blue solution in 0.15 M K_{2}HPO_{4} (pH = 8.2), because a higher intensity of fluorescence was desired. 2 N NaOH was used as a softening and clearing agent instead of 1 N (Kho and Baer, 1968) or 8 N (Martin, 1959) to reduce the consumption of NaOH as well as to obtain proper timing for clearing. The technique applied in the study of pollen-tube
germination and growth in the natural and artificial cross-pollination experiments permitted rapid screening of numerous pistils. This was almost a necessity because of the extremely low frequency of pollen-tube growth in the material under study.

In summary, several reports have addressed the problems of genetic male-sterile ms1 line in soybean. Nevertheless, at least seven male-sterile ms lines are known to show the same phenomenon of failure of cytokinesis after telophase II of meiosis. Five of them already are verified to be at the ms1 locus (Palmer et al., 1978; Yee and Jian, 1983). As previously mentioned, the ms1 mutant also is characterized by the production of polyembryonic seedlings, haploids, and polyploids in their progeny. Since previous reports included only the North Carolina ms line, I wanted to know whether the male-sterility character associated with the occurrence of polyembryony, haploidy, and polyploidy was genotype-specific. Furthermore, using triploids, obtained from screening the progeny of homozygous recessive ms1 ms1 plants, for cytological study could provide some information about the meiotic behavior in triploids. Also, aneuploids for further trisomic study might be obtained among progeny from triploids. Finally, how effective coenocytic pollen grains are in the role of fertilization is another point of our interest. In my dissertation, a separate section addresses
each of these three aspects.

**Explanation of dissertation format**

In this dissertation, I have given a brief review of male-sterility systems in higher plants, sterile mutants in soybean, some rationale of techniques applied, and the objectives in my research as a general introduction. Three sections are included in the dissertation. First, Studies on the frequency of polyembryonic seedlings and polyploids from ms1 soybean. Second, Cytological studies of triploids and their progeny from male-sterile ms1 soybean, and third, Pollen germination and pollen-tube growth in male-fertile and male-sterile ms1 soybean. Research on the first and third problems provide more genetic information on the effect of the ms1 allele on reproductive function. The second is a cytological study of triploids derived from the ms1 system and the frequency of aneuploids derived from progeny of these triploid plants.

Section I, frequency of polyembryonic seedlings and polyploids from four spontaneous and independent ms1 source populations and two derived populations, which were the F4 seed of the crosses of two ms1 lines to two homozygous chromosome interchange lines, were reported. Data obtained in this study included some from previous studies of Dr. Reid G. Palmer and Hollys E. Heer, as well as my own
research. Statistical tests were conducted to detect differences in frequency of polyembryony and polyploidy among different source populations. This paper was published in Theoretical and Applied Genetics in January, 1985. We thank Drs. Arnel R. Hallauer, Oscar S. Smith, and Thomas S. Cox in the Agronomy Department for reviewing this manuscript.

In section II, cytological studies on the meiosis and pollen fertility of male-fertile and male-sterile triploid plants were mainly conducted by myself. Chromosome numbers of triploid progeny were provided by Dr. Reid G. Palmer's earlier work, together with some from my study. We thank Hollys E. Heer for helping with chromosome counts of these triploid progeny. This paper recently was accepted for publication in Theoretical and Applied Genetics. We also thank Dr. Walter R. Fehr in the Agronomy Department, Dr. Henry Hadley, University of Illinois, Urbana, Illinois, Dr. Wayne W. Hanna, USDA ARS, Tifton, Georgia, and Dr. Robert A. Graybosch, Monsanto Agricultural Products Co. for their reviews and suggestions on this manuscript.

In section III, research on pollen germination and pollen-tube growth in male-sterile $ms1$ lines continues the previous observations made by Dr. Marc C. Albertsen, now at Pioneer Hi-bred International, and by Dr. R. G. Palmer. Jack C. Kennell, a former graduate student of Dr. Harry T. Horner
in the department of botany, Iowa State University, now a Ph.D. student in Florida, also observed, by electron microscopy, some coenocytic pollen-grain germination in his study. Therefore, we will write a co-authored paper on this subject for publication. However, the results presented in this section are solely derived by myself under the supervision of Dr. Palmer.

Finally, a summary and discussion of my research and implications of my research are addressed.
SECTION I: STUDIES ON THE FREQUENCY OF POLYEMBRYONIC SEEDLINGS AND POLYPLOIDS FROM ms1 SOYBEAN

ABSTRACT

Seed from homozygous recessive ms1 ms1 genetic male-sterile soybean (Glycine max (L.) Merr.) plants were studied for frequencies of polyembryonic seedlings and different levels of polyploidy among abnormal seedlings from six different source populations: Ames ms1 (Ams), North Carolina ms1 (NCms), Tonica ms1 (Tms), Urbana ms1 (Ums), and F4 generation seed obtained from crosses of ms1 to two chromosome interchange lines (Ams x Clark T/T and Ums x KS-172-11-3). Frequencies of polyembryony observed in Tms, Ums, Ams, NCms, F4 seed from Ams x Clark T/T, and F4 seed from Ums x KS 172-11-3 were 3.6%, 2.4%, 3.1%, 2.5%, 2.2% and 0.1%, respectively. Frequencies of abnormal seedlings from these six sources varied from 1.7% (Ums x KS-172-11-3) to 16.8% (Ams x Clark T/T). Frequencies of polyploids among the abnormal seedlings ranged from 6.8% in Ums x KS-172-11-3 to 66.7% in Tms. On the average, the frequency of polyploid individuals from monoembryonic seedlings was 1.22%. Chromosome number of these seedlings varied from 20 to 200. Variation of the frequencies of polyembryonic seedlings and polyploid progeny among abnormal seedlings suggested that the
mechanism(s) controlling the characters of polyembryony and formation of polyploids was associated with the \textit{ms1} gene and was affected by other gene(s) or environmental factors.
INTRODUCTION

The first report of male-sterile, female-sterile soybean (Glycine max (L.) Merr.) plants was by Owen (1928a). Synaptic male-sterile mutants were reported by Hadley and Starnes (1964), Palmer (1974), and Palmer and Kaul (1983). Partial male-sterility systems are known: Arkansas partial male-sterile (Caviness et al., 1970) and msp (Stelly and Palmer, 1980a, b). Completely male-sterile, female-fertile mutants are msi (Brim and Young, 1971; Palmer et al., 1978; Yee and Jian, 1983), m2 (Graybosch et al., 1984), m3 (Palmer et al., 1980) and m4 (Delannay and Palmer, 1982). A structural male-sterile mutant fs1 fs2 was described by Johns and Palmer (1982). Within the msi and m4 sterile systems, the formation of coenocytic microspores is due to the failure of cytokinesis after telophase II of meiosis. In m4 there is either early degeneration of coenocytic microspores or further division of the microspore (Delannay and Palmer, 1982), whereas in msi most coenocytic microspores do not undergo complete cell division, but differentiate into large grains (Albertsen, 1976; Albertsen and Palmer, 1979). Similar phenomena also were noted in two male-sterile lines by Rubaihayo and Gumisiriza (1978).

In addition to the formation of coenocytic microspores, homozygous recessive msi msi plants also were associated with
the production of polyembryonic seedlings, haploids, and polyploids in their progenies (Kenworthy et al., 1973; Cutter and Bingham, 1977; Beversdorf and Bingham, 1977; Crane et al., 1982). Polyembryony, haploidy, and polyploidy, as explained by Cutter and Bingham (1977), result from abnormal embryo-sac development, producing supernumerary nuclei and restitution of these extra nuclei before fertilization.

On the average, the frequency of polyembryony reported among progeny of homozygous recessive ms1 ms1 plants is 4.0%, 2.3%, and 2.2%, respectively (Kenworthy et al., 1973, Beversdorf and Bingham, 1977; Sorrells and Bingham, 1979). The seed for these three studies, however, all came from North Carolina ms1 (Brim and Young, 1971). There are at least five independent mutations at the ms1 locus in soybean. Four mutants, designated the North Carolina, Urbana, Tonica, and Ames male steriles, arose spontaneously and independently of each other in the United States (Palmer et al., 1978). Another mutation at the ms1 locus is the Shennong male sterile recently reported in China (Yee and Jian, 1983).

Occurrence of twin seedlings from nonmale-sterile soybeans was reported by Owen (1928b) in the study of inheritance of cotyledon colors in a Chinese variety; the frequency of double-embryo seeds was 0.44%. Shorter and Byth (1975) reported relatively high frequencies (6.3% to 13.0%) of twin seedlings in three Australian cultivars. However,
from their limited number of chromosome counts, all twin seedlings examined were diploid-diploid except for one diploid-haploid.

The objective of our study was to examine the relationships among polyembryonic seedlings, polyploids, haploids, and male sterility. To provide more genetic information on the action of the \textit{ms}^1 gene, we report frequencies of polyembryonic seedlings and polyploids from \textit{ms}^1 \textit{ms}^1 male-sterile progeny among different seed-source populations. The samples examined were from four independent source populations and two derived populations.
MATERIALS AND METHODS

Seed harvested from homozygous recessive \textit{ms1} \textit{ms1} plants was obtained from six different source populations: North Carolina \textit{ms1} (NCms) T260, Urbana \textit{ms1} (Ums) T266, Tonica \textit{ms1} (Tms) T267, Ames \textit{ms1} (Ams) T268, and \(F_4\) seeds from the cross of \textit{ms1} to two homozygous chromosome interchange lines (Ams x Clark T/T and Ums x KS-172-11-3). Seed from T260 were obtained from Dr. C. A. Brim, North Carolina State University. Seed from the other populations were grown at Ames, Iowa. The T number refers to the Soybean Genetics Type Collection number.

The origin of the four independent \textit{ms1} mutants was as follows:

\textbf{North Carolina \textit{ms1}} was found in a farmer's field by inspectors from the North Carolina Certified Seed Growers' Association in 1966 (Brim and Young, 1971).

\textbf{Urbana \textit{ms1}} was found in an \(F_3\) row from the cross of 'Clark-2' x SRF 300 at Urbana, Illinois, 1971 (Boerma and Cooper, 1978).

\textbf{Tonica \textit{ms1}} was found in a field of 'Harosoy' by a farmer at Tonica, Illinois in 1955 (Palmer et al., 1978).

\textbf{Ames \textit{ms1}} was found in a row segregating for desynaptic mutant \textit{st4} at Ames, Iowa, in 1970 (Palmer et al., 1978).

The two interchange lines used for crosses with \textit{ms1}
lines were: Clark T/T, a near-isogenic cultivar 'Clark' with an interchange from PI 101404B (a *Glycine soja* accession from Northeastern China) and KS 172-ll-3, an interchange involving the satellite chromosome from an irradiated population of the cultivar 'Hodgson' (Sadanaga and Newhouse, 1982). The derived populations (Ams x Clark T/T and Ums x KS-172-ll-3) have trisomic segregation for white flower (\(w1\)) and \(ms1\) both of Linkage Group 8.

Seed were germinated and chromosome number determined by use of the procedure of Palmer and Heer (1973). Frequencies of polyembryonic and abnormal seedlings and of polyploids among the polyembryonic seedlings, the abnormal seedlings and total number of seed planted from the six source populations were tested for homogeneity by the formula: \(X^2 = \left( \sum p_i a_i - \overline{pA} \right) / \overline{p} \overline{q} \). Differences among populations were tested, on the basis of \( \frac{P_1 - P_2}{\sqrt{\overline{p} \overline{q} (1/n_1 + 1/n_2)}} \) (Snedecor and Cochran, 1980)

Where \( \overline{p} \) = frequency of events over all seed sources;
\( \overline{q} = (1-\overline{p}) \)
\( P_i = \) frequency of events in \( i \) source;
\( a_i = \) number of events in \( i \) source;
\( n_i = \) number of observations in \( i \) source; and
\( A = \) total number of events over all sources.
RESULTS AND DISCUSSION

A total of 159 sets of polyembryonic seedlings (146 twins, 12 triplets, and a quadruplet) was found among 9,153 seed planted from the six sources (Table 1). Chromosome numbers of the twins included diploid-diploid, haploid-diploid, diploid-triploid, diploid-tetraploid, triploid-triploid, triploid-tetraploid, and tetraploid-tetraploid. Figure 1 shows chromosomes of a 2n=20 plant (a haploid) as one member of a twin. From the 12 triplets, only one set showed a triploid-triploid-unknown, while all others manifested 2n=40. The quadruplet had a chromosome number of 100 in each individual seedling. In this study, the majority of the twins were at the diploid chromosome level, which agrees with the findings of Beversdorf and Bingham (1977) and Cutter and Bingham (1977).

