CYTOLOGY AND SEED SET STUDIES IN THE PANSY, VIOLA TRICOLOR HORTENSIS L.

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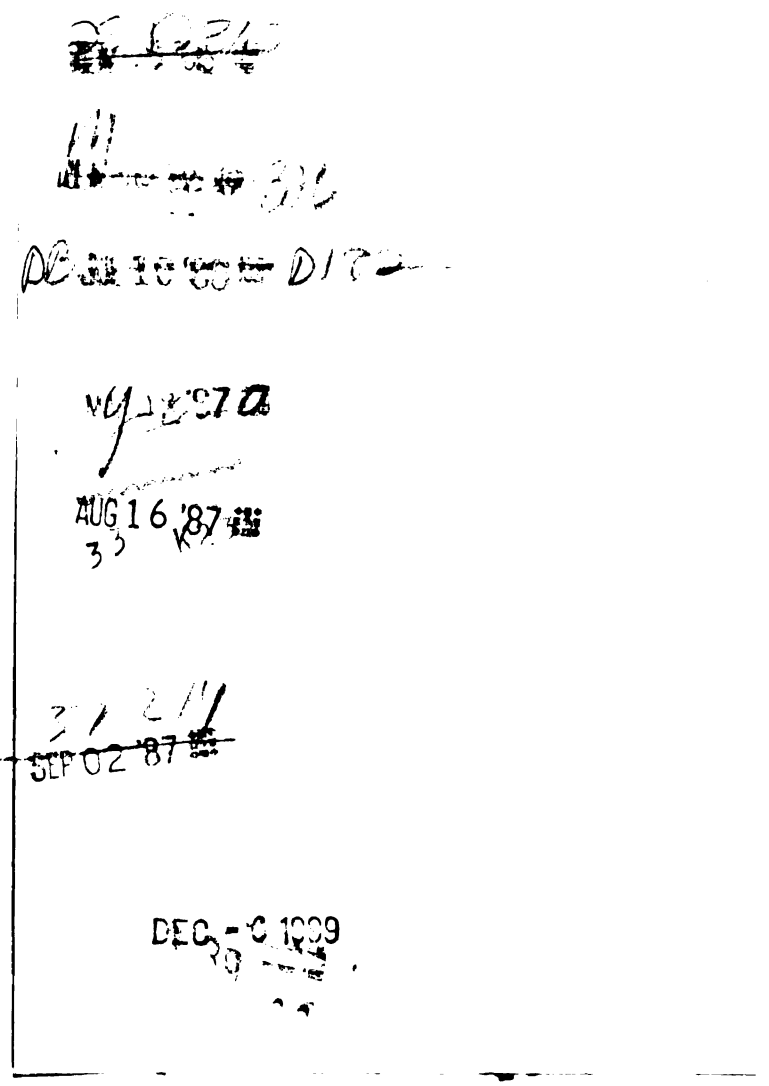
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ABSTRACT

CYTOLOGY AND SEED SET STUDIES IN THE PANSY,

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by Everett R. Emino

The pansy, *Viola tricolor* Hortensis L., is a popular bedding plant; however problems of low seed set have hindered genetic studies, plant breeding efforts and the production of commercial F_1 hybrids. Twenty-nine commercial inbred pansy lines were investigated cytologically to establish a possible relationship between chromosome number and pollen fertility. The somatic chromosome number of all lines studied was found to be 2n=48, meiosis appeared regular and pollen fertility was above 90 percent in greenhouse and controlled environment grown plants.

Additional investigations on low seed set suggested a self-incompatibility system. Selected inbred lines were classified as low or high seed set when self-pollinated. When low seed set lines were used as the male parent on high seed set lines, the seed set was always improved. This indicated the male parent was fertile as previously shown by high pollen fertility, and the female parent or the interaction between the male and female parents may be the site of an incompatibility system which results in low seed set.

CYTOLOGY AND SEED SET STUDIES IN THE PANSY, *VIOLA TRICOLOR HORTENSIS* L.

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

The pansy (*Viola tricolor* Hortensis L. or *V. Wittrockiana* Gams.) is an important horticultural member of the *Violaceae*. It has been grown for many years in European and American gardens as a spring bedding plant. Bailey (1947) stated that it is an old garden flower with a report of its being grown as early as 1629. Frost (1947) suggested that the pansy was first improved in England where the cool climate was conducive for its cultivation. About 1900, three French seed specialists, Bugnot, Cassier, and Triardeau, studied the pansy and made improvements in color and flower size (Frost 1947).

In recent years interest in the pansy as a high quality bedding plant has increased. The pansy cultivar, 'Giant Majestic with Blotch,' an F_1 hybrid, won the All American Selection Award for 1966. This gain in popularity was due to many desirable plant characteristics such as, numerous flowers, good growth, relatively disease and insect free, increased adaptability, and wide range of flower colors. For the pansy to compete with present bedding plants such as the petunia, which is currently the most popular bedding plant, solutions to maintaining inbred lines and breeding F_1 hybrids must be found. The inability of inbred lines to set seed in repeated inbreeding and in cross-pollinations to produce hybrids must be overcome for further improvement of inbreds, and to determine inheritance patterns.

The purpose of this study was to obtain information on developing a method for selecting and maintaining desirable inbred lines which will

be genetically stable, show high pollen fertility, and set viable seed from self- and cross-pollinations for economical seed production.

To accomplish this purpose the following objectives were undertaken:

1. To determine an appropriate chromosome smear technique for pollen mother cells and root tips.
2. To determine the chromosome number of selected inbred lines and examine meiotic divisions in pollen mother cells of these lines for presence or absence of fragments, bridges, and chromosome associations.
3. To relate cytological observations to pollen fertility and seed set under controlled environmental conditions.
4. To determine which lines or types can be used to produce genetically stable and fertile inbred lines for hybrid seed production.

II. LITERATURE REVIEW

A. Classification

The pansy, V. tricolor Hortensis L. is a member of the Violaceae (Bailey, 1947). He stated that this family included many attractive herbaceous perennials which generally thrive best in partial shade and fairly rich soil.

