Cytological Investigation of Parents, Offspring and Backcross Derivatives Involved in the Interspecific Cross *Phaseolus lunatus* L. X *P. polystachyus* (L.) B.S.P.

By
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INTRODUCTION

Phaseolus lunatus L., the lima bean, is an important vegetable legume of the United States. Florida once used to be one of the leading states in the production of the lima bean but over the past several years there has been a considerable decline in its acreage in this state. Still nearly two thousand acres are planted to this crop annually (12).

There are the large-seeded (macrospermus) and the small-seeded (microspermus) varieties of lima beans. The large, thick-seeded type represented by the commercial variety Fordhook is regarded as having the best table quality (51). One of the problems encountered in the production of Fordhook type lima beans in encrusted or impacted soil is the mechanical injury to the seedling at the time of emergence. Due to epigeal germination of the lima bean, the cotyledons have to be pushed out of the soil as the hypocotyl elongates (Figure 4). During this germination process the massive cotyledons are often broken away from the axis, resulting in "bald heads" breakage of hypocotyl, and reduced stand and vigor of seedlings.

The damage at germination might be avoided if it were possible to breed a Fordhook-type lima bean with a hypogeal character of germination. With this as the primary object,

attempts have been made to produce interspecific crosses between the lima beans and other closely related species having the hypogeal character of germination (16, 26). It is conceivable that the lima bean has affinities with the other New World species of Phaseolus. This view is supported by the recent evidence presented by Mackie (31) to the effect that lima bean is a native of Guatemala rather than of Africa as was believed by Linneaus.

Most of the crosses so far attempted between the lima bean and the other American species of Phaseolus have not been successful. There was one, however, the primary success of which was reported by Lorz (26). It involved Phaseolus lunatus L. var. Fordhook x Phaseolus polystachyus (L.) B. S. P. The latter species is the wild thicket bean which is a native of the eastern United States. According to Small (44) its geographical distribution extends from Florida northwards to Maine and westward from the Atlantic seaboard to Texas and Nebraska. The cotyledons of this species remain entirely underground during germination and all the growth of the shoot takes place by the elongation of epicotyl and the plumule (Figure 4). Lorz (26) also considered the possibility that in addition to the valuable germination character other desirable characters might be transferred in such a cross: e.g., possible unrecognized disease and pest resistance, vegetative vigor, perennial cold-hardy rootstock and other physiological attributes of survival value.

The hybrid between these two species expressed nearly completely hypogeal germination (Figure 4). It also showed a high degree of self- and cross-sterility. Following the primary success with the cross reported by Lorz (26), a cholchicine-induced chimera was obtained by Lorz (29) in F_1 vegetative material in which the amount of apparently viable pollen was increased from approximately 5 per cent in the untreated to approximately 90 per cent in the chimera and the average volume of the individual, apparently viable pollen grains was doubled. Lorz (27) also described fifteen individuals resulting from Fordhook pollinated by the F_1 and observed what he regarded as an evidence of linkage involving an association of the character of hypogeal germination and that of vine habit, both characters derived from P. polystachyus. All this material was subsequently lost without any further work (29). Following a repeat of the original cross two F_2 plants were, however, derived as a result of open pollination. One seed was also produced in pollinating the F_1 with Fordhook type pollen, thus establishing a backcross with Fordhook lima ancestry. From one of the two F_2 plants and from the single backcross individual further progenies and backcrosses were derived.

The need for cytological study of this material was indicated by the lack of any marked homology between the chromosomes of the two species, as exhibited by cytological examination of F_1 (10), and also by the high fertility of F_2 and later generations. The determination of the chromosomal mechanisms involved in this material should be of considerable value in designing a breeding

program for the utilization of this material.

It is now unquestionably recognized by biologists that cytology is as great an ally of taxonomy as it is of genetics. At present there is little information on the chromosome morphology of the species involved. Therefore, besides the above mentioned practical considerations, cytological analyses of these derivatives and karyological studies, per se, would be highly valuable from the point of view of taxonomy.

This study is thus primarily concerned with the cytological investigation of two Phaseolus species, namely, P. lunatus and P. polystachyus; the F_1 to F_4 generations from this cross, and the backcross generations from the hybrid with P. lunatus (recurrent). An attempt has been made to establish the karyotype of the two parents, the chromosome numbers of the derived material and the homologies and association affinities as indicated by meiotic behavior. These studies were also supplemented by pollen counts and size measurements.

REVIEW OF LITERATURE

Interspecific hybridization is not a new phenomenon in plants. Cytotaxonomical studies have revealed that hybridization is of widespread occurrence in nature. Several instances have also been found where a cross between two species has been followed by a spontaneous reduplication of the chromosome complement of the diploid hybrid giving rise to an allopolyploid or an amphidiploid. According to some authors this phenomenon has played a significant role in the process of evolution (11, 47). There is now ample evidence to show that some of our important crop plants, wheat, cotton and tobacco have originated in this way (11).

Interspecific hybrids have also been produced artifically. Plant breeders often resort to interspecific hybridization in order to incorporate "new" genes in the cultivated plants. Such hybrids are also produced experimentally to augment cytotaxonomical studies for tracing relationships between species, genera or other taxonomic groups.

In this section the cytological aspects of hybridization and amphidiploidy will be surveyed briefly. A brief review will also be made of the available literature on interspecific hybridization in the genus Phaseolus.

Allopolyploids

Though there is a series of intermediates, allopolyploids at the tetraploid level have been classified into two general types by Stebbins (46). The first of these types is called the typical allopolyploid or amphidiploid and the second as segmental allopolyploid or segmental amphidiploid. A typical allopolyploid is one in which there is no intergenomic pairing of the chromosomes and the identity of parental genomes is preserved. Most representatives of this type are highly constant because of allosyndesis or pairing between homologous chromosomes.