Seeds that were slow in germinating usually were either very small or very large and seedlings had visibly abnormal cotyledons and/or roots and were classified as abnormal seedlings. Although Beversdorf and Bingham (1977) found a haploid plant among a population of 800 plants from monoembryonic seed produced by early-maturing male-sterile plants, no haploid plants were found in our study among progeny from abnormal seedlings. In most cases, these abnormal seedlings had 40 chromosomes and we suspect the
Table 1. Total number of polyembryonic seedlings and abnormal seedlings with different ploidy levels from six male-sterile (ms1) seed source populations in soybean

<table>
<thead>
<tr>
<th>Chromosome no.</th>
<th>Polyembryonic seedlings</th>
<th>Abnormal seedlings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of sets observed</td>
<td>No. of plants observed</td>
</tr>
<tr>
<td>40-40</td>
<td>113</td>
<td>20</td>
</tr>
<tr>
<td>20-40</td>
<td>5</td>
<td>40</td>
</tr>
<tr>
<td>40-60</td>
<td>19</td>
<td>60</td>
</tr>
<tr>
<td>40-80</td>
<td>3</td>
<td>80</td>
</tr>
<tr>
<td>60-60</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>60-80</td>
<td>3</td>
<td>120</td>
</tr>
<tr>
<td>80-80</td>
<td>2</td>
<td>140</td>
</tr>
<tr>
<td>40-40-40</td>
<td>11</td>
<td>160</td>
</tr>
<tr>
<td>60-60-?</td>
<td>1</td>
<td>180</td>
</tr>
<tr>
<td>100-100-100-100</td>
<td>1</td>
<td>200</td>
</tr>
</tbody>
</table>

Total 159 Total 376

^Total number of seed planted was 9,153.

Includes 10 trisomic (2n=41) plants segregating from the two chromosome interchange populations.

Chromosome number unconfirmed, but above diploid level.
observed abnormalities were the result of natural variation. However, about 30% of the abnormal seedlings proved to be polyploids (Tables 1 and 3). Chromosome numbers of these \textit{ms1} progenies varied from 2n=40 to 2n=200 (Fig. 2 to Fig. 8). This is the highest ploidy level reported from \textit{ms1} homozygous recessive progenies. In previous studies, Palmer and Heer (1976) reported a 2n=180 plant among the progeny of Tonica male-sterile (\textit{ms1 ms1}) soybean plants.

Cutter and Bingham (1977) suggested that polyembryony and polyploidy in \textit{ms1 ms1} progenies resulted from abnormal embryo sac development, with subsequent supernumerary nuclei and the restitution of these extra nuclei before fertilization. Haploids could arise from egg sacs by parthenogenetic development of one of these nuclei with the gametophytic chromosome number or perhaps androgenetically. Sorrells and Bingham (1979) reported the predominance of diploids and of polyploids in F\textsubscript{1} plants from crosses between \textit{ms1} haploids and diploids. Only 4 of 67 F\textsubscript{1} plants proved to be trisomics (2n=41). They suggested that the restitution gamete production associated with the \textit{ms1} allele carried by the haploid reduced the efficiency of isolating aneuploids. In our studies, the ploidy levels are multiples of 20 instead of 40, i.e., the multiple of the gametic, not the sporophytic, chromosome number. Albertsen and Palmer (1979) reported that male sterility in the various \textit{ms1} mutants was caused by the
Figure 1. Mitotic chromosomes of a haploid (2n=20) seedling obtained from progeny of Ames ms1 source, A75-1187 sterile plant (X 1950)

Figure 2. Mitotic chromosomes of a diploid (2n=40) seedling obtained from Ames ms1 source, A75-1180 sterile plant (X 1100)

Figure 3. Mitotic chromosomes of a triploid (2n=60) seedling from progeny of Tonica ms1 source, P327-1 sterile plant (X 1950)

Figure 4. Mitotic chromosomes of a tetraploid (2n=80) seedling from progeny of Tonica ms1 source, P13 sterile plant (X 1950)

Figure 5. Mitotic chromosomes of a hexaploid (2n=120) seedling from progeny of Tonica ms1 source, P8 sterile plant (X 1950)

Figure 6. Mitotic chromosomes of a pentaploid (2n=100) seedling from progeny of Ames ms1 source, A75-1187 sterile plant (X 1950)

Figure 7. Mitotic chromosomes of an octoploid (2n=160) seedling from progeny of North Carolina ms1 source, A77-336-16 sterile plant (X 1100)

Figure 8. Mitotic chromosomes of a decaploid (2n=200) seedling from progeny of Tonica ms1 source, A75-1189 sterile plant (X 1200)
failure of cytokinesis following telophase II of meiosis. The four nuclei become enclosed in a single-celled structure, followed by either degeneration or the occasional development of extensions resembling pollen tubes. Whether these pollen-like tubes can effect fertilization and/or occurrence of polyploidy is not known. Sterile ms1 ms1 plants occasionally produced seed in the greenhouse where pollinating vectors seemed to be absent (Cutter, 1975; Palmer and Heer, 1976).

The frequency of polyembryonic seedlings was 2 to 4% for five male-sterile source populations (Table 2) which agrees with the results of Kenworthy et al. (1973), Sorrells and Bingham (1979), and Beversdorf and Bingham (1977). The F4 seed from Ums x KS-172-11-3 gave an extremely low frequency (0.1%) of polyembryonic seedlings. Incidence of seedlings with chromosome number other than the diploid level among polyembryonic seedlings was 19.0%, 13.3%, 13.5%, 9.4%, 25.0%, and 0% in Tms, Ums, Ams, NCms, F4 seed of Ams x Clark T/T, and F4 seed of Ums x KS 172-11-3, respectively.

Frequencies of abnormal seedlings among male-sterile progeny were much more uniform among the four original ms1 seed-source populations [Ams (5.4%), Tms (5.1%), NCms (4.4%), and Ums, (3.6%)] than between F4 seed of the two interchange crosses Ames x Clark T/T (16.8%) and Ums x KS 172-11-3 (1.7%) (Table 3).

The percentage of polyploids found among these abnormal
Table 2. Frequency of polyembryonic seedlings and number of diploid seedlings vs. non-diploid seedlings in polyembryonic seedlings from six male sterile (msl) seed source populations in soybean

<table>
<thead>
<tr>
<th>Seed source</th>
<th>No. of seed planted</th>
<th>Polyembryony sets</th>
<th>Number of polyembryonic individuals</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No.</td>
<td>%</td>
<td>Total</td>
<td>Diploid</td>
<td>Non diploid</td>
</tr>
<tr>
<td>Tms</td>
<td>1051</td>
<td>38</td>
<td>3.6ac³</td>
<td>79</td>
<td>64</td>
<td>15</td>
</tr>
<tr>
<td>Ums</td>
<td>905</td>
<td>22</td>
<td>2.4b</td>
<td>45</td>
<td>39</td>
<td>6</td>
</tr>
<tr>
<td>Ams</td>
<td>784</td>
<td>24</td>
<td>3.1ab</td>
<td>52</td>
<td>45</td>
<td>7</td>
</tr>
<tr>
<td>NCms</td>
<td>2474</td>
<td>61</td>
<td>2.5b</td>
<td>127</td>
<td>115</td>
<td>12</td>
</tr>
<tr>
<td>Ams X Clark T/T</td>
<td>464</td>
<td>10</td>
<td>2.2bc</td>
<td>20</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>Ums X KS-172-11-3</td>
<td>3475</td>
<td>4</td>
<td>0.1d</td>
<td>8</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>9153</td>
<td>159</td>
<td>1.7</td>
<td>331</td>
<td>286</td>
<td>45</td>
</tr>
</tbody>
</table>

¹Chi-square (df=5) for homogeneity = 98.01, P < 0.01.
²Chi-square (df=5) for homogeneity = 6.43, P = 0.50-0.25.
³Frequencies followed by the same letter are not significantly different from one another at the 5% level.
Table 3. Frequency of abnormal seedlings and number of diploids and polyploids among the abnormal seedlings from six male-sterile (ms1) seed source populations in soybean

<table>
<thead>
<tr>
<th>Seed source</th>
<th>No. of seed planted</th>
<th>Abnormal seedlings</th>
<th>Number of polyploids</th>
<th>% polyploid among no. of seed planted^2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tms</td>
<td>1051</td>
<td>54</td>
<td>5.1b^3</td>
<td>36</td>
</tr>
<tr>
<td>Ums</td>
<td>905</td>
<td>33</td>
<td>3.6b</td>
<td>10</td>
</tr>
<tr>
<td>Ams</td>
<td>784</td>
<td>42</td>
<td>5.4b</td>
<td>10</td>
</tr>
<tr>
<td>NCms</td>
<td>2474</td>
<td>110</td>
<td>4.4b</td>
<td>16</td>
</tr>
<tr>
<td>Ams X Clark T/T</td>
<td>464</td>
<td>78</td>
<td>16.8a</td>
<td>36</td>
</tr>
<tr>
<td>Ums X KS-172-11-3</td>
<td>3475</td>
<td>59</td>
<td>1.7c</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>9153</td>
<td>376</td>
<td>4.1</td>
<td>112</td>
</tr>
</tbody>
</table>

^1Chi-square (df=5) for homogeneity =249.66; P < 0.01.

^2Chi-square (df=5) for homogeneity of frequency of polyploids among abnormal seedlings and among number of seed planted are 72.9 and 248.26, respectively; P < 0.01.

^3Frequencies followed by the same letter are not significantly different from one another at the 5% level.
36

_progenies varied with seed source (Table 3). Tonica_ms1_ had the highest percentage (66.7%) of polyploidy among abnormal seedlings, and F4 seed of Ums x KS 172-11-3 had the lowest (6.8%). However, when the frequency of polyploidy among the total number of seed planted is considered, Ams x Clark T/T had the highest frequency (7.76%) and the F4 seed for Ums x KS 172-11-3 had the lowest frequency (0.12%). This is a more conservative estimation of polyploidy because polyploids also were observed among normal-appearing seedlings but records were not made.

From our unpublished data, progenies of Ams x Clark T/T and Ums x KS 172-11-3 had trisomic inheritance for w1 (white flower) and_ms1_. In this study, three trisomic plants were found in abnormal seedlings from F4 seed of Ams x Clark T/T and seven in Ums x KS 172-11-3.

Statistical tests on homogeneity are presented in Tables 2 and 3. Only the frequency of nondiploid ploidy levels among the polyembryonic progenies was homogeneous. The other parameters showed a heterogeneity among the six source populations. Thus, differences between each two source populations for the frequency of polyembryonic seedlings, abnormal seedlings, and polyploidy among the abnormal seedlings and among total number of seed planted were compared. Results indicated that F4 seed of crosses of Ums x KS 172-11-3 had a significantly lower frequency than the
other five seed sources in the occurrence of abnormal seedlings and polyploid progeny. Progeny of Tonica \textit{ms1} differ in frequency of polyembryonic seedlings from Ums, NCms, and Ums x KS-172-11-3 (Table 2) and from all five other sources in frequency of polyploids from their abnormal seedlings (Table 3). No significant differences in frequency of polyploidy among total number of seed planted were found among the Ums, Ams, and NCms (Table 3). However, when each of the above three sources was compared with the other three populations respectively, the difference is statistically significant (Table 3).