Wittrock, according to Clausen (1926), stated the cultivated pansy was produced in the 1830's by English gardeners from the cross of V. lutea and V. tricolor. In the 1860's the pansies thus produced were crossed with V. cornuta to give the perennial pansy. Because of Wittrock's description, Clausen (1927) suggested the name of the pansy be V. Wittrockiana Gams which according to Clausen (1927), Gams had described and suggested a year earlier. This is the accepted name of several foreign workers, (Kondo, et al., 1956; Endo, 1959; and Huziwara, 1966). The pansy is also referred to as V. tricolor maxima, (Horn, 1956).

Clausen (1922a) first stated V. tricolor L. as a collective species but upon cytological observation distinguished between V. tricolor L. and V. arvensis Murr. Clausen (1927) later stated that V. tricolor is a member of the Melanium section of the Violaceae. He included the following species in the Melanium section: V. cenisia, elegantula, declinata, valderia, heterophylla, cornuta, orthoceras, orphanidis, tricolor, alpestris, arvensis, rothomagersis, and lutea. Since the cultivated pansy has as its parents, species of the Melanium

section (Clausen, 1926), it follows that the pansy is also a member in this section.

Clausen (1931) gives detailed description of some members of the Melanium section as follows:

> "Melanium Ging. Style capitate, hollow, stipules large and foliaceous, often divided, lateral petals turned upwards.
>
> a. Grandiflorae
>
> 1. V. tricolor L., n=13. Petals larger than sepals with labellum under the stigma, corolla deep blue or yellow, pale blue or purely white.
>
> Subspecies genuna Wittr. annual or biennial, large leaves, erect-ascending stems. Cultivated grass fields. A perennial type grows in open coniferous forests.
>
> Subspecies maritima Schweigg. Perennial (hemicryptophyte) leaves smaller than in the first one, more fleshy, flowers also smaller; aspetose growth and often with long prostrate stems. In dunes along the coast most extreme along the western coast of Jutland.
>
> b. Parviflorae
>
> 2. V. arvensis Murr., n=17. Petals smaller than sepals, no labellum under the style, corolla yellowish white. In grass fields."

B. Chromosome number and cytology

Darlington and Wylie (1955) reported the following numbers for

Violas in the Melanium section: V. lutea, 2n=48; V. tricolor, 2n=26; V. saxatalis (alpestris), 2n=26; V. tricolor X lutea, 2n=26-53; V. arvensis, 2n=34.

Clausen (1922 a,b) cytologically investigated Viola and stated that V. tricolor had n=13 and V. arvensis had n=17. He (1924) further studied crosses between these two species, n=13 X n=17, and observed that six pairs of chromosomes conjugate and 18 are unpaired, 17 of which divide at the first metaphase and one remained unsplit. He cited lack of conjugation and splitting of unpaired chromosomes as evidence for increased chromosome numbers and stated a new species can arise from crosses between already existing ones.

Clausen (1922a) explained that this might occur as follows:

> "A species with 13 chromosomes haploid might come into existence from one with 12 chromosomes in the following way: two 12 chromosome forms cross with each other. For one reason or another only 11 of the chromosomes are able to unite in pairs. Therefore the diakinesis of the F_1 may exhibit 11 double chromosomes and two single ones, which split up. After self-fertilization or mutual fertilization in the F_1 there might be a chance to obtain an F_2 with 26 chromosomes diploid, and it might be consistent in the future."

Also following from this idea, Clausen (1927) stated the basic number of the series to be six and other species in the section resulted from crosses followed by deletions or additions of chromosomes within

the groups of hybrids.

Clausen (1927), in further studies, crossed the older garden V. tricolor hortensis, n=13 with the wild type of V. lutea, n=24; the bivalent associations could not be distinguished at metaphase I, but nine univalents were observed. At anaphase I one univalent was observed 10 were splitting or had split. Only one remained undivided. As a result the progeny will have a chromosome number of n=24, similar to the V. lutea Huds. parent.

Clausen (1926) stated cultivated pansies exhibit irregular distribution of chromosomes. There are usually from n=22-26 chromosomes in metaphase I and this thereby agrees in chromosome number with V. lutea Huds. Later Clausen (1927) stated cultivated pansies have as a rule n=24 (approximately), but the gametes in the same plant may have different chromosome numbers.

Gershoy (1928) reported the V. tricolor as 2n=24, V. arvensis as 2n=36, V. cornuta as 2n=42, V. lutea as 2n=48, and the cultivated pansy as 2n=48.

Fothergill (1938) studied wild populations of hybrids of V. tricolor L. and V. lutea Huds. and noted the offspring either resembled V. lutea or were intermediate and also showed hybrid vigor. He stated this was in agreement with Wittrock and his description of the origin of the cultivated pansy. The hybrids were shown to have a chromosome number of 2n equal about 48 with gametes of n=23 to n=26. He illustrated how univalents divided to increase the chromosome number. The univalents that divided were presumed to be of lutea origin, thereby giving more lutea character to the hybrid. He found the plant

tended to stabilize mostly at 2n=48 and some 2n=52. These numbers are the same as one parent and double the other.

More recently, Horn (1956, 1958) investigated the chromosome number of 44 commercial pansy varieties from German, Swiss, English, and American sources. He found a haploid chromosome number of n=24 in pollen mother cells of current varieties as well as older smaller flowered ones. He observed meiotic configurations and found one to eight quadrivalents were present at diakinesis. The average frequency of univalents was less than two percent. When present, univalents split during anaphase I, sometimes a second time during metaphase II. A constant haploid chromosome number of n=24 was found with only these few exceptions.

Horn (1956, 1958) regarded the pansy cytogenetically as V. lutea Huds. with V. tricolor L. characters. The genome construction is AAAABBBB, A and B being the basic complement of six chromosomes, thereby being an auto-allo-octoploid.

Kondo, Matsunami, and Hagiwara (1956) reported in some preliminary notes, working on a limited number of plants, that in the Pansy 'Swiss Giant' of nine plants, eight had 2n=52 and one 2n=46. For Pansy 'Trimardeau' five plants had 2n=52. They also reported V. lutea as 2n=48; V. cornuta, two plants of 2n=44 and one plant of 2n=34; V. cornuta hybrida, two plants of 2n=46 and one plant of 2n=52; V. nigra, 2n=26; and V. arvensis, 2n=34.

Endo (1959) investigated the inheritance of flower color in 'Swiss Giant Pansy' and observed root tip chromosomes and found all varieties had the same chromosome number, 2n=48. Two chromosome pairs had

somewhat globular satellites, that are not always seen because of their small size.