The second type of amphidiploid or allopolyploid classified by Stebbins involves a partial or complete intergenomal pairing of chromosomes.

Typical allopolyploids

Karpechenko's (22) Raphanobrassica derived from the intergeneric hybrid Raphanus sativus L. ($n = 9$) x Brassica oleracea L. ($n = 9$) is a classical and perhaps the earliest recognized example of an experimental amphidiploid. The F_1 from this cross had 18 univalents, 9 from one parent and 9 from the other. During metaphase of the first division the chromosomes were distributed at random to the two poles. The F_1 plants were highly sterile and only a few seeds were produced. Cytological examination showed that there were 36 chromosomes in F_2 somatic cells. The F_2 was assumed to have arisen from the union of unreduced gametes. Meiosis in the tetraploid plant was found to be regular. Eighteen bivalents appeared

at metaphase I, 9 originating from the pairing of Raphanus homologs and the other 9 from Brassica homologs, and disjunction was normal. The gametes produced by the tetraploid carried 9 Raphanus and 9 Brassica chromosomes. The tetraploid plants were fully fertile. All the progeny from this cross were uniform in appearance and no segregation was observed.

A similar case of spontaneous amphidiploidy was reported by Buxton and Newton (4) while investigating the cytology of F_2 from the species cross Digitalis ambigua x Digitalis purpurea. The n and $2n$ number of both the species was 28 and 56, respectively. It was discovered that while the F_1 was a diploid the F_2 had 56 and 112 chromosomes in gametic and somatic cells, respectively. It was concluded that the doubling of the chromosomes had occurred due to the union of unreduced F_1 gametes. The unreduced gametes were assumed to result from failure of reduction division and the formation of restitution nuclei. The F_2 generation differed from F_1 principally in size and fertility. There was no segregation of parental characters.

Segmental allopolyploids

A great deal of work has been done on Primula kewensis, a diploid hybrid of P. floribunda x P. verticillata ($2n = 18$) and its derivatives. Newton and Pellew (34) have studied the cytology and genetics of the fertile tetraploid progeny of Primula kewensis with $2n = 36$ chromosomes. Their investigation shows that doubling took place in the somatic tissues of the hybrid.

Meiosis, both in the diploid P. kewensis and its tetraploid derivative was found to be regular but only the tetraploid was fertile. The high degree of fertility and constancy of the tetraploid has been explained by Winge's hypothesis of interspecific pairing of the chromosomes. A sporadic variation of the tetraploid was observed and has been explained as the possibility of some intraspecific pairing of chromosomes. It has been found to be associated with the loss or gain of a chromosome. There was also some variability in the tetraploid which was not so associated and was probably allelic.

They also observed a plant with 26 somatic chromosomes. It was derived from a presumed triploid by self-fertilization. In meiosis it formed 10 bivalents and 6 univalents. It was used as a male parent for a backcross with floribunda, and produced descendants with combined characters of verticillata and floribunda. Artificial triploids were also obtained by crossing diploid varieties of floribunda with the tetraploid.

Ichijima (19) investigated the cytology of tetraploids originating from selfed progeny of an F_1 plant of the cross Fragaria bracteata Heller ($n = 7$) x Fragaria vesca rosea Rost. ($n = 7$). In the tetraploids 14 bivalent chromosomes were found at metaphase I. Root tip counts showed 28 somatic chromosomes. It was believed by Yarnell (53) that this F_1 plant originated as a chromosomal chimera.

Meiosis of F_2 tetraploid was found to be irregular. Four chromosomes were occasionally found to be associated at

metaphase I. It was deduced from genetical evidence that a chromosome of F. vesca rosea seems to pair as readily with the corresponding one from F. bracteata as with the F. vesca rosea homolog. Anaphase I showed occasional lagging. The second anaphase sometimes showed an early disjunction of a chromosome pair. Meiosis in some of the F_3 plants showed an increased amount of irregularity, especially at anaphase I.

Jones and Clarke (20) reported the discovery of a natural, fertile amphidiploid from a cross between Allium cepa L. var. Australian Brown x Allium fistulosa L. type Nebuka. It shows greater vegetative vigor than either parent and is perennial like type Nebuka. Meiotic behavior is fairly regular. Sixteen bivalents ($2n = 16$) were generally formed at metaphase I. Fragments, micronuclei and chromatin bridges were observed but were not as common as in the diploid hybrid.

Another example of natural segmental amphidiploid is that derived from the hybrid Nicotiana glutinosa ($n = 12$) x Nicotiana tabaccum ($n = 24$) reported by Clausen and Goodspeed (6). In the diploid hybrid there were bivalents as well as univalents at metaphase I. During anaphase I the bivalent partners approach the poles while the univalents remain in the equatorial zone either dividing or preparing to divide. In the tetraploid, on the other hand, there appeared to be no univalent chromosomes and the bivalent partners (36_{II}) moved in regular fashion to the poles. The amphidiploid was uniform and constant. The authors believed that the F_1 must have arisen from a doubling of

the chromosome number immediately or soon after fertilization by which a tetraploid hybrid with 36 pairs of chromosomes was produced. This plant might be represented by the chromosomal formula 12 GG + 24 TT.