Genetic control of the occurrence of polyembryonic seedlings in soybean is not clearly understood. Owen (1928b) found a frequency of 0.44% of twins in 5000 seed of a Chinese cultivar; he found no twins in similar examination of a number of other cultivars. The same tendency toward twinning also was found among F$_2$ seed and F$_4$ seed from crosses between the Chinese cultivar and other cultivars. Shorter and Byth (1975) reported that frequencies of twin seedlings from three Australian cultivars were 13.0%, 12.0%, and 6.3%. They observed twin seedlings among progenies of both normal plants and twin plants, but the frequency varied when seed were produced in different environments. Only diploid-diploid twins and one haploid-diploid twin were found.
Beversdorf and Bingham (1977) also detected a lower frequency (0.7% in 800 seed planted) of polyembryony from maturity group I in male-sterile (ms1) progeny than from later maturity groups. Boerma and Cooper (1978) reported that sterile plants from Ums tended to have a higher seed set and greater frequency of 2- and 3-seeded pods than did NCms sterile plants, which had predominantly 1-seeded pods. This suggested that Ums, on the basis of its female fertility, was phenotypically distinct from NCms, and they suggested either that Ums is a different allele from NCms or that Ums has closely linked modifier gene(s) that affect female fertility. Therefore, it is not known whether the genetic control of polyembryonic seedlings from non-male-sterile sources was the same as that of male-sterile sources.

In this study, measurement of differences in the frequency of polyembryonic seedlings and polyploids among different ms1 seed source populations provided some evidence on the effect of different genetic background on the action of the ms1 gene. Use of two derived populations (crosses of two ms1 lines with two chromosome interchange lines) enabled us to see if any linked factors involved with ms1, resulting from the interchange, could have an effect on the occurrence of polyembryony and polyploidy.

This study showed that variation occurred in frequencies of polyploidy and polyembryony when different
source populations were used (Tables 2 and 3). Although the difference in frequency of polyembryony between $F_4$ seed of Ams x Clark T/T and the original Ams populations was not significant, a substantial decline in frequency was observed when comparing the $F_4$ seed of Ums x KS-172-11-3 to the Ums populations (Table 2). In comparing the two original $ms1$ populations with the $F_4$-seed-derived $ms1$ populations, the occurrence of polyploids is either increased ($F_4$ seed of Ams x Clark T/T vs. Ams) or decreased ($F_4$ seed of Ums x Ks-172-11-3 vs. Ums) (Table 3). Therefore, the change of frequency of polyembryony and(or) polyploidy associated with the male-sterility ($ms1$) character could be either intensified or reduced when different genetic background materials are introduced. This could be explained by a linkage of $ms1$ with modifying genes.

The occurrence of polyembryony and polyploidy from all six $ms1$ populations in this study confirmed the pleiotropic effects of the $ms1$ gene on the frequency of polyembryony and polyploidy. As previously mentioned, frequency of polyembryonic seedlings was reported to vary when seed was produced in different environments (Shorter and Byth, 1975). The NCms population was grown in North Carolina while the other five populations were grown in Iowa. Therefore, the variation in frequency of polyembryony and polyploidy
displayed by the difference sources of the ms1 gene suggests that the action of the ms1 gene on polyembryony and polyploidy might be modified by some other gene(s) when different genetic backgrounds were used, and might be affected by environmental conditions where the plants were grown.
ACKNOWLEDGMENT

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REFERENCES


SECTION II: CYTOLOGICAL STUDIES OF TRIPLOIDS AND THEIR PROGENY FROM MALE-STERILE ms1 SOYBEAN

ABSTRACT

Triploids (2n=3X=60) were obtained from genetic male-sterile (ms1 ms1) soybean [Glycine max (L.) Merr.] plants. Meiosis, pollen fertility, and chromosome number of their progeny were studied. Studies of meiosis in fertile and sterile triploids revealed no distinguishable differences in chromosome associations. Male-sterile plants formed coenocytic microspores characteristic of the ms1 mutant. Restitution of some dyad and tetrad nuclei were observed in male-sterile plants. Chromosomes of the triploids tended to occur in trivalents during diakinesis and metaphase I (MI), but multivalents, bivalents, and univalents also were observed. Average types and frequencies of chromosome associations per cell in diakinesis and MI from 542 pollen mother cells were 0.004 IX + 0.06 VI + 0.002 V + 0.005 IV + 16.99 III + 1.79 II + 5.03 I. Some secondary associations, nonhomologous pairing, and aberrant nucleolar distributions occasionally were observed. Such behavior support the hypothesis of duplicated genomes and the polyploid origin of soybean. Pollen fertility in male-fertile triploid plants (Ms1 ms1 ms1) varied from 57% to 82%, with an average of
about 71%. Chromosome numbers of progenies obtained from these fertile triploids varied from 2n=40 to 2n=71, and exhibited a near-random distribution, with the majority (about 60%) being between 56 and 65. Progenies of the fertile triploids gave segregation ratios for the ms1 alleles, which confirmed the Ms1 ms1 ms1 genotype.
INTRODUCTION

Triploids are one of the primary sources of aneuploids in most plant species (Khush, 1973). In soybean [Glycine max (L.) Merr.], previous attempts to produce triploids (2n=3X=60) through artificial cross-pollination and natural cross-pollination between autotetraploids and diploids were unsuccessful (Porter and Weiss, 1948; Sadanaga and Grindeland, 1981). Sadanaga and Grindeland (1981) noted that the failure to obtain triploids from crosses between diploids and induced tetraploids could be explained either by the hypothesis that endosperm development depends on a 2:1 ratio of female to male genomes (Nishiyama and Inomata, 1966), or by the endosperm balance number (EBN) hypothesis (Johnston et al., 1980). Under either hypothesis, crosses between diploid and induced autotetraploid soybean fail to produce triploids because the maternal: paternal genome or EBN ratio is 4:1 when the autotetraploid is the female parent and 1:1 when the diploid is the female parent.

Haploids and polyploids (including triploids) can be obtained spontaneously by screening progeny of homozygous recessive male-sterile (ms1 ms1) soybean (Kenworthy et al., 1973; Cutter and Bingham, 1977; Beversdorf and Bingham, 1977; Chen et al., 1985).

Homozygous recessive ms1 ms1 soybean plants are
characterized by formation of coenocytic microspores, which result from failure of cytokinesis after telophase II of meiosis (Albertsen and Palmer, 1979). Observations of abnormal embryo-sac development and multiple nuclei in megagametophytes (Cutter and Bingham, 1977; Kennell, 1984) provided some clue to the origin of these polyploids. Kennell (1984), in a study of megasporogenesis and megagametogenesis of the ms1 lines with light and electron microscopy, indicated that partial or complete failure of cytokinesis at meiosis resulted in four-nucleate functional megaspores. These four nuclei may lead to mature megagametophytes with four times the normal number of nuclei. Kennell (1984) pointed out that degeneration and/or nuclear fusion of developing nuclei result in mature gametophytes varying in nuclear number from 8 to 32. Thus, spontaneous triploids occurring among progeny of ms1 ms1 plants could be the fusion product of a 2n egg fertilized by an n sperm.

Our objectives were to study the meiotic chromosome associations in triploid soybeans occurring among progeny of male-sterile ms1 ms1 plants and to determine chromosome numbers of progenies of these triploids.
MATERIALS AND METHODS

Triploid soybeans obtained from a previous study (Chen et al., 1985) were used. Chromosome numbers were determined from root tips of polyembryonic and abnormal seedlings in progeny of homozygous recessive male-sterile \( ms1 \ ms1 \) plants (Palmer and Heer, 1973). Triploid plants of these polyembryonic and abnormal seedlings were grown in the greenhouse.

Coenocytic microspores from male-sterile plants generally are large and darkly stained by \( I_2KI \). Pollen grains from fertile plants stain a dark golden brown with \( I_2KI \). At the time of flowering, fresh open flower buds were collected from each triploid plant, fixed in 70% ethanol, and classified as male sterile or male fertile on the basis of pollen stainability. At least two flower buds were collected per plant, and at least 400 coenocytic microspores or pollen grains were classified per sample. Pollen diameters were measured on 100 grains per sample for 10 male-fertile triploid plants, 10 male-sterile triploid plants, two male-fertile diploid plants, and two male-sterile diploid plants.

For meiotic studies, young flower buds from 12 male-fertile triploid plants and eight male-sterile triploid plants were fixed in 6:3:2 ethanol:chloroform:propionic acid, and placed under a vacuum to enhance penetration of the
fixative. Samples were fixed for 48 hours and then stored in 70% ethanol at 4°C. For slide preparation, flower buds were dissected, smeared with a drop of 45% acetic acid or propionic acid, and stained with a drop of either aceto-carmine or propio-carmine.

Seeds obtained from fertile triploids were germinated and chromosome numbers were determined from root tips following the method of Palmer and Heer (1973). These seedlings also were transplanted and maintained in the greenhouse and classified as male fertile or male sterile on the basis of stainability and morphology of pollen grains and coenocytic microspores.
RESULTS

Of the 68 triploids obtained, 32 were classified as male fertile and 36 were male sterile, which fit the expected 1:1 segregation ratio ($X^2=0.24; P=0.75-0.50$), because fertile plants are maintained as heterozygotes (Ms1 ms1) and seed are harvested only from the male-sterile plants. Diploid progeny of sterile ms1 ms1 plants should be either Ms1 ms1 or ms1 ms1. Seed obtained from these sterile plants probably are the result of fertilization of an ms1 ovule by an Ms1 or ms1 gamete, from the Ms1 ms1 fertile sib plants. Thus, the genotype of male-fertile triploids is Ms1 ms1 ms1, and that of male-sterile triploids is ms1 ms1 ms1. These plants are the result of fertilization by an Ms1 or ms1 male gamete with an ms1 ms1 ovule.

Meiotic studies

In general, meiotic observation of pollen mother cells (PMC) among male-fertile and male-sterile triploids revealed no distinguishable differences, except for the formation of coenocytic microspores after telophase II of meiosis, which characterized the ms1 ms1 ms1 genotype, and restitution of some dyads and tetrads in the male-sterile plants.

Study of the pachytene stage is difficult because of the large number of chromosomes involved and the overlapping of most chromosomes throughout their entire length. However, in
the limited number of cells observed, some chromosomes were loosely paired and association of homologues was not always complete. Frequently, segments of chromosome strands were unpaired, or interpaired with a third chromosome. This allowed for formation of trivalents.

Diakinesis through metaphase I (MI) provided cells with more clear figures for interpretation of chromosome associations (Fig. 1 to Fig. 6). Chromosomes of the triploids tended to occur as trivalents from diakinesis to MI. From a total of 542 cells observed, 28% exhibited 20 trivalents and 20% had 19 trivalents (Table 1; Figs. 1 and 2). About 4% of the cells showed no chromosome pairing. Multivalents other than trivalents were observed; they might be the result of nonhomologous pairing or of close secondary association (Figs. 3 and 4). As indicated by superscript a in Table 1, 10% of the cells observed in male-fertile triploids involved nonhomologous pairing or secondary association, compared with 5% in male-sterile plants. The difference may be due to the number of cells observed. The average types and frequencies of chromosome associations per cell in diakinesis or MI were 0.004 IX + 0.06 VI + 0.002 V + 0.005 IV + 16.99 III + 1.79 II + 5.03 I.

Anaphase I chromosome distribution showed that gametic chromosome numbers between 35 and 26 were most frequent.
Figures 1-6. Meiotic diakinesis or metaphase I cells from triploid soybean plants.

(1) 20 trivalents (III). x1930.

(2) 19 III + II (arrow) + I (arrow) x2130.

(3) 17 III + II + 3 I and 1 possible VI or close secondary association (arrow) x2480.

(4) 13 III (including 2 III overlapped) + 1 VI (arrow) + 1 V (arrow) + 3 II + 4 I. x2310

(5) Two associations of 3 III (long arrows) and three associations of 2 III. (short arrows). x1920.