Most recently Huziwara (1966) reported that 'Swiss Giant Pansy' did not have a constant number, but only 10 plants were examined -- three plants had 2n=52, three plants 2n=55 and the remaining four plants 2n=54, 51, 50, and 49 respectively. Also irregularities were found in meiotic divisions.

Investigations into the chromosome number of cultivated pansies can be summarized as follows: n=about 24 (22-26), (Clausen, 1926); 2n=48, (Gershoy, 1928); 2n=about 48 in V. tricolor X V. lutea hybrids, (Fothergill, 1933); 2n=52, 46, (Kondo et al., 1956); 2n=48, (Horn, 1956); 2n=48, (Endo, 1959); and 2n=55, 54, 51, 50, and 49, (Huziwara, 1966).

Horn (1956) speculated on the development of a 2n=48 constant chromosome number as follows: As evidenced by early workers, hybrids between V. tricolor X V. lutea had as well as an enlargement group, a reduction group, that is, garden pansies with n=13. The lack of these plants is due to selection by the breeder toward larger flowering plants. The forms with n=13 chromosomes would probably be smaller blooming ones than the ones with n=24 chromosomes and would be eliminated in selection. Therefore, as the hybrid species adjusted to an increase or decrease in chromosome number from either parent the most desirable were selected resulting in a stable number of n=24.

C. Pollen Morphology and Fertility

Clausen (1922b) described the V. tricolor pollen as ellipsoidal

with a protuberant equatorial belt when seen from the edge. It is the protuberant equitorial belt that gives the edged appearance ranging from three to six but mostly four and five. Clausen (1922b) further stated that V. tricolor was four edged with some plants three to five edged and V. arvensis was five edged with some four or six edged types. Some plants with equal four and five edged types may be hybrid pollen.

Horn (1956) studied pollen fertility by using glycerine gelatine of phenol and methyl green and found fertility to be very high. Horn (1958) stated that not more than five to seven percent of the grains aborted.

Iodine, cotton blue, and aceto-carmine have been used for pollen viability studies but King (1960) believed these to be only a measure of pollen maturity.

Hauser and Morrison (1964) criticized the cotton blue method since the mechanism is not well understood. They suggested the use of a test that will indicate the capacity to carry on oxidative metabolism.

King (1960) reported a new test for determining pollen viability. The test was based on the oxidation of benzidine by peroxidase in the presence of hydrogen peroxide. The reaction in the pollen grains was accompanied by the release of oxygen which was liberated from the hydrogen peroxide by catalase. Two types of viable reactions were reported. In sweet potato (Ipomoea batatas) and sugar cane (Saccharum spp.) intensities of blue color indicated various degrees of viability while non-viable and aborted grains did not become blue. Pollen of irish potato (Solanum tuberosum) and tomato (Lycopersion esculentum) quickly enlarged in size and remained colorless. Non-viable grains became blue

and did not enlarge in size.

Brewbaker (1957, 1959) stated that pollen cytologically are of two types, binucleate or trinucleate. Binucleate grains have a generative and tube nucleus while in trinucleate grains the generative nucleus divides before the pollen is shed giving the three nuclei. In a survey of many plant families Brewbaker (1957, 1959) noted Violaceae as binucleate.

D. Self-Incompatibility

According to Maheshwari (1949), Jost in 1907 working with Cytisus laburnum first described self-incompatibility when he noticed pollen of artifically selfed plants did not germinate.

Brewbaker (1957) stated that self-incompatibility is the inability of a plant producing functional male and female gametes to set seed when self-pollinated. Brewbaker (1959) reported that self-incompatibility is known in 71 families thereby providing them with an outbreeding mechanism.

Sears (1937) described and gave examples of three types of incompatibility as follows: a) before the pollen germinates; b) while the pollen tube is growing in the style; and c) when the tube reaches the ovule. For (a) he noted in Pelargonium hortorum that the germination of incompatible pollen was completely suppressed or if some did germinate they burst soon after penetrating the stigma. For (b) as in Petunia violaceae most incompatible types stop shortly below the stigma and become abnormally thick walled. For (c) with Gasteria he noted compatible and incompatible tubes grow at the same rate and fertilization

takes place but in the incompatible reaction the ovule degenerates.

Brewbaker (1957, 1959) cited two types of self-incompatibility; a) gametophytic, in the pistil usually within the first few hours of pollen growth and associated with an S allele system; and b) sporophytic, on the stigma or soon after germination. He showed binucleate grains are associated with gametophytic self-incompatibility and trinucleate grains with sporophytic self-incompatibility.

Liskens (1959) cited four areas of incompatibility which are: a) at the germination of the pollen grain on the surface of the stigma and the subsequent process of penetration; b) during the growth of the pollen tube through the conducting tissue of the style; c) at the discharge of the contents of the pollen tube into the embryo sac; d) after fertilization by aborting of the fertilized egg cell of the young embryo.

Sears (1937) reported earlier researchers had found immature stigmas more receptive to pollen. He found this true for Brassica. While more recent workers, Ascher and Peloquin (1966) noted that floral aging was correlated with longer pollen tube growth of incompatible pollen in lily.

Smith (1942) reported good pollen tube growth at 25 C on agar with several species. Auxins improved germination and elongation, vitamin B_1 was not helpful, and colchicine was a depressant. He used ten and three percent sugar and 0.75 percent agar medium for the tests.

According to Brewbaker and Majumder (1959) Straub in 1947 developed a semi-vitro technique with excised styles of Petunia inflata L. Straub placed the excised pollinated styles on agar and observed the

tubes that grew out the end. Kwack (1965) adapted Straub's techniques on Oenothera organensis. She found that in the incompatibility reaction calcium was important and also implied the technique was adaptable to incompatibility work in other plants. Linskens (1959) suggested incompatibility should be investigated concerning sugars, amino acids, peptides, and organic salts.

III. MATERIALS AND METHODS

A. Plant material and culture

Twenty-nine inbred breeding lines, obtained from commercial seed companies, were used as the research material. These are described in Table 1. Accessions of Viola spp. and the cultivar 'Swiss Giant' were also used.