Skirm (43) experimentally produced a polyploid by thermal treatment of the natural hybrid Tradescantia canaliculata x T. humilis. One of the 4n derivatives from this treatment showed primarily bivalent chromosomes pairing during meiosis. The origin of this plant was attributed to chromosome doubling following fertilization. The argument presented in favor of this view is that most Tradescantias are heterozygous in respect to chromosome structure. The production of a tetraploid from such a stock by meiotic irregularities resulting in diploid gametes would produce a plant in which no 2 of the 4 genomes would be identical. Thus a lack of preferential pairing would favor quadrivalent formation. In this particular plant, however, the meiotic behavior suggested that chromosome doubling occurred at the time of fertilization or during embryonic development. In other words it had been a case of somatic doubling.

Kostoff (23) made extensive studies on the cytological behavior of Nicotiana glauca (n = 12) x Nicotiana langsdorffii (n = 9) amphidiploid. He reported differences in the chromosome morphology of the two parental species. The F_1 hybrids usually had 21 somatic chromosomes. It was reported that N. glauca chromosomes had small segments homologous with portions of N. langsdorffii chromosomes.

Another important observation made by the author was the formation of chromosomes with new genetic contents. This was supposed to be due to exchange of parts in bivalent and trivalent groups during the meiosis of F_1 hybrids following allosyndesis and, in exceptional cases, following autosyndesis between homologous segments of the partially homologous chromosomes and probably between heterochromatic regions of nonhomologous or partially homologous chromosomes.

The F_1 hybrids were self-sterile. Most of the backcrosses to N. langsdorffii had 30 somatic chromosomes. The meiosis of these backcrosses varied from plant to plant in irregularity. These plants also showed variation in morphology and fertility. One amphidiploid was also obtained in the backcross. The original amphidiploid type was studied through six generations and it was discovered that the viability of the pollen increased with each successive generation till it was 99.5 per cent in F_6. This was correlated with a corresponding decrease in the number of multivalent and univalent associations.

Euploid chromosome alterations led ultimately to changes in the size of nuclei and cells. The addition of each genom led to a significant increase in size. It was also observed that aneuploidy conditioned changes in the size of nuclei and cells but such changes were not always significant. Also, the amphidiploids originating from the asyndetic F_1 had normal syndesis and were highly constant, while those originating from F_1 hybrid with complete or partial allosyndesis were not

constant. It was postulated that changed mutation rate and chromosome rearrangements in the amphidiploid were responsible for the gradual increase in its fertility. The amphidiploids and their derivatives were physiologically isolated. They were reported to cross either with difficulty or not at all with other species and species hybrids or with the parental species.

Interspecific Hybrids in the Genus Phaseolus

A number of interspecific crosses have been attempted in the genus Phaseolus. The earliest record of such a cross is that made by Mendel in 1866 (15), between Phaseolus vulgaris L. and Phaseolus multiflorus, Lem., a runner bean. This cross was later repeated by other workers. Lamprecht (24) made extensive genetical and cytological study of the cross P. vulgaris x P. multiflorus. He discovered that though meiosis of the F_1 ran a normal course, there was a high degree of pollen sterility. The degeneration of the pollen grains occurred following telophase. As a result of this, the F_2 showed a loss of multiflorus characters and was consequently matroclinous to a great extent. This was explained by the fact that the new gene combinations were not viable in the existing plasma.

In the later generations it was found that a combination of vulgaris and multiflorus properties met with greater resistance as attempts were made to introduce more genes into the combination. Eventually, from some of the later generations could be selected

entirely constant and fertile lines containing combinations of taxonomic characters of both species. Thus a bridge was thrown across the species boundary between P. vulgaris and P. multiflorus.

A cross between P. vulgaris L. and P. mungo L., the Urd bean, an oriental species, has been reported by Strand (48). The main objective in this breeding program was to secure an offspring that was resistant to the Mexican bean beetle. The cross was successful and an F_3 generation was raised.

Three different interspecific crosses in Phaseolus have been made by Honma and Honma and Heeckt. The first of these crosses made in 1956 (15) involved P. vulgaris x P. acutifolius. Chromosome smears of the root tips showed no morphological differences between the complements of the hybrids and the parents. The second cross reported in 1958 involved P. coccineus x P. lunatus (16). This was an effort to transmit the hypogeal germination habit into P. lunatus, the lima bean. The F_1 seedlings exhibited the hypogeal germination habit and, in general, the vegetative characters appeared more like P. coccineus parent. The segregation for germination habit ranged from epigeal to hypogeal suggesting a polygenic inheritance. The third cross attempted in 1959 (17) was between P. vulgaris and P. lunatus. The object was to combine a darker seed coat color with green cotyledons in snap beans.

Several interspecific crosses were attempted by Lorz (28) in the genus Phaseolus from time to time. They are enumerated below

P. vulgaris x *P. coccineus*
_____ x *P. polyanthus*
_____ x *P. xanthotrichus*
_____ x *P. glabellus*
_____ x *P. acutifolius*
_____ x *P. lathyroides*
_____ x *P. atropurpureus*
_____ x *P. aureus*
_____ x *P. mungo*
_____ x *P. angularis*
_____ x *P. calcaratus*
_____ x *P. helvolus*
_____ x *P. speciosus*
_____ x *P. lunatus* (and reciprocal)
P. lunatus x *P. polystachyus*
_____ x *Phaseolus* sp. (PI No. 201205)

Many of the above noted crosses were not successful. In several cases sterility was encountered in the F_1 hybrid.