(6) one association of 3 III (long arrow) and six associations of 2 III (short arrows) x2390.
Table 1. Frequencies and types of chromosome associations in pollen mother cells of triploid soybean at diakinesis or metaphase I of meiosis

<table>
<thead>
<tr>
<th>Type of chromosome associations</th>
<th>Number of cells observed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male-fertile</td>
</tr>
<tr>
<td>20 III</td>
<td>120</td>
</tr>
<tr>
<td>19 III + II + I</td>
<td>60</td>
</tr>
<tr>
<td>19 III + 3 I</td>
<td>5</td>
</tr>
<tr>
<td>18 III + VI^</td>
<td>5</td>
</tr>
<tr>
<td>18 III + 3 II</td>
<td>5</td>
</tr>
<tr>
<td>18 III + 2 II + 2 I</td>
<td>38</td>
</tr>
<tr>
<td>18 III + II + 4 I</td>
<td>3</td>
</tr>
<tr>
<td>17 III + VI + II + I^</td>
<td>4</td>
</tr>
<tr>
<td>17 III + IV + II + 3 I^</td>
<td>1</td>
</tr>
<tr>
<td>17 III + 3 II + 3 I</td>
<td>25</td>
</tr>
<tr>
<td>17 III + 2 II + 5 I</td>
<td>1</td>
</tr>
<tr>
<td>17 III + II + 7 I</td>
<td>1</td>
</tr>
<tr>
<td>16 III + 2 VI^</td>
<td>2</td>
</tr>
<tr>
<td>16 III + VI + 2 II + 2 I^</td>
<td>6</td>
</tr>
<tr>
<td>16 III + 2 IV + 2 II^</td>
<td>1</td>
</tr>
<tr>
<td>16 III + 6 II^</td>
<td>1</td>
</tr>
<tr>
<td>16 III + 4 II + 4 I</td>
<td>18</td>
</tr>
<tr>
<td>16 III + 3 II + 6 I</td>
<td>3</td>
</tr>
<tr>
<td>16 III + 2 II + 8 I</td>
<td>1</td>
</tr>
</tbody>
</table>

^Associations involved either nonhomologous pairing or secondary chromosome associations.
Table 1. (continued)

<table>
<thead>
<tr>
<th>Types of chromosome associations</th>
<th>Number of cells observed</th>
<th>Male-fertile</th>
<th>Male-sterile</th>
<th>Total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 III + VI + 3 II + 3 I\textsuperscript{a}</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 III + 6 II + 3 I\textsuperscript{a}</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>25</td>
<td>4.6</td>
</tr>
<tr>
<td>15 III + 5 II + 5 I</td>
<td>15</td>
<td>5</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 III + 2 II + 11 I</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 III + 3 VI\textsuperscript{a}</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 III + VI + 2 IV + 2 II\textsuperscript{a}</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 III + VI + 6 II\textsuperscript{a}</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>22</td>
<td>4.1</td>
</tr>
<tr>
<td>14 III + 7 II + 4 I\textsuperscript{a}</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 III + 6 II + 6 I</td>
<td>10</td>
<td>4</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 III + 5 II + 8 I</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 III + 4 II + 10 I</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13 III + VI + V + 3 II + 4 I\textsuperscript{a}</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>6</td>
<td>1.1</td>
</tr>
<tr>
<td>13 III + VI + 6 II + 3 I\textsuperscript{a}</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13 III + 9 II + 3 I\textsuperscript{a}</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>13 III + 7 II + 7 I</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 III + 10 II + 4 I\textsuperscript{a}</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 III + 9 II + 6 I\textsuperscript{a}</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 III + 8 II + 8 I</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>7</td>
<td>1.3</td>
</tr>
<tr>
<td>12 III + 6 II + 12 I</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12 III + 3 II + 18 I</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 1. (continued)

<table>
<thead>
<tr>
<th>Type of chromosome associations</th>
<th>Male-fertile</th>
<th>Male-sterile</th>
<th>Total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>11 III + 2 IX + VI + 3 I&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>11 III + 10 II + 7 I&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>10 III to 3 III&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4</td>
<td>2</td>
<td>6</td>
<td>1.1</td>
</tr>
<tr>
<td>8 II to 1 II&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7</td>
<td>0</td>
<td>7</td>
<td>1.3</td>
</tr>
<tr>
<td>60 I</td>
<td>19</td>
<td>3</td>
<td>22</td>
<td>4.0</td>
</tr>
<tr>
<td>Total no. of cells observed</td>
<td>373</td>
<td>169</td>
<td>542</td>
<td></td>
</tr>
<tr>
<td>Average number of III's</td>
<td>16.7</td>
<td>17.6</td>
<td>17.0</td>
<td></td>
</tr>
<tr>
<td>Number of cells with non-</td>
<td>37</td>
<td>9</td>
<td>46</td>
<td></td>
</tr>
<tr>
<td>homologous pairing or</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>secondary association</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent %</td>
<td>9.9</td>
<td>5.3</td>
<td>8.5</td>
<td></td>
</tr>
</tbody>
</table>

<sup>b</sup>Combined data from 10 III to 3 III plus some II's and I's.

<sup>c</sup>Combined data from no III to 1 II plus some I's.
Chromosome laggards were found in both male-fertile and male-sterile plants. About 65% of the anaphase I cells manifested laggards, the number varying from 1 to 6. However, only one to two laggards were observed most frequently. In prophase II of male-sterile plants, some dyads seemingly tended toward fusion or restitution. This phenomenon was not found in male-fertile plants. About 66% of the cells observed in prophase II, metaphase II, and anaphase II exhibited lagging chromosomes. Laggards were observed infrequently after telophase II, possibly because they formed micronuclei. Coenocytic microspores formed in the male-sterile plants, and a tendency toward restitution of the four daughter nuclei was observed in some cells. In tetrads, occurrence of more than two nucleoli in microspores and two nucleoli in more than two microspores of a quartet were observed frequently in both male-sterile and male-fertile triploid plants. This is not unexpected because diploid meiocytes with more than one nucleolus in member of a quartet were observed occasionally in our study.

Secondary associations

Groups of trivalents, which are similar in size and in configuration and tend to lie in close approximation in MI, are considered as secondary associations (Figs. 5 and 6). Studies on secondary chromosome associations in soybean are
difficult because of the lack of any chromosome morphology marker, the similarity of the small chromosomes, and the obstruction by other chromosome pairs and univalents. Therefore, records were made only from 40 clear MI figures (Table 2).

Types of secondary chromosome associations include association of three trivalents and two trivalents (Figs. 5 and 6). The number of associations of three varied from 0 to 2 per cell, while the number of associations of two varied from 0 to 6 per cell. Most of the cells observed exhibited no association of three, but had some degree of associations of two trivalents. Furthermore, the apparent nonhomologous associations found in some cells might actually have been close secondary associations.

Pollen fertility

Pollen and coenocytic microspores can be distinguished easily by I₂KI stainability as well as diameters from both diploids and triploids (Figs. 7-10). Average pollen diameters from diploid male-fertile and male-sterile plants were 19.2+0.1μm and 33.1+0.4μm, respectively. Pollen grain diameter from male-fertile triploid plants ranged from 19.0 to 33.3μm, with an average of 23.8±2.8μm. Coenocytic microspore diameters from the male-sterile triploids varied from 28.6 to 47.6μm with an average of 38.1±3.8μm.
Table 2. Frequency of possible secondary chromosome associations in metaphase I of meiosis in triploid soybean plants

<table>
<thead>
<tr>
<th>Type of association</th>
<th>Number of cells observed</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>2(3) + 5(2) + 4(1)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2(3) + 4(2) + 6(1)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>2(3) + 3(2) + 8(1)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2(3) + 2(2) + 10(1)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>2(3) + 1(2) + 12(1)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>8</strong></td>
<td><strong>20.0</strong></td>
</tr>
<tr>
<td>1(3) + 6(2) + 5(1)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>1(3) + 5(2) + 7(1)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>1(3) + 4(2) + 9(1)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>1(3) + 3(2) + 11(1)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>1(3) + 2(2) + 13(1)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>1(3) + 1(2) + 15(1)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>9</strong></td>
<td><strong>22.5</strong></td>
</tr>
<tr>
<td>0(3) + 6(2) + 8(1)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>0(3) + 5(2) + 10(1)</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>0(3) + 4(2) + 12(1)</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>0(3) + 3(2) + 14(1)</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>0(3) + 2(2) + 16(1)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>0(3) + 1(2) + 18(1)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20</strong></td>
<td><strong>50.0</strong></td>
</tr>
<tr>
<td>0(3) + 0(2) + 20(1)</td>
<td>3</td>
<td><strong>7.5</strong></td>
</tr>
<tr>
<td><strong>Grand Total</strong></td>
<td><strong>40</strong></td>
<td></td>
</tr>
</tbody>
</table>

*Number in parentheses indicates number of chromosome trivalents associated; e.g., 2(3) + 5(2) + 4(1) indicates two groups of associations of three trivalents (18 chromosomes) plus five groups of associations of two trivalents (30 chromosomes) plus four individual trivalents (12 chromosomes).
Figure 7. Pollen from a male-fertile diploid plant. x825.

Figure 8. Coenocytic microspores from a male-sterile diploid plant. x825.

Figure 9. Pollen from a male-fertile triploid plant. x825.

Figure 10. Coenocytic microspores from a male-sterile triploid plant. x825.
In triploid male-fertile plants, four classes of pollen grains were noted: plump and fully stained; plasmolyzed and partially stained; darkly stained; and empty or aborted (unstained) (Fig. 9). The plump pollen grains represent the viable pollen grains. The plasmolyzed pollen grains suggest degeneration or deficiency. Pollen fertility in these male-fertile triploid plants varied from 57% to 82%, with an average of about 71%. A small portion (5%) of darkly stained pollen, similar to msl pollen, also was observed. The percentage of darkly stained pollen grains in male-fertile plants varied from 0.8 to 9.3. This might be due to the incomplete penetrance of the Ms1 allele, genotypic difference, or to dosage effect of ms1. Coenocytic microspores of male-sterile triploid plants were either darkly stained, plasmolyzed with partially dark stain, or aborted (Fig. 10). The frequencies of these types were 48%, 17%, and 35%, respectively.

**Chromosome number**

Number of seeds obtained from 32 fertile triploid plants varied from 1 to 13, with an average of 4.4 per plant. Seeds from these male-fertile triploids were germinated and root tip chromosome number determined. A total of 140 viable progeny was obtained. Ninety-nine of these 140 progeny were grown to maturity. Among these 99 plants, 23 were male
sterile and 76 were male fertile. A chi-square test for a 3:1 segregation ratio was nonsignificant at the 5% level ($X^2=0.16$; $P=0.50-0.75$), indicating that the male-fertile triploid parents were $Ms1\, ms1\, ms1$.

Chromosome numbers of the 140 progeny of these fertile triploid plants varied from 40 to 71, with a modal value around 60 (Figs. 11 and 12; Table 3). Only four aneuploid plants set seed and their progenies segregated for different chromosome numbers (Fig. 13; Table 3).
Figures 11-13. Mitotic chromosome number of some aneuploids obtained from triploids and their progenies.

(11) 50-chromosome plant from a triploid progeny. 
     x2420.

(12) 64-chromosome plant from a triploid progeny.
     x2110.

(13) 43-chromosome plant obtained from a 45-chromosome plant of a triploid progeny.
     x1900.
Table 3. Frequency and chromosome numbers from progenies of triploid soybean plants

<table>
<thead>
<tr>
<th>Chromosome number</th>
<th>No. of plants</th>
<th>%</th>
<th>Chromosome number</th>
<th>No. of plants</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>2</td>
<td>1.4</td>
<td>58</td>
<td>10</td>
<td>7.1</td>
</tr>
<tr>
<td>44</td>
<td>1</td>
<td>0.7</td>
<td>60</td>
<td>26</td>
<td>18.6</td>
</tr>
<tr>
<td>45&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4</td>
<td>2.9</td>
<td>61</td>
<td>5</td>
<td>3.6</td>
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<tr>
<td>47</td>
<td>1</td>
<td>0.7</td>
<td>62</td>
<td>14</td>
<td>10.0</td>
</tr>
<tr>
<td>49</td>
<td>2</td>
<td>1.4</td>
<td>63</td>
<td>6</td>
<td>4.3</td>
</tr>
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<td>52</td>
<td>5</td>
<td>3.6</td>
<td>65</td>
<td>1</td>
<td>0.7</td>
</tr>
<tr>
<td>53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5</td>
<td>3.6</td>
<td>66</td>
<td>10</td>
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<tr>
<td>55</td>
<td>5</td>
<td>3.6</td>
<td>68&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>4.3</td>
</tr>
<tr>
<td>56</td>
<td>5</td>
<td>3.6</td>
<td>70</td>
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<td>0.7</td>
</tr>
<tr>
<td>57</td>
<td>6</td>
<td>4.3</td>
<td>71&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1</td>
<td>0.7</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>140</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>One 45-chromosome plant set 23 seed of which 15 germinated; three progeny had 41 chromosomes; six had 42 chromosomes; four had 43 chromosomes; and two had 44 chromosomes.