Seeds were sown in a sterile medium of one part each of sand, soil, peat, and perlite in sterile pots. They were placed under mist to germinate at 70 F. When the first true leaf expanded the seedlings were transplanted into two and one quarter inch peat pots in the same medium and placed under a bank of fluorescent lights, on a 24 hour regiem, particularly during the winter months. Actively growing young plants were then planted to sterilized five inch clay pots using a soil mixture of one part each of sand, soil, peat, and perlite and placed in the greenhouse at 65 F day and 60 F night temperatures. Ten or twelve plants of each line were maintained in the greenhouse. As the experiments required, selected plants were moved to controlled environment chambers for further study. The plants were fertilized weekly with a 20-20-20 soluble fertilizer applied with a 'Hozon.' at 1/3 oz. per gallon.

B. Microtechnique

Buds of various sizes were killed and fixed in Carnoy's fluid (Baldwin, 1938) and treated according to Johansen (1940) for standard histological sectioning. The sections were stained with Heidenhain's haematoxylin (Clausen, 1927) and microscopically examined to determine when meiosis occurred.

Table 1. The code number and flower color description of the inbred lines investigated.

Number	Description	Generation inbred
V-1	Lavender with streaked petals	P_4
V-2	Solid purple	P_5
V-3	Magenta purple	P_5
V-4	Light creamy ivory with large purple blotch	P_4
V-5	Light lavender with large blotch	P_3
V-6	Medium blue with large blotch	P_4
V-7	Light ivory blue with blotch	P_4
V-8	Medium dark blue with blotch	P_4
V-9	Medium dark blue with blotch	P_5
V-10	Light ivory blue	P_4
V-11	Ivory blue with blotch	P_5
V-12	Slate blue with slight lines	P_5
V-13	Dark rose red with purplish blotch	P_3
V-14	Dark rose with blotch	P_4
V-15	Rose with blotch	P_4
V-16	Dark red with blotch	P_5
V-17	Scarlet red	P_5
V-18	Red and yellow bicolor with blotch	P_1
V-19	Red and yellow bicolor with blotch	P_3
V-20	Plain yellow	P_5
V-21	Yellow with lines	P_5
V-22	Yellow with lines	P_5
V-23	Yellow with dark blotch	P_4
V-24	Yellow with a line blotch	P_4
V-25	Yellow with a line blotch	P_3
V-26	Golden yellow with brown blotch	P_4
V-27	Ivory white	P_3
V-28	Dark rose with blotch	P_4
V-29	Pure white	P_6

Several methods of killing and fixing material for cytological investigation were tried. The most successful technique was that of Ewart's (1957) root tip smear technique modified by Milbocker (1966) for shoot apices. Either apical meristems or developing buds about one mm were placed in vials containing a modified Carnoy's fluid (Baldwin, 1928) consisting of one part glacial acetic acid, two parts 95 percent ethyl alcohol, and three parts chloroform. Flower buds were used for examining PMC meiosis and the shoot apices for somatic chromosomes. The shoot apices were more convenient than root tips and had larger cells. Huziwara (1966) stated he used cells of corolla tissue because the chromosomes were shorter and therefore easier to count. This was also true of shoot apex cells as compared to those found in root tips. The buds or apices were treated in Carnoy's solution for at least 15 minutes to 24 hours. They were removed and hydrolized in a mixture of 1:1 95 percent ethyl alcohol and concentrated hydrochloric acid. The buds or apices were removed after 10 minutes and washed in distilled water for 10 to 15 minutes. Developing anthers were dissected from the buds and placed on a clean glass slide with a drop of aceto-orecin stain (La Cour, 1941). A small section of the shoot apex was placed on a slide in a similar fashion. The tissue was spread with a smearing needle and another drop of aceto-orecin added. A cover slip was placed over the tissue. After several minutes the slide was placed between a paper towel and pressed with the thumb and then tapped with the end of a dissecting needle. The edges of the cover slip were sealed with vaseline to prevent evaporation. Slides were stored up to three days in a refrigerator at 2 C. The slides were examined with a compound

microscope under oil immersion at X1250. Plates that appeared exceptionally clear were photographed for a permanent record.

C. Pollen fertility

Pollen grains were collected by removing the spur petal from the flower and shaking the pollen that had collected on the spur petal onto a slide. A drop of cotton blue stain was added which was made up of equal parts phenol crystals, lactic acid, glycerine, and distilled water (Johansen, 1940). A cover slip was placed over the drop and allowed to stand for 10 minutes. The preparation was scanned at X537 on a compound microscope. Plump, blue stained pollen grains were counted as viable and shrunken or light stained grains were counted as non-viable. Two counts of at least 100 grains each were made for each observation.

The peroxidase reaction (King, 1960) was also used to measure pollen fertility. The test medium sufficient for one petri dish consisted of 10 ml of two percent agar, five ml of hydrogen peroxide, and 1.8 ml of one percent benzidine base dissolved in 60 percent ethyl alcohol. The medium was prepared one hour prior to collecting pollen. The pollen was dusted on the medium as described for cotton blue and allowed to stand at room temperature for approximately 20 minutes. Pollen grains that were plump, and appeared dark blue to black were considered viable while the light blue, orange, shrunken, or distorted ones were counted as non-viable. Two counts of at least 100 grains each were made for each observation under X250 of a stereomicroscope. Three observations were made per inbred line.

Five lines were randomly selected and the flowers tagged an anthesis. The cotton blue test was applied at one, three, five, seven, and nine days after anthesis to determine the effect of aging on pollen viability.

To determine the effect of the environmental variation on fertility, four lines were grown in controlled environment chambers under a 14 hour light regiem and a temperature of 60 F night - 65 F day or a 55 F night and 70 F day. The fertility was measured by the cotton blue test on pollen three days after anthesis.

D. Nucleate condition of pollen grains

Pollen grains were collected by removing the spur petal of the flower and dusting the pollen onto a microscope slide. As described by Sink (1963), a crop of aceto-carmine stain was added and a cover slip placed on top. The cover slip was gently tapped with the wooden handle of a dissecting needle. Observations were made at X537 on a compound microscope. Photomicrographs were taken to verify the observations.

E. Pollen grain length and morphology

Pollen was collected and measured with an ocular micrometer at X125 on a compound microscope. Selected lines were used for these measurements. Large vigorous lines were compared with smaller flowered lines that may have been of a different polidy. Each observation consisted of 50 measurements, and three plants were measured per line.