A preliminary report on the success of *P. lunatus* x *P. polystachyus* appeared in 1952 (26). The F_1 was vigorous but almost completely self-sterile. Following the primary success of the above cross, a colchicine-induced chimera was obtained by Lorz (29) in the F_1 vegetative material. The chimeral tissue

produced pollen grains having approximately double the volume of those produced by the untreated sector. It also showed about 90 per cent apparently viable pollen as compared to about 5 per cent in the untreated tissues. These observations constituted presumptive evidence of a successful induction of polyploidy in the chimera. Few good pollen grains from F_1 were used to obtain backcrosses to P. lunatus var. Fordhook, the chief objective was to introduce hypogeal germination into P. lunatus.

Dhaliwal et al. (10) investigated the cytology of the hybrid P. lunatus var. Fordhook x P. polystachyus and the parent species. It was found that both parents show a normal meiotic behavior without any irregularity and almost all the pollen grains were capable of germination. In the hybrid, on the other hand, a number of meiotic irregularities such as nonpairing of chromosomes, early disjunction, the presence of bridges and laggards with unequal distribution of chromosomes at anaphase I, and polyspory were observed. In a majority of pollen mother cells, 6 bivalents and 10 univalents were observed at metaphase I. An occasional quadrivalent was also observed. Some of the chromosomes did not become oriented on the equatorial plate. The anaphasic distribution of the chromosomes ranged from 5/17 to 8/14 in abnormal pollen-mother-cells. Pollen was extremely variable in size. Apparently fertile pollen grains were rarely found. It was concluded that these meiotic irregularities were responsible for the high pollen sterility

observed in the hybrid plants.

Dana (8) has produced several interspecific hybrids in Phaseolus. Some of these hybrids have been treated with colchicine in an attempt to produce amphidiploids. These were:

P. aureus x P. mungo
_____ x P. ricciardianus
_____ x P. calcaratus
_____ x P. trilobus
P. auricus x P. ricciardianus

A fertile sector appeared on the colchicine treated hybrid P. mungo x P. auricus. An amphidiploid branch of spontaneous origin was also reported in the hybrid P. aureus x P. trilobus. Cytological studies revealed the chromosomes mostly forming 22 bivalents. One or 2 quadrivalents were observed in a few cells. The progenies of natural and artificial amphidiploids were wholly alike in morphological character.

MATERIALS AND METHODS

For the purpose of convenience this section is subdivided into separate subsections, each dealing with a particular material, equipment or technique.

Plant Material

All the materials studied were derived from the reinvestigation, begun in 1958, of the parent stocks of Phaseolus lunatus L. var. Fordhook x Phaseolus polystachyus (L.) B. S. P., and the pure line and backcross (Fordhook recurrent) resulting from crosses between these two parent stocks. This material was available for cytological studies from the Phaseolus interspecific hybridization program in progress in the Department of the Vegetable Crops, at the University of Florida Agricultural Experiment Station.

The material studied falls into 11 categories as described below and as illustrated in the accompanying pedigree (Figure 1). The number identifying each category will be used henceforth for further reference for the sake of convenience.

1. Phaseolus lunatus var. Fordhook progeny.
2. Phaseolus polystachyus. Wild individuals presumed to be identical to the original polystachyus parent because of conformity to the species description given in Small (44). This species

is a native of this region as it is to the other parts of eastern United States. It grows abundantly in patches in the woods around the Horticultural Unit of the Agricultural Experiment Station. The growing season extends from summer to early fall with flowering beginning with the start of the short days in fall. The bud material was collected early in fall and the seeds were collected later for obtaining root tip smears.

3. P. lunatus var. Fordhook x P. polystachyus, the F_1 hybrid. The original cross was made in 1952. This material was subsequently lost. The cross was repeated in 1958 and again in 1961. The plants utilized in the present study were those produced in 1961. Three F_1 individuals were available for study.

4. P. lunatus var. Fordhook x P. polystachyus F_2, "fertile." There was a single individual in this category. It was relatively fertile and was found to be an amphidiploid.

5. P. lunatus var. Fordhook x P. polystachyus, F_2, "sterile." This F_2 individual, was diploid and was a highly sterile plant.

6. F_1 (No. 3 above) x P. lunatus var. Fordhook, the first backcross was made in 1958 using Fordhook as the pollen parent. There was a single individual representing this backcross. It was a perennial which produced seeds sparingly.

7. The progeny from presumed selfing of the first backcross (No. 6 above). There were six individuals in this progeny.

8. Number 6 x P. lunatus var. Fordhook is the first generation from the second backcross (Fordhook recurrent). Twenty-seven individuals from this generation were available for study.

9. Progeny of No. 8 is the second generation from the second backcross (Fordhook recurrent). This category comprised of 14 plants derived from several different BC_2 individuals.

10. P. lunatus var. Fordhook x P. polystachyus, the F_3 progeny consisting of 7 plants derived from the amphidiploid F_2 plant.

11. P. lunatus var. Fordhook x P. polystachyus, the F_4 progeny derived from the F_3 amphidiploid. This generation is contained by 10 plants.

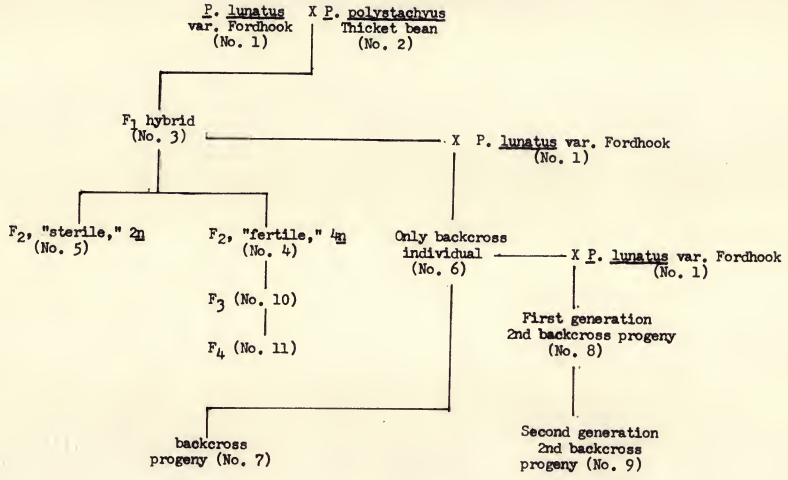

Figure 1.--Pedigree of material investigated. Numbers in parenthesis refer to text description (page 17 and 18) and will be used henceforth for the purpose of identification.