<sup>b</sup>One 53-chromosome plant gave one progeny with 50 chromosomes.

<sup>c</sup>One 68-chromosome plant gave one progeny with 73 chromosomes.

<sup>d</sup>The 71-chromosome plant gave four progeny, two with 72 chromosomes, and two with 74 chromosomes.
DISCUSSION

Meiotic studies; secondary associations

Meiotic studies of male-fertile and male-sterile triploids revealed no distinguishable differences in chromosome associations at diakinesis or metaphase I. The numbers of trivalents observed varied from 0 to 20, with an average of near 17 (Table 1). Two enneavalents, associations of three trivalents, were observed in one cell. Several hexavalents, pentavalents, and quadrivalents also were noted in some cells at diakinesis or metaphase I. The occurrence of multivalents other than trivalents and the sum of groups of trivalents and bivalents not equivalent to the basic number 20 might be due to either 1) occurrence of nonhomologous association by chance, 2) close secondary association, 3) partial homology among the chromosomes associated, or 4) artifact of chromosome stickiness.

In our studies, about 9% of the pollen mother cells in diakinesis or metaphase I exhibited either multivalents or groups of trivalents + bivalents in excess of the basic number 20. This indicates that pairings could occur between nonhomologous chromosomes (Table 1, Figs. 3 and 4). Crane et al. (1982) described the occurrence of bivalents and secondary associations in haploid soybean (2n=20) derived from the genetic male-sterile (ms1 ms1) lines. They observed
from 0 to 5 bivalents per cell at diakinesis or metaphase I. Our observation of from 0 to 3 hexavalents in triploids seems to be consistent with their finding of bivalents in haploid soybean. Cultivated soybean (Glycine max), which behaves cytogenetically and genetically as a diploid, has been suggested to be a tetraploid (Bernard and Weiss, 1973; Hadley and Hymowitz, 1973; Palmer, 1976; Bingham et al., 1976; Crane et al., 1982; Lee and Verma, 1984). However, no direct evidence has been provided. Recently, Jackson and Casey (1982) developed models to analyze meiotic configuration for classification of types of polyploidy. Nevertheless, we found it difficult to determine the exact chiasma frequency from our materials for the use of their model.

We believe that, if soybean is of polyploid origin or has a duplicated genome, the occasional occurrence of multivalents might be possible. Another indication is the occurrence of secondary chromosome associations. Secondary associations at meiosis have been interpreted as indicating distant relationships between different sets of chromosomes in allopolyploids (Darlington, 1931; Lawrance, 1931). This assumption has been proved to be accurate in wheat (Triticum aestivum [2n=42]) (Riley, 1960; Kempanna and Riley, 1964). Stebbins (1950) stated that, in most cases, secondary associations have been considered to be an indication of the polyploid nature of a species or genus; however, it might
also be due to modification by segmental interchange, duplication of chromosomal segments, or other phenomena not at all related to polyploidy. In soybean, secondary association of three and two chromosomes were reported by Crane et al. (1982). In our studies, the maximum number of associations of three and two trivalents are two and six, respectively (Table 2). This also might correspond to the nonhomologous pairing observed in diakinesis or metaphase I.

Our finding of multivalents and secondary chromosome association in this study supports the allopolyploid or segmental allopolyploid origin of soybean. Complete verification of this hypothesis would be facilitated if certain genetic systems regulating specific pairing, such as the Ph locus in wheat (Sears and Okamoto, 1958), were available in soybean.

Sorrells and Bingham (1979) pointed out that some Ms1 ms1 plants produced restitution gametes in various stages of cytokinesis, although in the majority of plants microspore development seemed normal. In our study, most microspores in fertile triploids (Ms1 ms1 ms1) looked normal, whereas in sterile plants some restitution coenocytic microspores did occur. Thus, we believe that if restitution gametes did occur in fertile heterozygous Ms1 ms1 ms1 plants the probability might be too low to be recognized in this study,
or it might be affected by some other factors such as different genetic background or environmental conditions.

Pollen fertility

On the average, 71% of the pollen in male-fertile triploids is normally well-stained; however, seed set was generally low in these triploids. Lin and Lee (1979), studying triploids in Rhoeo, observed an average of 45% normally stained pollen by using cotton blue stain; found low seed set in these triploids. They suggested that the low fertility was due either to the fact that the cotton blue stain was probably not specific enough to differentiate between viable and nonviable pollen, or to the failure of zygote formation or development. Although I₂KI stainability did provide good differential between the msi and non-msi pollen, I₂KI stainability may not be a good indicator of fertility.

Schulz-Schaeffer (1980) pointed out that most of the gametes produced by autotriploid individuals do not have balanced chromosome complements and are not viable. Brink and Cooper (1947) noted that success of the embryo depends on the normal development of the endosperm in almost all species. Therefore, the low fertility in these triploids in our study could be attributed to 1) imbalance of gamete chromosome number, or 2) failure of zygotic development due
to chromosome or genomic imbalance, or 3) failure of endosperm development.

**Chromosome number**

Both random and preferential chromosome segregations in meiosis were reported in triploid organisms (see Schulz-Schaeffer, 1980). As shown in Table 3, the pattern of distribution in chromosome number in our selfed progeny of triploids seems very close to a random distribution with a majority (about 60%) falling between 56 and 65. The lack of chromosome numbers between 72 and 80 may be explained by 1) the chance of obtaining gametes with high hypodiploid or diploid chromosome number being lower than that of obtaining gametes with intermediate chromosome numbers, and 2) the competitive disadvantage of gametes with higher hypodiploid chromosome number being greater than that of hypermonoploid gametes.

Triploid progeny manifested 3:1 segregation for male fertility versus male sterility. This might be the result of random segregation of the chromosome involved in the male-sterile phenotype. Thus, it provides evidence to verify the genotype of the triploid Ms1 ms1 ms1 plants that result from the fertilization of an ms1 ms1 ovule by Ms1 pollen. This is further supported by the finding of potential fusion of nuclei in female embryo sacs of ms1 ms1 plants (Kennell,
1984), providing some evidence for the formation of
polyploids found in the homozygous recessive \textit{ms1 ms1} progeny.

Most of the aneuploids produced from self progeny of
these triploids had chromosome number higher than the diploid
level, precluding their use in genetic studies. However,
among the self progeny of a 45-chromosome plant were three
plants with 41 chromosomes, six with 42, four with 43, and
two with 44 chromosomes. These aneuploids could be used for
establishing trisomic lines and in genetic studies. The
possible random distribution of chromosome segregation
observed in this study suggests that crosses of fertile
diploids with these triploids might produce trisomic plants.
ACKNOWLEDGEMENT

This work was supported in part by grant 59-2191-1-1-760-0 from the USDA Genetic Mechanisms for Crop Improvement Program.
REFERENCES


SECTION III: POLLEN GERMINATION AND POLLEN-TUBE GROWTH IN
MALE-FERTILE AND MALE-STERILE ms1 SOYBEAN

ABSTRACT

Using light and fluorescence microscopy to study in vivo and in vitro pollen-tube germination, a low percentage of coenocytic pollen-tube growth was observed from male-sterile plants. Anther squashes from male-sterile plants revealed that natural pollen-tube germination occurred in male-sterile anthers of four independent ms1 lines [NCms (T260), Ums (T266), Tms (T267), and Ams (T268)]. However, the Ums line seemed to have a higher percentage of pollen-tube germination than the other three lines. Abnormalities such as giant tubes, branched tubes, tubes with swollen areas, and multiple tubes were observed from coenocytic pollen grains. Flowers of the Urbana line from both male-fertile and male-sterile plants, as well as gynoecia pollinated with pollen grains from fertile or sterile plants, were used for in vivo studies. In male-fertile plants, or in female gametophytes of male-sterile plants pollinated with pollen from fertile plants, pollen-tube growth appeared normal. However, some heterozygote effect on cross-compatibility was noted. Pollen from heterozygous male-fertile plants reacts differently than that from homozygous male-fertile plants when pollinated on
stigmas of different genotypes. Coenocytic pollen tubes were observed rarely in gynoecia from male-sterile plants or gynoecia from male-fertile plants as a result of artificial cross-pollinations with ms1 ms1 plants as male parents. Few pollen tubes from coenocytic pollen grains were observed in the vicinity of the microphylar region. It could not be determined from our results whether or not these tubes released male-gametes that could effect fertilization.
INTRODUCTION

The ms1 mutant is a male-sterile, female-fertile soybean mutation inherited as a single-gene recessive (Brim and Young, 1971). Male-sterility of this ms1 mutant results from the formation of coenocytic microspores after telophase II of meiosis (Albertsen, 1976; Albertsen and Palmer, 1979). Previous studies have indicated that the male-sterility of the ms1 mutant is associated with the occurrence of polyembryony, haploidy, and polyploidy in the progeny of homozygous recessive (ms1 ms1) male-sterile plants (Kenworthy et al., 1973; Cutter and Bingham, 1977; Beversdorf and Bingham, 1977; Crane et al., 1982; Chen et al., 1985). At the present time most explanations for the formation of polyembryonic seedlings, haploids, and polyploids come from studies of megasporogenesis and megagametogenesis (Cutter and Bingham, 1977; Kennell and Horner, 1985). Although the observation of abnormal embryo sac development that results in multiple nuclei in the megagametophytes, and of fusion of these nuclei provides some evidence on the occurrence of polyembryony and polyploidy, it is difficult to fully explain the high ploidy levels (up to 2n=200) found during our studies (Chen et al., 1985). The origin of male-sterile plants among progeny from male-sterile (ms1 ms1) plants grown in the absence of pollinators is not understood (Brim and Young,
1971; Cutter, 1975; Palmer and Heer, 1976). Brim and Young (1971) noted that at least 99% of the seed set on the male-sterile plants resulted from natural crossing, but they did not exclude the possibility of some self-pollinated progeny from the sterile plants. Cutter (1975) and Palmer and Heer (1976) also reported seed set on $ms_1$ $ms_1$ plants in the greenhouse where pollinating vectors seemed to be absent. Albertsen and Palmer (1979) observed that the coenocytic microspores in $ms_1$ $ms_1$ plants either degenerated or developed structures resembling pollen tubes. Skorupska and Nawracala (1980) observed the growth of pollen tubes in the style of male-sterile plants with enlarged pollen grains in the Urbana male-sterile $ms_1$ line. However, only a single pollen tube was noted in the style. This observation indicates that at least some of the coenocytic pollen grains might germinate, grow, and possibly participate in fertilization.

Objectives of my study were to determine the frequency of coenocytic pollen grain germination and pollen-tube growth in stylar tissue. If, indeed, coenocytic pollen tubes are capable of germination and growth, they might also be responsible for fertilization and the formation of polyembryonic seedlings and polyploids in the progeny of male-sterile plants. To exclude the possibility of genotype specificity, I report the occurrence of natural pollen tubes in the male-sterile anthers of four spontaneous and
independent male-sterile (ms1) mutants designated Ames ms1, Tonica ms1, North Carolina ms1, and Urbana ms1 (Palmer et al., 1978a). The efficiency of these coenocytic pollen tubes in natural self-pollination and artificial cross-pollination was studied. The Urbana ms1 source was evaluated more extensively because of its comparatively high percentage of coenocytic pollen-grain germination and pollen-tube growth.
MATERIALS AND METHODS

Four methods, anther squash, pollen germination in culture medium, natural pollen-tube germination and growth in the gynoecium, and artificial cross-pollination for pollen-tube germination and growth in the gynoecia, were used in this study. Seeds from four male-sterile ms1 lines that originated from four independent and spontaneous male-sterile ms1 mutants designated North Carolina ms1 (NCms) T260, Tonica ms1 (Tms) T267, Ames ms1 (Ams) T268, and Urbana ms1 (Ums) T266 (Palmer et al., 1978a), were planted for the anther squash study of natural occurrence of pollen-tubes in male-sterile anthers. (The T number refers to the Soybean Genetic Type Collection number.) All the plants were grown and maintained either in the field or in the greenhouse except the NCms (T260) line which was induced to flower in the growth chamber under a 12-hour photoperiod, due to the difference in maturity group, before being maintained in the greenhouse. Only the Ums (T266) line grown in the greenhouse was used to conduct the study of pollen germination in culture medium, natural pollen-tube growth in gynoecia, and artificial cross-pollination for the growth of pollen tubes. Only the Ums (T266) line was used for these studies are because previous reports have indicated that the Ums line has 1) higher female productivity (Boerma and Cooper, 1978); 2) a lower percentage
of ovule abortion (Kennell and Horner, 1985); and 3) a higher percentage of natural occurrence of pollen tubes in anthers of male-sterile plants observed in this study. Thus, the information on the Ums line should be more valuable than that from the other three sources.