Morphology was studied by both a compound microscope at X537 and a stereoscope at X250. Photomicrographs were made to illustrate the results.

F. Self-incompatibility and seed set

Self-pollinations were made by removing the spur petal of the flower and inserting the pubescent area where the dehisced pollen had collected, into the stigma and covering it with pollen.

Cross-pollinations were made in a similar manner. Flowers of the female parent were emasculated prior to anthesis by removing the spur petal and taking out the anthers with tweezers. All flowers were tagged at anthesis and all self- and cross-pollinations were made three days after anthesis. Lines V-1, V-3, V-7, V-12, V-13, and V-25 were used in cross- and self-pollination. V-1 and V-13 were very low seed set types. V-3 and V-7 average, and V-12 and V-25 were high.

G. Stylar tissue culture

A stylar culture or semi vitro technique was modified from Kwack (1965) who worked with Oenothera organensis. Flowers were collected the day of anthesis. The styles were dissected out and either self- or cross-pollinated and placed on a drop of culture medium. The medium consisted of one percent agar, 10 percent sucrose, 100 mg/l boric acid, 300 mg/l $Ca(NO_3)_2 \cdot 4H_2O$, 200 mg/l $MgSO_4 \cdot 7H_2O$, and 100 mg/l KNO_3. A large drop of the culture medium was placed on a glass slide and the style placed on it after the medium had cooled. The slide was placed on moist

filter paper in a petri dish for incubation at room temperature for six to eighteen hours. The styles were examined under a stereo-microscope at X250. Photomicrographs were taken for verification.

IV. RESULTS AND DISCUSSION

A. Chromosome number

The chromosome number of the cultivated pansies investigated in this research had a somatic number of 2n=48. Ten lines, V-1, 6, 12, 15, 16, 18, 25, 26, 27, and 28 were counted rigorously. Ten plates of somatic chromosomes were counted for each line and in all cases the chromosome number was 2n=48. The somatic number of 2n=48 is illustrated in Figure 1, A-F. Smears were made of the remaining lines to confirm their number by counting either one or two clear preparations.

Observations of PMC's indicated meiosis occurred in a regular fashion. This is illustrated in Figure 2 A-D. Meiosis was observed on plants growing in controlled environment chambers under a 14 hour light regeim and a temperature of 60 F night and 65 F day or a 55 F night and 70 F day. Meiosis appeared regular in PMC's obtained from plants in both environments.

Cytological preparations were made of accessions designated as V. tricolor and V. lutea to verify their chromosome number and species. All plants labeled V. tricolor or V. lutea had a 2n=26 somatic chromosome number. Since plants with a 2n=48 were not found among the species accessions, the plants designated as V. lutea must have been mislabeled.

The finding of a 2n=48 constant chromosome number in the cultivated pansy is in agreement with Horn (1956, 1958) and Endo (1959) and in contradiction with the findings of Kondo et. al. (1956) and Huziwara (1966).

Figure 1. Somatic chromosomes of pansy breeding lines.