Technique

The material is rather difficult to handle cytologically. No standard technique has been evolved so far. Several workers have employed and proposed different schedules for fixing, staining and mordanting or for otherwise treating difficult plant materials. Since different materials do not respond alike to different treatments and reagents, a preliminary trial was conducted to determine the most satisfactory combination of treatments both for the pollen-mother-cells and the root tips. The procedures used for these 2 kinds of materials are discussed separately as follows.

Pollen-mother-cells

Preliminary examination showed that the anthers of *Phaseolus* are quite small, and that each anther contains a relatively small number of pollen-mother-cells. Microsporogenesis takes place when the buds are quite small (< 2 mm long). The buds were fixed at 2-hour intervals during the day from 6:00 A.M. to 4:00 P.M. in order to determine whether a particular time was most favorable for optimum production of meiotic division. It was found that the divisions were not restricted to any particular time and no single period was conspicuously more favorable than any other. However, there seemed to be a relatively larger number of dividing cells at about 6:00 A.M. during the months of June and July. There was an apparent shift in this time towards 8:00 A.M. with the approach of

colder weather. Consequently an attempt was made to fix as much of the bud material as possible close to the 8:00 A.M. hour.

Fixatives

The most common fixative recommended for pollen-mother-cells intended for acetocarmine squash preparations consists of a mixture of three parts of absolute ethyl alcohol to one part of glacial acetic acid (Belling 2). The original formula of Belling has since been modified and adapted for more intense staining of small chromosomes. It was thought desirable to make a preliminary trial with 3 different fixatives, namely, Belling and the modifications proposed by Swaminathan et al. (49) and by Hyde and Gardella (18). Hyde and Gardella's modification was found to give the most intense staining as well as increased differentiation of details. It was, therefore, used with slight changes as a basis for the following preparation of the fixative most widely used in this investigation. The procedure used was as follows:

1. An excess of 5 gms of ferric chloride was dissolved in 300 ml of distilled water until a thick precipitate of $Fe(OH)_3$ resulted.
2. The precipitate was thoroughly washed in a Buchner funnel, dried in a desiccator and stored in a refrigerator in a sealed container.
3. Two grams of the $Fe(OH)_3$ thus prepared was dissolved in 50 ml of propionic acid by gently heating.
4. This was filtered and 50 ml absolute ethyl alcohol was then added to the cooled filtrate. The addition of a few drops of acetocarmine to the above mixture, as recommended by Hyde and Gardella (18), was omitted as it invariably precipitated iron.

The whole inflorescence containing buds in a graded series of stages of maturity was placed in the fixing fluid, so that it was easily possible to determine which size was near optimum for meiosis. Consequently, it was possible to select for squashing only those buds which were near the optimum. The fixation was quite satisfactory with such small buds and it was not necessary to remove the floral coverings.

The optimum period of fixation was found to be from 12 to 24 hours. If the buds were not immediately used they were placed in 70 per cent ethyl alcohol and stored in the refrigerator.

Staining

Two different stains were tried; a 1.5 per cent orcein solution in 45 per cent glacial acetic acid prepared according to Vosa (52) and acetocarmine in which iron was introduced as a mordant. The latter was found to give decidedly superior preparations with respect to intensity as well as differentiation. Acetocarmine was prepared by dissolving 1 gm of carmine in 100 ml of boiling 45 per cent glacial acetic acid and refluxing for one hour. To half of this was added a few drops of $Fe(OH)_3$ solution in 45 per cent acetic acid until the liquid became bluish red without any visible precipitate. This was then combined with the rest of the untreated acetocarmine. Acetocarmine staining was adopted for all subsequent work with the pollen-mother-cells. Except for a few camera lucida sketches, all other drawings and photomicrographs were prepared using this procedure.

Squashing

All the buds were detached from the axis and placed on a flat bottomed watch glass containing 70 per cent ethyl alcohol. Those of near optimum size were sorted out for squashing. In order to obtain as many as possible of the pollen-mother-cells, all the anthers of a bud were squashed under a single coverslip. The bud was dissected under a binocular microscope in a drop of the stain fixative on a slide and all the anthers taken out with the help of a needle. The anthers were then mashed thoroughly with the blunt end of a scalpel in as small a quantity of the stain fixative as practicable because an excess of the stain would not only allow the anthers to float and escape squashing but would dilute the suspension of pollen-mother-cells and permit many to escape from under the coverslip.

All anther debris was excluded from the film beneath the coverslip by touching one edge of the coverslip to one edge of the drop containing the squashed anthers (Figure 2). Then, as the coverslip was allowed to rest flat against the slide, all the supernatant liquid along with the suspended pollen-mother-cells was drawn underneath by capillarity while the coarser debris remained behind. The slide was then heated gently over the flame of a spirit lamp, pressed lightly between bibulous papers and sealed with a 1:1 mixture of paraffin and Canada balsam. These preparations were made permanent by the quick freezing method of Conger and Fairchild (7).