Plants were classified as male fertile or male sterile by I2KI stainability of pollen grains or coenocytic pollen grains at the beginning of flowering. All the studies were made with light and fluorescence microscopy. A Zeiss standard WL microscope with ultraviolet light source from HBO 200 mercury lamp and filters (BG38 for heat filter, UG1 for exciter filter, and 410 nm barrier filter) was used for fluorescence microscopy. Aniline blue (0.1 % in 0.15 M K2HPO4 at pH 8.2) (modification of Jensen, 1962, and Martin, 1959) was used as the fluorescent dye for staining callose in pollen tubes in all methods.

Anther-squash method

Freshly opened flowers from male-sterile plants were collected and placed in petri dishes with Drierite (anhydrous CaSO4) for 2-3 hours at room temperature. Anthers were dissected from the flower on a slide and squashed in a drop of aniline blue and after 5-10 minutes were observed with fluorescence microscopy. The percentage of pollen-tube germination that occurred in the sterile anther was recorded
from at least 10 randomly selected flowers from different individuals at R2 to R3 stage of flowering (stages refer to Fehr and Caviness, 1977) from each source.

**Pollen germination in culture medium**

Freshly opened flowers were collected from both male-fertile and male-sterile plants in the Ums line and desiccated over Drierite for 4–5 hours before dusting on depression slides with drops of culture medium. The culture medium used followed Brewbaker and Kwack (1963), except that 20% sucrose was used. This medium has been reported to be productive for soybean pollen growth (Tilton and Russell, 1983). Pollen grains were germinated at room temperature for 1 hour and observed with fluorescence microscopy with aniline blue stain. Percentage germination was counted for at least 600 pollen grains in each male-fertile flower and from the whole slide in male-sterile flowers because of its generally low number of coenocytic pollen grains. Records were made from 10 to 20 male-fertile and male-sterile flower samples, which were randomly collected from different individuals at R2 to R3 stage during flowering, as in the anther-squash method.

**Natural pollen-tube germination and growth in the gynoecium**

Starting one day postanthesis, varying numbers of flowers were sampled from each different genotype at the same time of day on each collecting date during flowering and
fixed in FAA (Berlyn and Miksche, 1976) for 24 hours at room temperature. The gynoecia then were dissected and cleared in 2N NaOH for 7-8 hours (modification of Martin, 1959). They were stained in aniline blue for 10-20 minutes before observation.

**Artificial crosses for pollen-tube growth in gynoecium**

Sibling male-fertile lines not segregating for male-sterility served as the homozygous male-fertile \textit{Ms1 Ms1} (FH) plant source. Seeds obtained from male-sterile plants will produce either male-fertile \textit{Ms1 Ms1} (Fh) or male-sterile \textit{ms1 ms1} (SH) plants. Different combinations of cross-pollinations were made among these three genotypes for the study of pollen germination and pollen-tube growth in gynoecia. For each crossing date, artificial crosses were made in all combinations during the same time period, but varied in number of crossings in each combination. Gynoecia were collected from each artificial crossing combination of greenhouse-grown plants in summer 1984 at 2, 4, 8, 20, 24, and 30 hours after pollination and fixed in FAA for 24 hours, but were fixed 24 hours after pollination for the study of winter 1985. Fixation and clearing procedures were the same as described previously in the section on natural pollen-tube germination and growth in the gynoecium.

Seeds obtained from the male-sterile plants in the
greenhouse were germinated. Chromosome numbers of these seedlings were counted following the procedure of Palmer and Heer (1973). Fertility of these progeny of male-sterile plants also was determined by staining pollen with $I_2KI$.

Chi-square test for occurrence of pollen-tube growth in natural self-pollination and artificial cross-pollination among different crosses was based on the analysis of frequency (Snedecor and Cochran, 1980).
RESULTS AND DISCUSSION

Pollen germination in culture medium and squashed anthers

Germination percentage of coenocytic pollen grains from the male-sterile ms1 ms1 plants in culture medium was not very high (Table 1). Few coenocytic pollen grains with pollen-tube growth were observed. On the contrary, male-fertile pollen grains can be germinated easily in the culture medium (Fig. 1a). As shown in Table 1, the percentage of pollen germination in culture medium of male-fertile plants varied from 72% to 87%, with an average of about 80%, whereas, in the male-sterile plants, only about 1.8% of coenocytic pollen grains germinated. Whether these germinated coenocytic pollen grains from the male-sterile plants were induced to germinate by the culture medium is questionable. As indicated in Table 2, the percentage of coenocytic pollen grains germinated from the squashed sterile anthers is obviously higher than that germinated in the culture medium (Table 1). Therefore, germinated coenocytic pollen grains seen in culture medium are probably the result of already germinated pollen being dusted onto the medium. The culture medium method was abandoned because the coenocytic pollen grains were generally difficult to dust onto the medium due to the poor dehiscence of sterile anthers and this method was not as efficient as the anther-squash method.
Albertsen and Palmer (1979), using scanning electron microscopy, observed that coenocytic microspores often were found within a matrix and were not released easily. From our observations, the coenocytic pollen grains released in the culture medium tend to clump, and frequently only very few coenocytic pollen grains were dusted onto the culture medium. The anther-squash method for observing natural pollen-tube germination in sterile anthers in soybean was first reported by Albertsen (1976). From our routine classification of the male-fertile and male-sterile plants by I$_2$KI pollen stain, occasionally some pollen-tube-like materials were clumped with coenocytic pollen grains. However, under light microscope, it is difficult to determine whether it was matrix-like materials described by Albertsen and Palmer (1979) or true pollen tubes. The use of fluorescence microscopy with aniline blue stain enabled us to verify that there is natural pollen-tube germination in male-sterile anthers.

Our results indicated that natural coenocytic pollen tubes occurred in all four sources. However, the percentage of naturally occurring pollen tubes in sterile anthers of the Ums source is generally higher than in the other three sources under our growing conditions (Table 2). However, variation within source also was noted. Various types of abnormalities of coenocytic pollen tubes, such as giant
Table 1. Average percentage of pollen germination in culture medium of Ums male-fertile and male-sterile plants in greenhouse plantings of summer 1984

<table>
<thead>
<tr>
<th>Class</th>
<th>No. samples</th>
<th>Percent of pollen germination Mean ± S.D. of mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male fertile</td>
<td>13</td>
<td>80.5 ± 1.2</td>
<td>72.4 - 87.2</td>
</tr>
<tr>
<td>Male sterile</td>
<td>20</td>
<td>1.8 ± 0.3</td>
<td>0.0 - 5.8</td>
</tr>
</tbody>
</table>
Table 2. The means, standard deviation of means and range of percentage of pollen-tube germination in squashed anthers of male-sterile *ms1* plants from different sources and environments.

<table>
<thead>
<tr>
<th>Source</th>
<th>Seasons</th>
<th>No. of samples</th>
<th>Mean ± S.D. of mean</th>
<th>Range</th>
<th>No. of samples</th>
<th>Mean ± S.D. of mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ums</td>
<td>1984 S</td>
<td>10</td>
<td>8.2 ± 1.9</td>
<td>2.2-21.0</td>
<td>20</td>
<td>15.2 ± 1.9</td>
<td>3.4-32.4</td>
</tr>
<tr>
<td></td>
<td>1984 F</td>
<td>23</td>
<td>2.7 ± 0.4</td>
<td>0.8-7.4</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1985 W</td>
<td>20</td>
<td>4.1 ± 0.8</td>
<td>0.6-13.8</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tms</td>
<td>1984 S</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>12</td>
<td>2.5 ± 0.9</td>
<td>0.0-11.0</td>
</tr>
<tr>
<td></td>
<td>1984 F</td>
<td>13</td>
<td>1.2 ± 0.3</td>
<td>0.3-4.9</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1985 W</td>
<td>10</td>
<td>1.4 ± 0.5</td>
<td>0.3-4.9</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ams</td>
<td>1984 S</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>0.6 ± 0.5</td>
<td>0.0-2.7</td>
</tr>
<tr>
<td></td>
<td>1984 F</td>
<td>23</td>
<td>2.0 ± 0.3</td>
<td>0.0-5.5</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1985 W</td>
<td>20</td>
<td>1.5 ± 0.5</td>
<td>0.2-10.1</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NCms</td>
<td>1984 S</td>
<td>28</td>
<td>2.1 ± 0.4</td>
<td>0.0-6.5</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1984 F</td>
<td>11</td>
<td>1.1 ± 0.3</td>
<td>0.0-2.9</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Seasons*: S: Summer; F: Fall; W: Winter.

*Sampled at late stage of flowering.*
pollen tubes (Fig. 2a), tubes with swollen areas (Fig. 2b), branched tubes (Fig. 2c), and multiple pollen tubes from one coenocytic pollen grain (Fig. 2d) were observed. Tubes with swollen areas usually were retarded in growth. Branching of coenocytic pollen tubes could occur at the beginning of germination or after growth for a certain length of time. Multiple pollen tubes could come from the same pore of a coenocytic pollen grain or from different pores. The abnormalities found in coenocytic pollen tubes suggested that the four nuclei in the coenocytic microspore might act independently or cooperatively. Therefore, if the coenocytic pollen tubes were able to grow down to the ovule, various sorts of fertilization, such as multiple fertilization by different sperm nuclei with extra eggs and fused sperm nuclei fertilized with fused eggs etc., might occur. This might lead to the formation of polyembryonic seedlings and polyploids in the progeny of male-sterile plants. A similar mutant (jp) caused by failure of postmeiotic cytokinesis in alfalfa (Medicago sativa L.) recently was reported (McCoy and Smith, 1983). Frequency of germinated jumbo pollen grains varied from 3.1% to 37.8% in a culture medium with 10% sucrose and 50 ppm boric acid. However, their cross-pollination study indicated that jumbo pollen was incapable of effecting fertilization.
Natural pollen-tube growth in the gynoecium

The aniline blue fluorescence (ABF) method used on localizing callose response was reviewed by Dumas and Knox (1983). The ABF method provides a useful way to determine the incompatibility system, gametophytic competition, and stigma and ovule viability. In this study, the ABF method was used to study the growth of coenocytic pollen tubes in gynoecia of the ms1 line. Soybean is a self-pollinated species. Therefore, flowers collected about a day postanthesis were used for the study of natural pollen germination and pollen-tube growth in the gynoecia. Observations were made on two greenhouse plantings, summer 1984 and winter 1985, of the Ums line. From 54 gynoecia of male-fertile (Ms1 Ms1) plants in the summer 1984 planting, about 83% of gynoecia manifested pollen-tube growth (Figs. 1b, c, d) and 17% showed no pollen-tube growth (Table 3). On the contrary, from 285 gynoecia of male-sterile plants, no pollen germination or pollen-tube growth in the gynoecia (Fig. 2e) were observed on about 93% of gynoecia, 6% showed pollen tube degeneration or retardation in growth in the stigma (Figs. 2f, g) or style area; only 1% showed pollen-tube growth down the style (Fig. 2 h).