A. Line V-26 showing 2n=48 from apical meristem tissue. X2820.

B. Interpretation of 1-A. X2820.

C. Line V-12 with 2n=48. X4225.

D. Interpretation of 1-C. X8450.

E. Line V-18 showing 2n=48. X4225.

F. Interpretation of 1-E. X5070.

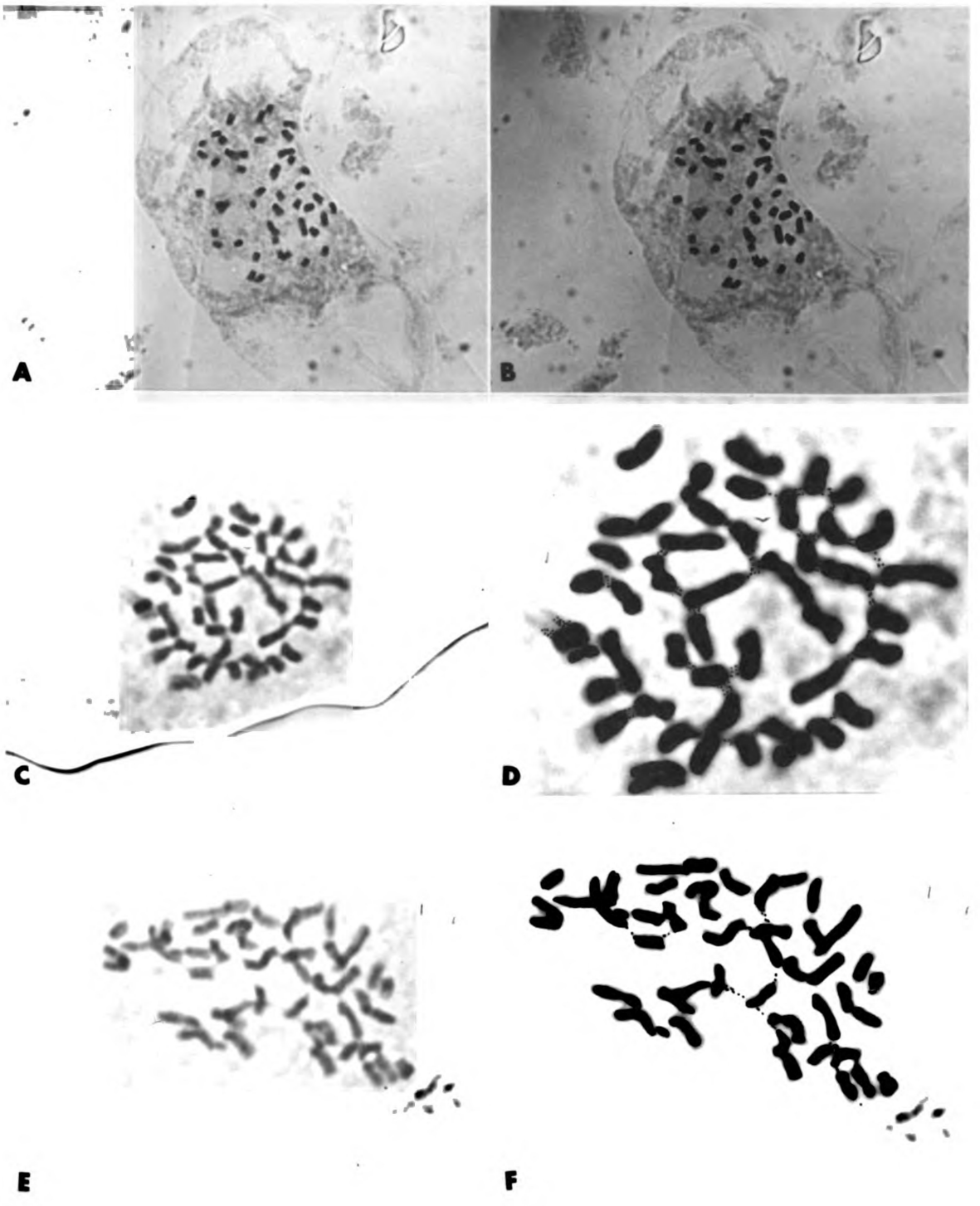

A
B
C
D
E
F

Figure 2. Meiosis of several inbred pansy lines.

A. Anaphase I of line V-6 showing normal meiosis. X4225.

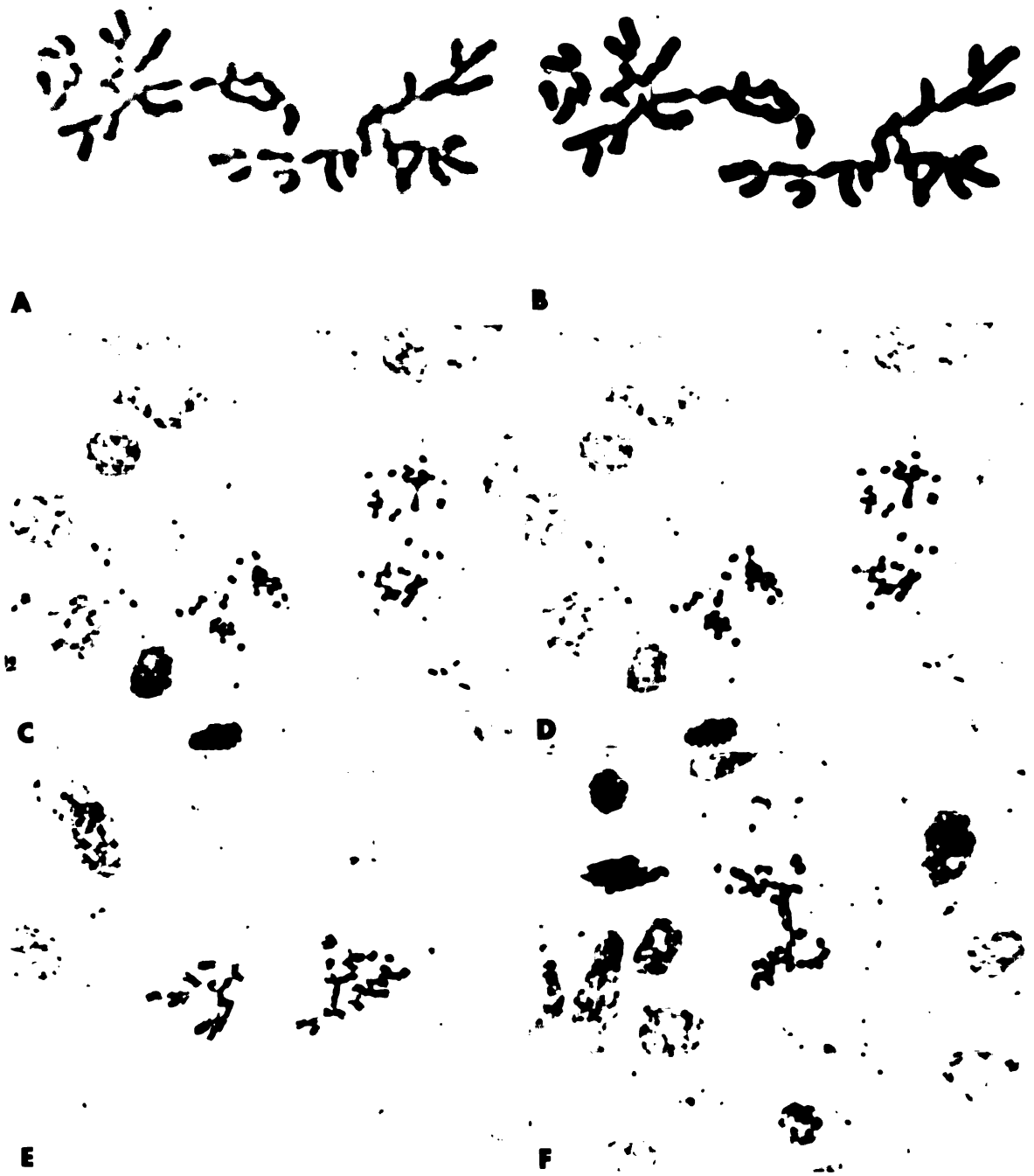
B. Interpretative drawing of 2-A. X4225.

C. Anaphase II showing normal meiosis in line V-18. X2820.

D. Interpretation of 2-C. X2820.

E. Anaphase I of V-18 showing unequal division. Feulgin staining. X2820.

F. Anaphase I showing bridges in line V-18. X2820.


A
B
C
D
E
F

Unfortunately, both Kondo and Huziwara were using small populations for their study and the paraffin method was used for most of their observations. Horn, Endo, and this author used the smear technique and therefore obtained shortened, spread, clear preparations so there was no doubt to the somatic number of chromosomes.

The cytological investigations conducted showed that 2n=48 types occurred and not multiples of the base number nor were aneuploids found in the pansy breeding lines tested. The frequency of fragments and bridges in meiosis appeared to be low. Fig. 2-F shows such a case.

B. Pollen fertility

The pollen fertility was high among the inbred lines tested as indicated by cotton blue staining. The peroxidase test as suggested by King (1960) to measure oxidative metabolism of pollen was used. Percent pollen fertility by both methods was high and in most cases above 90 percent. For example, using the peroxidase reaction, line V-3, had 93.8, 100.0, and 99.5 percent fertility and V-25 had 97.0, 94.8, and 97.6 percent fertility.