For studying the prophase chromosomes the postmordanting method suggested by MacDonald (30) was employed.

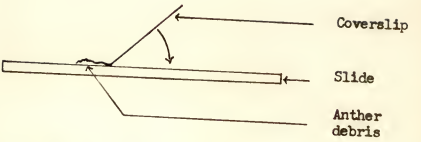

Figure 2.--Method for excluding anther debris.

Preparation of root tips

The root tips were obtained from two sources, germinated seedlings and rooted cuttings. The seeds were germinated between moist paper towels and the actively growing root tips were taken as they appeared. Later the seedlings were transferred to plastic pots about 4 inches square, 4 inches deep with a central hole in the bottom with a nylon wick inserted through the hole. The pots were filled with a well-prepared sterilized soil and the seedling planted in the center of the surface of the pot. Each pot was placed over a jar partially full of water with the nylon wick dipping in it. The bottom of the pot acted as a lid and a water-vapor saturated atmosphere was built up in the lower jar. This device caused the water to move up to the root zone by capillarity. The growing roots soon paralleled the course of the wick and grew into the water inside the jar (Figure 3). Thus more material for root tips was available and these were obtained before the seedlings were finally transplanted in the ground.

Figure 3.--Development of roots grown by wick device.

Cuttings

Cuttings were employed in order to obtain root tip material from adult plants. An effort was made to grow cuttings in the ordinary way and some success was achieved if the cuttings were grown in a well aerated medium. However, this was a slow and an unsatisfactory method. Some investigators (42) have used growth regulators to induce rooting of cuttings for cytological use. It was, therefore, decided to try a similar procedure. Satisfactory results were obtained by treating the cuttings with a 50 ppm aqueous solution of indolebutyric acid for 24 hours prior to planting. The cuttings were also planted in pots with the wick device. Treatment with indolebutyric acid resulted in an early and a more profuse rooting as compared to the untreated cuttings.

Pre-treatment, fixation and staining

The somatic chromosomes are quite small and it was difficult to get a well stained preparation. Several schedules of treatment and stains were tried as recommended (3, 32, 33, 35, 39). The most satisfactory results were obtained with the schedule used by Sagawa (38) with some modifications. This was as follows:

1. Fresh and actively growing root tips were pre-treated in a .003 M aqueous solution of 8-hydroxyquinoline for 3 1/2 hours at 6°C.
2. Fixed in a 3:2:1 mixture of absolute ethyl alcohol, glacial acetic acid and chloroform for 30 minutes at 60°C.
3. Hydrolized in 1 N HCl for 10 minutes at 60°C.
4. Washed with distilled water.

5. Stained with leuco-basic-fuchsin for 1 hour.

6. Brightly stained tips were cut and treated with 5 per cent aqueous solution of pectinase prepared according to Setterfield et al. (41) for 4 hours.

7. Six-seven root tips were squashed on a slide in a drop of 45 per cent acetic acid the coverslip was applied and the preparation was pressed hard between bibulous papers.

8. Sealed with 1:1 mixture of paraffin and Canada balsam.

9. Made permanent by the quick freezing method of Conger and Fairchild (7).

Pollen studies

Study of pollen grains was made with two main objectives in view. First, to learn whether their chromosome content is reflected in their volume, enabling the researcher to devise a rapid method of predicting changes in chromosome number by pollen grain measurement. Second, to use it as an indication of polyploidy when more suitable material was not available.

A heirarchial sampling design was used. Observations were recorded on the diameter of 20 randomly selected pollen grains from 5 randomly selected, fully mature buds on each plant. Variance was analyzed and comparisons between means were made using student's "t" test.

Equipment

Observations were made with a Zeis L, research type microscope equipped with acromatic objectives 8 N.A. 20, 20 N.A. 40, apochromatic oil immersion 90 N. A. 1.4 condenser. Drawings were made with an Abbe camera lucida. Some of the

photomicrographs were taken with a box micro-camera on Kodak panchro-press film sheets. Later it was possible to use Contaflex 35 mm single lens reflex camera in conjunction with a Lafayette "micro-daptor."

The other photographs were taken with either a Contaflex or other 35 mm camera using a variety of film types.

RESULTS AND OBSERVATIONS

Phaseolus lunatus var. Fordhook progeny

(Category 1)

Morphological characters

Since Fordhook is a well-known commercial variety of lima bean, its detailed morphological description is omitted here. Only those characters are mentioned which mainly contrast with those of P. polystachyus. These are: white flowers, forward projection of the wings, absence of pigmentation in hypocotyl and internodes, large lens-shaped seeds with white seed coat, large flat pods, epigeal germination, bush habit and an absence of any marked response to photoperiod (Table 1). Some of these characters are represented in Figures 4, 5, and 6.

Cytology

Cytological examination of the root tips showed that the somatic chromosome number was 22. This confirms the number reported by Karpechenko in 1925 (21) and later corroborated by Dhaliwal et al. (10).

A critical analysis of the karyotype revealed that there are the following types of chromosomes in this species (Figure 27).

Type A - A pair of comparatively long chromosomes. No primary or secondary constructions were visible in this pair.

Table 1

Comparison of unit characters of P. polystachyus, amphidiploid derivatives (categories 4, 10 and 11) and P. lunatus var. Fordhook

Unit Character	P. polystachyus	amphidiploid derivatives	P. lunatus var. Fordhook
Flowering response	short day	intermediate	day neutral
Corolla wings	laterally spread	laterally spread	forward projected
Hypocotyl length	none (hypogeal)	very slight (pseudo-hypogeal)	much (epigeal)
Flower color	purple	purple*	white
Perennial tendency	strong	strong**	weak
Pod size	small	medium	large
Seed coat color	dark specks on fawn ground color	uniform purplish black	white

*F_4 (Category 11) plant No. 2 is a white-flowered segregate.