In winter greenhouse 1985 planting, the average percentage of gynoecia with pollen-tube growth in the male-fertile (Ms1 Ms1 + Ms1 ms1) plants was about 44%, leaving 55%
Figure 1. Pollen germination and pollen-tube growth in the male-fertile plants of Ums line. [All pictures were taken with fluorescence microscopy except (c)]

(a) Pollen germination in culture medium. X540

(b) Gynoecium collected about one day postanthesis showing pollen tubes growing down the style through transmitting channels. X540

(c) and (d) Comparison of pollen germination and pollen-tube growth in gynoecia observed under phase contrast light microscopy (c) and fluorescence microscopy (d). X540

(e) Pollen tube (arrows) entering the micropyle from an artificial cross of FH X FH fixed 24 hours post-pollination. X540
Figure 2. Pollen germination and pollen-tube growth of coenocytic pollen grains from male-sterile plants of Ums line. [All pictures were taken with fluorescence microscopy except (f)]

(a) - (d) Abnormalities in coenocytic pollen tubes from squashed anthers showing (a) giant pollen tube, (b) pollen tube with swollen area, (c) branched tubes, and (d) multiple and branched pollen tube. X540

(e) Gynoecium one day postanthesis showing no pollen germination or pollen-tube growth in the stigma and style areas. X540

(f) and (g) Comparison showing degenerated pollen tube (arrows) in stigma of a male-sterile flower collected one day postanthesis observed under light (f) and fluorescence microscopy(g). X540

(h) A single pollen tube observed in style of a male-sterile flower collected one day postanthesis. X540

(i) and (j) Gynoecia from artificial cross-pollinations with coenocytic pollen grains showing slowly growing pollen-tube in stigma (i) and degenerated-tube-like structure (j) in style. X540
without pollen germination and pollen-tube growth. The difference in percentage of pollen germination and pollen-tube growth between male-fertile homozygous (Ms1 Ms1) plants and male-fertile heterozygous (Ms1 ms1) plants was statistically nonsignificant (Table 3). The difference between environments is due to the unfavorable greenhouse conditions in the winter. Heslop-Harrison (1957) demonstrated the effect of temperature and light quality and quantity on the percentage of pollen production. Palmer et al. (1978b) reported that differences in pollen production of different ms1 sources occurred in greenhouse-grown plants. Less than the optimum conditions in the winter, as compared with the summer, might lead to the generally low percentage of pollen-tube germination and growth in gynoecia of male-fertile plants. Nevertheless, no gynoecia with pollen-tube growth were observed from those of male-sterile plants, but there was about 8% of gynoecia with pollen-tube-like degeneration or retardation of growth in the stigma or style areas in the winter planting of 1985 (Table 3).

Observation of pollen-tube growth in male-fertile gynoecia indicated that most pollen tubes had grown down the style through channels of transmitting tissue, as those reported by Tilton et al. (1984). However, some pollen tubes growing into stylar tissue other than transmitting channels
Table 3. Percent and number of gynoecia with pollen-tube growth from natural self pollinations of male-fertile and male-sterile (ms1) soybean (Ums) collected one day postanthesis

<table>
<thead>
<tr>
<th>Classes</th>
<th>1984 Summer</th>
<th>1985 Winter(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>W/pollen tube</td>
<td>Pollen tube degenerated</td>
</tr>
<tr>
<td>Male-fertile (FH)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>54</td>
<td>45 (83.3)(^c)</td>
</tr>
<tr>
<td>Male-fertile (Fh)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Male-sterile (SH)</td>
<td>285</td>
<td>4 (1.4)</td>
</tr>
</tbody>
</table>

\(^a\)Chi-square test between FH and Fh against types of gynoecia observed is not significant at 5% level.

\(^b\)FH: male-fertile homozygous; Fh: male-fertile heterozygous; SH: male-sterile homozygous.

\(^c\) ( ) indicates percentage.
were observed. Most of these types of pollen tubes were degenerated in the style. From this study, it seems that germination and growth of coenocytic pollen grains is not very effective in gynoecia and might be precluded by environmental conditions. Skorupska and Nawracala (1980), using the Ums source, showed 63.6% plants with enlarged pollen grains but with no pollen-tube growth in the style. In our study, pollen-tube germination was observed in sterile anthers from most sterile plants, but percent of gynoecia with pollen-tube growth in male-sterile plants was very low and most were degenerated or were retarded in growth. Single pollen-tube growth (Fig. 2h) was found in only a few gynoecia of male-sterile plants. However, variation in number of pollen tubes (from few to numerous) growing down the style (Figs. 1b, d) was frequently noted in both homozygous and heterozygous male-fertile plants. Corresponding differences were observed in percentage of pollen tubes occurring in male-sterile anthers and percentage of gynoecia with pollen-tube growth in male-fertile plants between summer 1984 and winter 1985 greenhouse plantings.

Some pods were obtained from four male-sterile plants grown in the greenhouse, two plants with one-seeded pod, one plant with 2 one-seeded pods, and one plant with 2 one-seeded pods, 2 two-seeded pods and 1 three-seeded pod. Among
these 13 seeds, eight were shrivelled and five were normal in morphology. Only six seeds were capable of germination. Chromosome numbers of these six seedlings were 2n=40. Classification for male-sterility after flowering of these six plants indicated that four were male-sterile and two were male-fertile. This suggested that either outcrossing is possible in the greenhouse or there was possibly a reverse mutation of ms1 to Ms1, because if all these seeds were from self-fertilization of the male-sterile plants, no male-fertile progeny should be expected. Beversdorf and Bingham (1977) observed a member of a haploid-haploid twin set with a completely fertile sector. They suggested that this was a result of either androgenesis from Ms1 or the reversion of ms1 to the Ms1 allele. Although no polyploids or polyembryonic seedlings were found from these six plants, seven seeds among those eight ungerminated seeds were shrivelled. Beversdorf and Bingham (1977) also reported highly polyploid individuals resulted from monoembryonic seeds that were shrivelled prior to germination. Therefore, whether these shrivelled seeds that did not germinate in this study were polyploids is not known.

Artificial crosses for pollen-tube growth in the gynoecium

Our primary reasons for using artificial crosses in observing pollen-tube growth in gynoecia were 1) artificial pollination might enhance the contact of coenocytic pollen
grains with the stigma of male-sterile plants and 2) to see if gynoecia of male-sterile plants allowed growth of male-fertile pollen grains as efficiently as gynoecia of male-fertile plants. In the greenhouse, several types of artificial pollinations were conducted. Fixing of plant materials at different time intervals was done to determine the duration of pollen-tube growth from pollination to fertilization to select the optimum time of fixation for further study. Results indicated that time from pollination to fertilization varied from cross to cross, but generally it took at least 20 to 24 hours from pollination to pollen tube entering the micropyle (Fig. 1e). This is in contrast to the report of 8 to 10 hours from pollination to fertilization by Rustamova (1964) (see Carlson, 1973) and by Prakash and Chan (1976). The cross-pollination procedures used followed the techniques described by Fehr (1980). Whether the difference is due to the specific genotype, different environments, or an interaction of both is not known.

Results from summer 1984 showed that in only a few cases (an average 1.5%) were coenocytic pollen grains capable of growing down the style (Table 4). However, growth of these coenocytic pollen tubes is generally slower than growth of the male-fertile pollen tubes. Regardless of what genotype of female pistils were pollinated with the coenocytic pollen
grains, about 14\% of crossed gynoecia showed degenerated pollen-tube-like structures in the stigma or style area (Table 4; Figs. 21, j).

The male-fertile pollen grains germinated and grew in the pistil of the male-sterile plants very well. However, unexpected results were observed in the male-fertile heterozygote X heterozygote crosses (Fh X Fh) in summer 1984: only 54\% of artificial crosses among the heterozygote sib plants were observed with pollen-tube growth in the gynoecia. This compares with 88\%, 91\% and 84\% for the FH X FH, SH X FH, and SH X Fh crosses (Table 4). A Chi-square test among the FH X FH, Fh X Fh, SH X FH, and SH X Fh crosses indicated distribution of percentage of gynoecia with pollen-tube growth, pollen-tube degeneration, and without pollen-tube growth is significantly different at the 5\% level (Table 5). Most of the variation is contributed from the Fh X Fh crosses. The success rate in artificial crosses is known to vary from person to person and from environment to environment (Fehr, 1978). In our study, different cross combinations were made the same day with a varying number of crosses by the same person. Thus, these differences are not likely to be due to sampling error.

The artificial crossing study was repeated to determine if there was any heterozygote effect on cross compatibility.
Table 4. Percent and number of gynoecia with pollen-tube growth from artificial cross-pollinations of male-fertile and male-sterile \textit{ms1} (Ums) soybean

<table>
<thead>
<tr>
<th>Environment</th>
<th>1984 Summer</th>
<th>1985 Winter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of crosses ( \mathcal{Q} \times \mathcal{G} )</td>
<td>Total W/pollen tube</td>
<td>Pollen-tube degenerated</td>
</tr>
<tr>
<td>FH X FH</td>
<td>24</td>
<td>21 (87.5)</td>
</tr>
<tr>
<td>FH X Fh</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fh X FH</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fh X Fh</td>
<td>24</td>
<td>13 (54.2)</td>
</tr>
<tr>
<td>SH X FH</td>
<td>32</td>
<td>29 (90.6)</td>
</tr>
<tr>
<td>SH X Fh</td>
<td>37</td>
<td>31 (83.8)</td>
</tr>
<tr>
<td>Cross</td>
<td>FH</td>
<td>FH</td>
</tr>
<tr>
<td>-------------</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>FH X SH</td>
<td>19</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>(5.3)</td>
<td>(15.8)</td>
</tr>
<tr>
<td></td>
<td>(0.0)</td>
<td>(19.0)</td>
</tr>
<tr>
<td></td>
<td>(17)</td>
<td></td>
</tr>
<tr>
<td>Fh X SH</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(0.0)</td>
<td>(15.6)</td>
</tr>
<tr>
<td></td>
<td>(0.0)</td>
<td>(4.8)</td>
</tr>
<tr>
<td></td>
<td>(20)</td>
<td></td>
</tr>
<tr>
<td>SH X SH</td>
<td>82</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>(1.2)</td>
<td>(13.4)</td>
</tr>
<tr>
<td></td>
<td>(0.0)</td>
<td>(10.3)</td>
</tr>
<tr>
<td></td>
<td>(26)</td>
<td></td>
</tr>
</tbody>
</table>

\[a\] FH: male-fertile homozygous; Fh: male-fertile heterozygous; SH: male-sterile homozygous.

\[b\] ( ) indicates percentage.
Compared with the results from the summer of 1984, the percentage of gynoecia with pollen-tube growth from the crosses of FH, Fh, and SH pistils with male-fertile pollen grains is generally lower (Table 4). These results are compatible with the results of a lower percentage of gynoecia with pollen-tube growth in natural self-pollinated plants (Table 3). No gynoecia with pollen-tube growth were observed when coenocytic pollen grains were used as male parents. However, some pollen-tube-like structures and degenerated tubes occasionally were observed in the stigma or style area.

Chi-square test for R X C contingency on frequency distribution of gynoecia with or without pollen-tube growth and with degenerated tubes of the crosses with male-fertile pollen was significantly different at the 5% level. Partition of Chi-square into its subdegrees of freedom indicated no significant differences among the female genotypes when the same genotype of male parent was used. Nevertheless, differences between pollen from the homozygote male-fertile (FH) plants and from heterozygote male-fertile (Fh) plants were obvious (Table 5). Percentage of gynoecia without pollen-tube growth was generally lower when Fh pollen was used than when the same genotype was crossed with FH pollen (Table 4). Furthermore, percentage of gynoecia with pollen-tube degeneration in the stigma or style area of the same genotype was much higher when the Fh pollen was used.
Table 5. Summary of Chi-square test for the frequency of three classes of pollen-tube growth among crosses with male-fertile pollen as male source in Table 4

<table>
<thead>
<tr>
<th>Year</th>
<th>Sourcea</th>
<th>df</th>
<th>(x^2)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1984 summer</td>
<td>Overall (among FH X FH, Fh X Fh, SH X FH, and SH X Fh)</td>
<td>6</td>
<td>16.73</td>
<td>0.025-0.01*</td>
</tr>
<tr>
<td></td>
<td>(a) Among FH X FH, SH X FH and SH X Fh</td>
<td>4</td>
<td>1.87</td>
<td>0.90-0.75 NSb</td>
</tr>
<tr>
<td></td>
<td>(b) Between FH X FH + SH X FH + SH X Fh and Fh X Fh</td>
<td>2</td>
<td>14.56</td>
<td>&lt; 0.005**</td>
</tr>
<tr>
<td>1985 winter</td>
<td>Overall (among FH X FH, Fh X Fh, SH X FH, and SH X Fh)</td>
<td>10</td>
<td>19.75</td>
<td>0.05-0.025*</td>
</tr>
<tr>
<td></td>
<td>(a) Among FH X FH, Fh X FH, and SH X FH</td>
<td>4</td>
<td>1.35</td>
<td>0.90-0.75 NSb</td>
</tr>
<tr>
<td></td>
<td>(b) Among FH X Fh, Fh X Fh, and SH X Fh</td>
<td>4</td>
<td>5.43</td>
<td>0.50-0.25 NSb</td>
</tr>
<tr>
<td></td>
<td>(c) combined (a) vs. combined (b)</td>
<td>2</td>
<td>12.31</td>
<td>&lt; 0.005**</td>
</tr>
</tbody>
</table>

aFH: male-fertile homozygous; Fh: male-fertile heterozygous; SH: male-sterile homozygous.

bNonsignificant.