It was postulated that because of possible high meiotic irregularities the male gamete would not be fertile. Fig. 2-E shows an unequal meiosis of line V-26. The gametes thus produced because of the unequal number of chromosomes are assumed to be infertile. The male gamete was shown to be fertile as indicated by high viability of pollen with the peroxidase and cotton blue tests. The low frequency of meiotic irregularities and low percentage of inviable pollen seem to be in agreement with each other. These results agreed with those of

Horn (1958) who noted that meiosis was normal resulting in not more than five to seven percent aborted pollen grains.

Pollen sampled at different dates from anthesis showed a slight increase in fertility to the third day and a gradual decrease thereafter. These observations were studied by the cotton blue method. The increase in pollen fertility after it dehisced as seen on the third day is difficult to interpret but as King (1960) and Hauser and Morrison (1964) pointed out, this test is only a measure of pollen maturity. In all cases pollen fertility was high and ample pollen was dehisced by the third day.

Growing plants in controlled environmental chambers had little effect on pollen fertility. This is in accord with the observations of a low frequency of meiotic irregularities. Perhaps the fluctuating environment employed was not sufficient to disrupt meiosis and thereby result in decreased pollen fertility. For example, V-18 plants grown at 60 F night, 65 F day had a percent fertility measured by cotton blue every two days from flowers three days after anthesis as follows: 97.3, 94.2, 98.7, 96.2, 99.0, 91.2 percent. A V-18 plant grown at 55 F night, 75 F day sampled at the same time as the plants in the other environments had 98.5, 94.9, 99.1, 88.5, 97.4, 91.1 percent fertility. Similar results were obtained with the other three lines.

C. Nucleate condition of pollen

The nucleate condition of pansy pollen was difficult to determine due to the high amount of starch present in each grain.

Starch masked the other constituents of the grain. When nuclei were observed the binucleate condition existed, (Fig. 3A). This finding concurs with Brewbaker (1957, 1959) stating that the Violaceae is binucleate. Although only two nuclei were observed it does not eliminate the possibility of pansy pollen being trinucleate. The numerous starch grains in the pollen may have masked the vegetative nuclei and actually the sperm nuclei were being observed.

D. Pollen grain length and morphology

Four lines were selected for pollen grain morphology observations. Two large flowering types, V-15 and V-28; and two small flowering types, V-16 and V-29 were examined. The pollen grain is approximately 0.083 mm long. There were no differences in pollen grain size relative to flower size.

Pollen morphology of the lines observed was essentially as described by Clausen (1922) for V. tricolor and V. arvensis. When observed from the side, the pollen grain was somewhat elongated or oval in shape with small markings on each and as seen in Fig. 3. Polar view of the grains showed they were either four, five, or six sided with five sides the most frequent. This appearance was due to either four, five, or six knobs protruding from the equitorial belt as seen with the stereomicroscope. This is illustrated in Fig. 3-B.

E. Self-incompatibility and seed set

The results of cross- and self-pollination experiments are presented in Tables 2, 3, and 4.

The problem of low seed set was partially solved in that varying

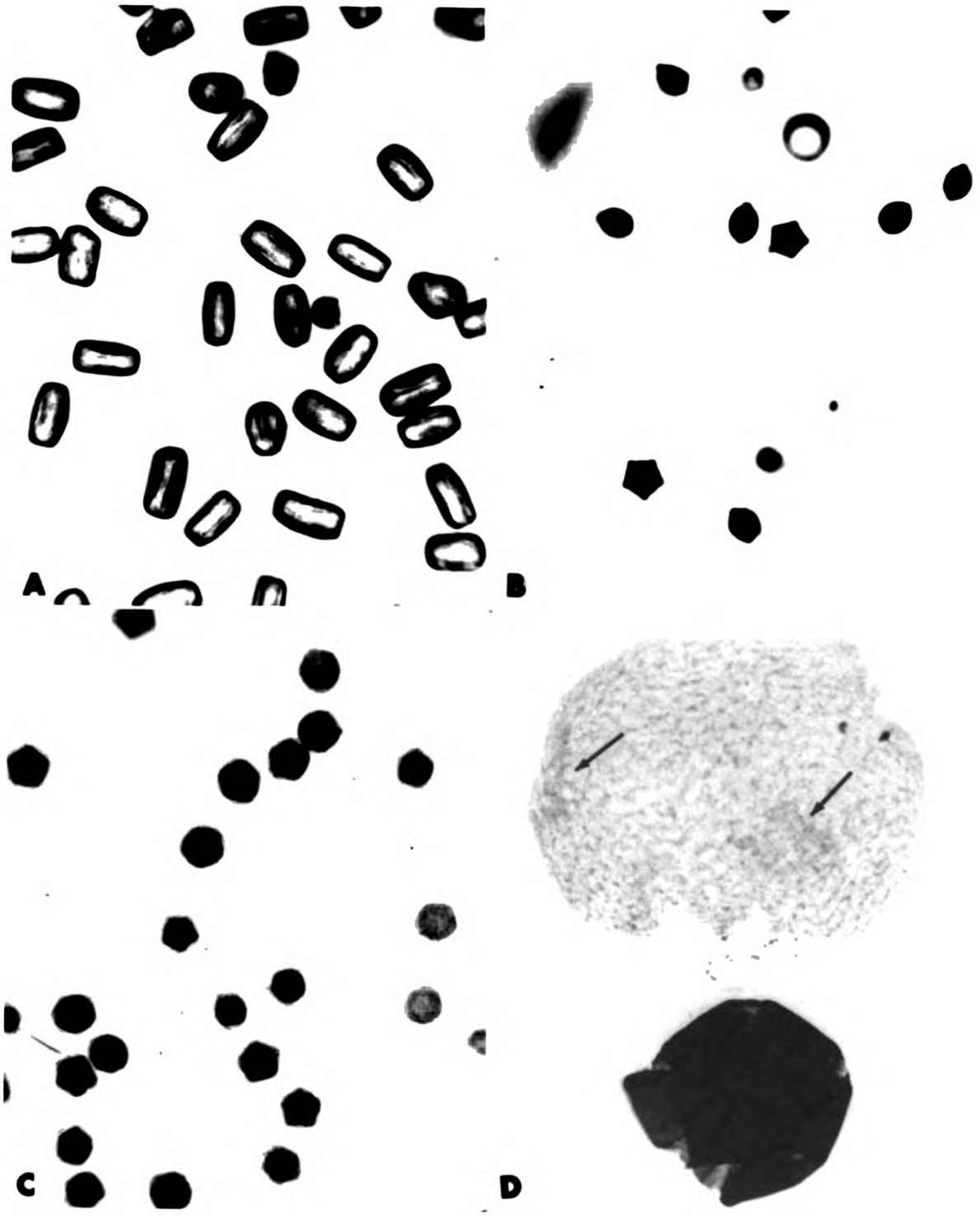
Figure 3. Pollen morphology.