**F_3 (Category 10) plant No. 4 survived the 1961-62 winter.

Figure 4.--Germinating seedlings of P. lunatus var. Fordhook, the F_1 and P. polystachyus.

Figure 5.—Leaves, flowers and pods of P. lunatus var. Fordhook, F_1 and P. polystachyus.

Figure 6.—Seeds and flowers of P. lunatus, F_1 and P. polystachyus.

Figure 7.--Metaphase plate from a root tip cell (above) and an idiogram (below) of P. lunatus var. Fordhook (Fuelgen; 3,500X)

Type B - A pair of comparatively long chromosomes with a submedian primary constriction and a submedian secondary constriction in the long arm.

Type C - Two pairs of comparatively medium sized chromosomes with a median or a near median centromere constriction.

Type D - A pair of comparatively medium sized chromosomes with a submedian primary constriction.

Type E - Two pairs of comparatively small chromosomes with median primary constriction.

Type F - Two pairs of comparatively small chromosomes in which constrictions were not apparent.

Type G - A pair of hookshaped chromosomes, the smallest in the complement.

An attempt was made to study the pachytene behavior of the chromosomes but it was found to be difficult to trace the chromosomes throughout their length as they were much tangled (Figure 8). Most of the studies of the pollen-mother-cells were, therefore, confined to diakinesis, metaphase I and anaphase I to determine the pairing relationship and disjunction of chromosomes. Table 2 shows the pairing relationship as observed at diakinesis and metaphase I (M_I).

Table 2

Frequencies of chromosome configurations in PMC's of P. lunatus var. Fordhook at diakinesis and M_I

Configurations	11_{II} 0_I	10_{II} 2_I	9_{II} 4_I	Total
Frequencies	12	5	1	18

It can be seen that 2/3 of the observed PMCs showed regular pairing of chromosomes to form 11 bivalents. A few cells with

10_{II} and 2_{I} (Figure 9) and a rare cell with 9_{II} and 4_{I} were also observed. A critical comparison of all the observed figures revealed that most of the univalents appeared due to an early disjunction of 1 or 2 bivalents. None of the cells observed in diakinesis showed any univalents. Figure 10 shows such a diakinesis stage. It can also be seen here that there is a single, spherical nucleolus attached to one pair of chromosomes. Though most of the anaphase I figures showed a regular separation of 11 chromosomes to each pole, a rare instance of irregularity with 10/11 separation and loss of 1 chromosome was also observed.

Over 90 per cent of the pollen grains were apparently normal. The mean diameter of the pollen grains was 38.87 microns (Table 2). Figure 11 shows an almost normal distribution of the diameters of the pollen grains.

Phaseolus polystachyus

(category 2)

Morphological characters

Morphologically P. polystachyus differs from P. lunatus var. Fordhook in some essential respects. These characters are: purple flowers, somewhat lateral extension of wings, purple pigmentation of hypocotyl and internodes, small seeds with mottled fawn seed coat, slender glabrous pods, hypogeal germination, vine habit and a marked flowering response to short days. It has a cold hardy rootstock by means of which

Figure 8.--Photomicrograph of a PMC of P. lunatus var. Fordhook showing pachytene chromosomes (1,875X).

Figure 9.--A PMC of P. lunatus, var. Fordhook showing 10_{II} and 2_{I} at M_{I} (1,700X).

Figure 10.--A PMC showing diakinesis with 11_{II} in P. lunatus, var. Fordhook (1,875X)

Table 3

Pollen grain diameters of P. lunatus var. Fordhook in microns

Observation Numbers	Buds				
	1	2	3	4	5
1	40.80	35.38	40.60	44.08	33.06
2	41.76	35.96	38.86	45.24	34.80
3	40.02	37.70	41.18	46.40	43.50
4	40.02	38.28	39.44	40.60	35.96
5	38.86	35.96	38.28	45.82	34.22
6	39.44	37.12	41.76	44.08	34.22
7	42.34	41.18	33.64	45.82	34.80
8	42.34	37.12	41.18	43.50	35.96
9	38.86	38.28	41.18	45.98	45.80
10	38.86	38.28	38.86	47.56	36.54
11	41.76	39.44	37.70	42.34	32.48
12	40.02	40.02	44.66	49.88	34.22
13	41.18	35.38	36.54	43.50	34.22
14	38.86	37.12	40.02	41.18	31.90
15	39.44	37.12	41.18	44.08	33.64
16	42.92	38.28	40.02	46.40	34.80
17	44.66	36.54	40.02	44.66	35.38
18	49.30	40.02	43.50	44.08	33.06
19	41.76	40.60	39.44	43.50	36.54
20	42.92	37.70	40.60	42.34	33.06
Total	826.12	757.48	798.66	807.36	697.16 = 3,886.78

$\bar{x}$ = 38.87

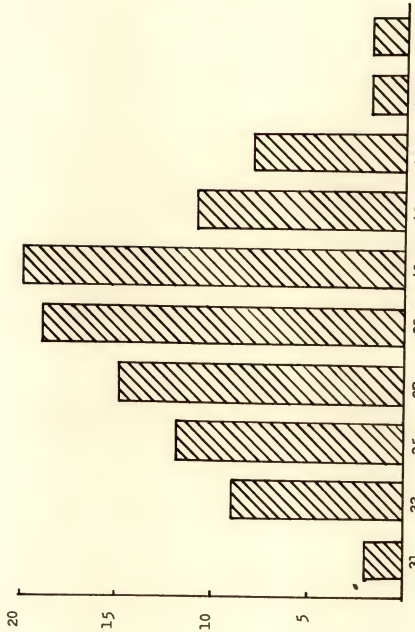

Figure 11.--Frequency distribution of pollen grain diameters of P. lunatus var. Fordhook.

it overwinters (Table 1). Some of these characters can be seen in Figures 4, 5 and 6.