*Significant at 5% level.

**Significant at 1% level.
than when FH pollen was used. Most variation was attributed to the difference between percentage of pollen-tube degeneration as well as percentage of gynoecia without pollen-tube growth.

Results obtained from artificial cross-pollination by using pollen from FH and Fh plants suggested pollen and style interaction. No pollen and style interactions in soybean have ever been reported. However, several reports in maize and other species have noted gamete competition between self pollen and cross pollen (Johnson and Mulcahy, 1978; Yamada and Murakami, 1983; Ottaviano et al., 1983; Currah, 1983; Sarr et al., 1983). As pointed out by Johnson and Mulcahy (1978), earlier studies on pollen-tube competition in maize revealed no consistent tendency. Three types of results were generally noted: 1) advantage of self pollen on mixed pollination in inbred lines, 2) no consistent pattern in competition between self and other pollen in F₁ hybrids, and 3) advantage of F₁ hybrid pollen in fertilization over pollen from inbred lines. Johnson and Mulcahy (1978) also noted that competitive ability of pollen from inbred plants in mixed pollinations in their study was not merely maintained but enhanced through successive generations of selfing. Yamada and Murakami (1983) confirmed the superiority in gamete competition of pollen derived from F₁ plants in maize. Ottaviano et al. (1983), in another study, indicated that
some inbred lines were more competitive in their own style and some less competitive, and that a tendency toward assortative mating was even more clear in hybrid combinations. Similar reports on pollen competition in onion (Currah, 1983) and in pearl millet (Sarr et al., 1983) also were documented. Because soybean is a self-pollinated species, through evolution, self-pollen might have some sort of advantage over cross pollen in optimum environment conditions. In this study, the result of Fh X Fh in winter 1985 was different from that of summer 1984. Our explanation is that there might be some heterozygote advantage under the unfavorable winter greenhouse conditions.

In conclusion, from this study, male-sterile ms1 lines from all four sources proved to have a low percentage of coenocytic pollen grains that were capable of germination. Among these four source populations, the Ums line tended to have higher percentage of natural pollen-tube growth in anthers of male-sterile plants. Based on the study of the Ums line, the coenocytic pollen tubes generally grew slowly in gynoecia and usually degenerated or collapsed. Only about 1% grew down the style. Therefore, coenocytic pollen grains are not effective in pollination.
ACKNOWLEDGEMENT

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REFERENCES


SUMMARY AND DISCUSSION

The male-sterile character \textit{ms1} is inherited monogenically (Brim and Young, 1971). Homozygous recessive male-sterile \textit{ms1} \textit{ms1} plants are characterized by: 1) formation of coenocytic microspores, due to failure of cytokinesis after telophase II of meiosis (Albertsen, 1976; Albertsen and Palmer, 1979); 2) production of polyembryonic seedlings; and 3) production of haploids and polyploids in both polyembryonic and monoembryonic progenies (Kenworthy et al., 1973; Cutter and Bingham, 1977; Beversdorf and Bingham, 1977; Chen et al., 1985). There are at least five independent mutations at the \textit{ms1} locus in soybean. Four mutants, designated North Carolina, Urbana, Tonica and Ames male steriles, arose spontaneously and independently of each other in the United States (Palmer et al., 1978). Another mutation at the \textit{ms1} is the Shennong male sterile recently reported in China (Yee and Jian, 1983).

Our primary interests reported in section I of this dissertation are to: 1) examine if there is any difference in frequency of polyembryony and polyploidy when using different source populations of the \textit{ms1} allele, 2) provide more genetic information on the relationships among the characters of male-sterility, haploidy, polyploidy, and polyembryony, and 3) obtain polyploids and their progeny for
further studies. Four independent ms1 sources (NCms, Tms, Ums, Ams) and two derived ms1 populations from F4 seeds of crosses of two ms1 lines to two homozygous chromosome interchange lines (Ams X Clark T/T and Ums X KS-172-11-3) were used for the studies on frequency of polyembryony and polyploidy. Results showed that variation occurred in frequencies of polyploidy and polyembryony when different source populations were used. Although the difference in frequency of polyembryony between F4 seed of Ams X Clark T/T and the original Ams population was not significant, a substantial decline in frequency was observed when comparing the F4 seed of Ums X KS-172-11-3 with the original Ums population. Furthermore, in comparing the original ms1 populations with the F4-seed-derived populations the occurrence of polyploids is either significantly increased (F4 seed of Ams X Clark T/T vs. Ams) or significantly decreased (F4 seed of Ums X KS-172-11-3 vs. Ums). The male sterile character of ms1 mutants from all sources was verified to be associated with the characteristic of producing polyembryonic seedlings and polyploids among homozygous recessive ms1 ms1 progeny. As discussed in section I, the variation in frequency of polyembryony and polyploidy displayed by the different sources of the ms1 gene suggests that the action of ms1 gene on polyembryony and polyploidy might be modified by some other gene(s) when different
genetic backgrounds were used, and might be affected by environmental factors where the plants are grown. From haploid 2n=20 up to 2n=200 plants were observed in the progeny of homozygous recessive male-sterile plants. This is the highest ploidy level ever reported for progeny from the \textit{ms}1 homozygote.

Cytological studies on triploids discussed in section II reveal no distinguishable difference in chromosome association between male-fertile and male-sterile triploids. Chromosomes of triploids tend to occur in trivalents during diakinesis to metaphase I, but multivalents, bivalents, and univalents also were observed. Some secondary chromosome associations, nonhomologous association, and aberrant nucleolar distributions (see Chen and Palmer, 1985a, b) occasionally were noted. Such behavior supports the hypothesis of duplicated genomes and the polyploid origin of soybean. Failure of cytokinesis after telophase II of meiosis resulting in formation of four-nucleate coenocytic microspores, which characterize the \textit{ms}1 mutant, were consistently observed in the male-sterile triploids.

Differences in pollen size and pollen stainability were notable between male-fertile and male-sterile plants. The average pollen stainability in male-fertile triploid plants is about 70%. However, a generally low seed set was observed
on the male-fertile triploids. Number of seeds obtained from 32 fertile triploid plants varied from 1 to 13, with an average of 4.4 per plant. The low fertility in these triploids could be attributed to: 1) imbalance of gametic chromosome number, 2) failure of zygote development due to chromosome or genomic imbalance, or 3) failure of endosperm development (Schulz-Schaeffer, 1980; Brink and Cooper, 1947). Chromosome number of these triploid progeny varied from 2n=40 to 2n=71 and exhibited a near-random distribution, with the majority (about 60%) being between 56 and 65. Progeny of fertile triploids gave segregation ratios for the \textit{ms1} allele that confirmed the \textit{Ms1 ms1 ms1} genotype. Nevertheless, only a few aneuploids from these triploid progeny set seeds and their progeny segregated for different chromosome numbers.

Pollen germination and pollen-tube growth studies are reported in section III. Natural pollen-tube growth in sterile anthers was observed in all independent \textit{ms1} source populations (NCms, Tms, Ams, and Ums). However, variation among samples within sources were noted. Ums seems to have a generally higher frequency of natural pollen-tube growth in sterile anthers than the other three sources. Abnormalities, including giant tubes, branched tubes, tubes with swollen areas, and multiple tubes, occasionally were observed in pollen tubes of coenocytic pollen grains. Investigations of
pollen-tube growth in natural self-pollination and artificial cross-pollination in gynoecia of Ums lines were conducted in both summer 1984 and winter 1985 plantings in the greenhouse. Coenocytic pollen-tube growth in style is generally very low in both natural self-pollination and artificial cross-pollination. No significant difference was noticed in pistil receptivity for pollen germination and pollen-tube growth between male-fertile (Ms1--) and male-sterile (ms1 ms1) plants when pollen was from the same genotype of male-fertile plants. However, difference between pollen from homozygous male-fertile and heterozygous male-fertile plants was noted.

Skorupska and Nawracala (1980) also observed single pollen-tube growth in styles of 12 out of 33 male-sterile plants observed. They reported that only two of these male-sterile plants set pods, one with a one-seeded pod and the other with four four-seeded pods. From our study, some seed pods also were obtained from sterile plants in the greenhouse in both summer 1984 and winter 1985. These seeds had a poor germination rate (6 out of 13 seeds planted) resulting in two male-fertile diploid plants and four male-sterile diploid plants. This indicated that either outcrossing is possible in the greenhouse or there possibly was a reverse mutation of ms1 to Ms1, because selfed seeds on the sterile plants should give rise to all male-sterile plants. Beversdorf and Bingham
(1977) also observed a member of haploid-haploid twin set with a completely fertile sector. They suggested either androgenesis from Ms1 pollen or the reversion of ms1 to the Ms1 allele. Though natural pollen-tube growth in sterile anthers frequently was observed, the coenocytic pollen grains were not effective in fertilization.

Previous explanations of occurrence of polyembryonic seedlings and polyploids are based on abnormal embryo-sac development (Cutter and Bingham, 1977; Kennell and Horner, 1985a). Kennell and Horner (1985b) observed several pollen tubes at the micropyle, but no more than one pollen tube was seen inside the megagametophyte in normal fertile plants. We did not observe more than one pollen tube entering the same micropyle in the male-fertile gynoecia or gynoecia of male-sterile plants pollinated with male-fertile pollen. Unless more than one pollen tube could enter the micropyle, multiple fertilizations required pollen-tube growth with two or more sperm nuclei discharged in an embryo sac with multiple eggs. This is possible if the four nuclei in the coenocytic microspore could act independently. Nuclear fusion in coenocytic microspore could result in sperm nuclei with chromosome numbers two to four times that of the haploid number. Fertilization of fused nucleate eggs, such as those observed by Cutter and Bingham (1977) and Kennell and Horner (1985a), with sperm nuclei from coenocytic pollen grains
might lead to the higher polyploidy level. Most highly polyploid progeny obtained generally are sterile.

In conclusion, results from my dissertation studies indicate that 1) the gene(s) controlling the characters of polyembryony and polyploidy are associated with the ms1 gene; 2) the occurrence of polyembryony and polyploidy could be modified by some other gene(s) when different genetic background is incorporated, or by environmental factors; 3) meiotic studies on triploids obtained from male-sterile ms1 ms1 progeny provide some cytological evidence of duplicated genomes or polyploid origin of soybean; 4) most aneuploids produced from fertile triploids had low fertility; however, some lower hyper-diploid aneuploids were obtained from fertile triploid progeny; 5) natural occurrence of pollen-tube growth in sterile anthers was observed in all four spontaneous and independent ms1 source populations; the Urbana male sterile line seems to have a higher frequency of natural pollen-tube growth among four source lines under our cultural conditions; 6) coenocytic pollen grains are not effective in fertilization; however, some are capable of germination; 7) pollen from the heterozygous male-fertile plants reacts differently from that of homozygous male-fertile plants when pollinated on stigmas of different genotypes.
ADDITIONAL LITERATURE


Kermicle, J. L. 1969. Androgenesis conditioned by a


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