A. Pollen observed from the side. X85.

B. Pollen on the peroxidase test medium showing the five protuberances from the equitorial belt. X85.

C. Pollen stained with aceto-carmine to show the five sided appearance when observed from the end. X85.

D. Pollen stained with aceto-carmine and smeared to show the binucleate condition. Note the abundant starch grains. X360.


A
B
C
D

chromosome numbers and low pollen fertility as indicated by staining tests did not appear to be the cause of low seed set. Additional investigation suggested self-incompatibility as a factor in the problem. It can be noted that cross-pollinations in most cases had higher seed set than self-pollinations. Also, when a line which had low seed in self-pollination such as V-1 or V-13 was used as a male parent on other lines, seed set was higher. This type of result may indicate a self-incompatibility system in the inbred lines tested. Seed set ranged from below 10 percent on some lines to above 90 percent on others indicating that if such a system exists it is only partial self-incompatibility.

Tables 2 and 3 show seed set results when line V-1 was used as a female parent and a male parent. Out of 84 crosses using 19 pollen sources, there were only 25 crosses or 30 percent set seed on V-1. Pollen of V-1 used on 12 different female parents making a total of 43 crosses, 37 crosses or 86 percent were successful indicating low seed set with the female parent.

Table 4 shows lines V-3 and V-13 with low self seed set and V-7 and V-12 with high self seed set. When pollinated by line V-25, seed set was increased in the low seed set lines and decreased in the high seed set lines. To gain further evidence on a possible mechanism for low seed set, 20 crosses each were made with V-3 and V-13 as the male parent with high self seed set line V-12 as the female parent. With V-3, 18 out of 20 set seed or 90 percent and with V-13, 17 out of 20 set seed or 85 percent. Both are high as compared to the self-pollination of 2.6 percent seed set on V-3 and 5.6 percent on V-13. Again

Table 2. Percent seed set when line V-1 was the female parent with several lines as the male parents.

Line	Number of crosses	Number set	Percent set
V-2	4	2	50
V-3	5	0	0
V-4	4	2	50
V-5	5	1	20
V-7	5	0	0
V-9	4	2	50
V-11	4	0	0
V-12	3	1	33
V-13	4	1	25
V-15	4	1	25
V-16	4	1	25
V-17	5	1	20
V-18	5	2	40
V-20	6	2	33
V-23	4	2	50
V-24	6	2	33
V-25	6	3	50
V-28	2	0	0
V-29	5	2	50
Total	84	25	

Average 30 percent seed set

Table 3. Percent seed set when line V-1 was the male parent with several lines as the female parents.

Line	Number of crosses	Number set	Percent set
V-2	2	2	100
V-3	6	4	67
V-4	2	2	100
V-7	3	2	67
V-9	2	1	50
V-12	5	5	100
V-15	3	2	67
V-16	6	6	100
V-18	1	0	0
V-20	6	6	100
V-23	5	5	100
V-28	2	2	100
Total	43	37	

Average 86 percent seed set

as in V-1, this data possibly indicates the female parent was responsible for low seed set.

F. Stylar tissue culture

Pollen placed on excised styles in semi vitro in most cases did not grow through the style into the agar medium. Tube growth was observed sporadically. Since no results occurred with Kwack's (1955)

Table 4. Percent self- and cross-pollination seed set on four lines with V-25 as the male parent.

Line	Number of self pollinations	Number set	Percent set	Number of cross pollinations	Number set	Percent set
V-3	39	1	2.56	33	4	12.12
V-7	38	12	31.58	34	5	14.71
V-12	40	27	67.50	39	23	58.97
V-13	36	2	5.56	27	12	44.44

method on Viola, investigations on the germination of pollen in vitro were made. The medium used by Kwack proved unsuccessful in germinating grains as did the other mediums.

It was noticed that grains placed on agar medium showed no sign of germination but after a time they aborted and the abundant starch grains were located outside the cell. Brewbaker (1957, 1959) noted lack of germination of pollen in vitro is a characteristic of trinucleate pollen grains while binucleate grains, which appeared to be the case here, germinate rather easily.

G. Suggestions for additional investigations

The problem of low seed set has not been resolved except that varying chromosome numbers apparently did not result in infertile gametes. The female parent appeared to be important in the problem. Further investigations into self-incompatibility may provide more

information on this problem. Specifically the four areas cited by Liskins (1959): pollen germination, pollen tube growth, the fertilization of the egg, and subsequent development of the zygote and endosperm: can be investigated both morphologically and physiologically.

The author does not attempt to explain the results of lack of pollen growth in semi vitro and in vitro but they could be employed as a starting point for further investigation.

V. SUMMARY AND CONCLUSIONS

1. The chromosome number of 29 inbred breeding lines of *V. tricolor* Hort L. was determined to be 2n=48. Meiosis was observed to be regular. Varying chromosome numbers apparently did not result in infertile gametes - or if they did the percentage was low.

2. The cotton blue and peroxidase tests indicated pollen fertility to be above 90 percent for the lines tested. This concurs with the observed frequency of meiotic irregularities. Pollen fertility studied in relation to fluctuating controlled environments was found to be similar under both environments.

3. The nucleate condition of the pollen appeared to be binucleate. Morphologically, the pollen grain was oval when seen from the side and usually four or five edged when observed from the end.

4. Observations on seed set indicated it varied among the inbred lines. In some cases it was high and others low for both inbreeding and crossing. Because of the higher seed set by out crossing it is proposed that an incompatibility mechanism exists.

5. Preliminary work on the incompatibility mechanism was attempted by semi vitro studies of excised styles and stigmas. Although these proved unsuccessful, they did indicate that pollen did germinate readily on the stigma surface and grow through the style.

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