Cytology

Cytological examination of the root tips showed that the somatic chromosome number is 22. This confirms the number previously reported by Allard and Allard in 1940 (1). A critical analysis of the karyotype showed the following types of chromosomes in this species (Figure 12).

Type A - A pair of comparatively long chromosomes which did not reveal any centromere or sedondary constrictions.

Type B - Four pairs of comparatively long or median chromosomes with a submedian primary constriction and a submedian secondary constriction in the long arm.

Type D - A pair of comparatively medium sized chromosomes with a submedian primary constriction.

Type F - Two pairs of comparatively small chromosomes in which constrictions were not apparent.

Type H - A pair of comparatively long chromosomes with a submedian primary constriction and a subterminal secondard constriction in the short arm.

Type I - A pair of comparatively long chromosomes with a median primary constriction and a submedian secondary constriction in one arm.

Type J - A pair of small chromosomes showing no constrictions of any kind.

On comparing the types of chromosomes in the two parent species it is apparent that the type H, I and J were possessed by P. polystachyus and not by P. lunatus. Similarly types C, E and G were found in P. lunatus and not in P. polystachyus. Types A, B, D and F occurred in both species. Thus on a visual

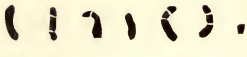

Figure 12.--Metaphase plate from a root-tip cell (above) and an idiogram (below) of P. polystachyus.

basis it might be possible to conclude that complete homology may exist between one or more of the chromosome pairs belonging to the types last mentioned. However, this does not preclude the possibility of homology between small segments of the types which are visually unrelated.

Another important observation concerns the total length of the chromosomes in each genom. Since the preparations were made with different treatments, it was considered that any absolute size comparison between the two genomes would not be valid. However, it can be seen that P. polystachyus had at least 3, possibly 4, pairs of comparatively long chromosomes within its complement. On the other hand, P. lunatus had only 2 pairs of comparatively long chromosomes. This might be taken as an indication of a greater total length of the P. polystachyus genom as compared to that of P. lunatus.

Table 4 shows the chromosome configurations as seen in diakinesis and metaphase I of the pollen-mother-cells. It can be seen that pairing was usually quite regular. Occasionally, there were 2 or 4 univalents, as were also observed in P. lunatus. Their origin was traced to an early disjunction.

Table 4

Frequencies of chromosome configurations of PMC's of P. polystachyus at diakinesis and M_I

Configurations	11_{II} 0_I	10_{II} 2_I	9_{II} 4_I	Total
Frequencies	18	5	2	25

Figure 13 shows M_I of a pollen-mother-cell. There were 2 univalents and 10 bivalents observable in this cell.

Over 95 per cent of the pollen grains appeared viable. The mean diameter of the pollen grains was found to be 41.89 (Table 5). Analysis of variance (Table 6) shows that significant differences exist between the pollen grain diameters of the parents and F_2, F_3 and F_4. A significant difference determined by "t" test was found between the mean diameters of P. lunatus and P. polystachyus pollen grains, the latter being larger (Table 7).

Statistical evidence regarding the greater volume of P. polystachyus pollen grains supports the cytological observation of a greater total length of chromosomes which could be responsible for the larger volume. The frequency distribution of the diameter shows a comparatively greater uniformity of diameter of the pollen grains of this species (Figure 14).

F_1 Hybrid

(Category 3)

Morphological characters

The hybrid showed a combination of characters of the two parents. In several respects it was intermediate between the two while in others it showed a stronger resemblance to P. polystachyus. These are: vine habit, colored flowers, lateral spread of wings and hypogeal germination. It also showed a tendency to short day flowering response. There was an apparent indication

Figure 13. A PMC showing M_I in P. polystachyus with 10_{II} and 2_I (1,700X)

Table 5

Pollen grain diameters of P. polystachyus in microns

Observation Numbers	Buds 1	2	3	4	5
1	41.18	41.76	40.60	44.08	42.92
2	40.60	45.29	43.50	46.98	43.50
3	37.70	39.44	45.82	41.18	42.34
4	38.28	40.02	37.12	45.82	42.34
5	42.34	40.02	41.18	44.08	42.34
6	42.34	40.02	39.44	42.92	42.34
7	38.28	41.18	38.28	48.72	43.50
8	41.18	37.70	38.28	48.72	43.50
9	42.92	42.34	40.60	43.50	42.92
10	41.76	40.60	38.28	45.24	42.34
11	40.60	41.18	39.44	44.08	39.44
12	36.54	42.92	40.02	46.40	35.38
13	41.18	41.76	36.54	50.46	42.34
14	40.60	41.18	38.44	34.22	44.08
15	46.98	47.56	41.18	47.56	49.50
16	38.28	40.02	40.02	44.08	40.60
17	41.76	40.60	38.28	42.34	42.92
18	41.18	45.24	38.28	43.50	43.50
19	44.08	46.98	39.44	46.40	38.28
20	40.02	40.60	40.02	46.40	38.86
Total	817.80	836.41	794.76	896.68	842.94 = 4,188.59

$\bar{x}$ = 41.89

Table 6

Analysis of variance of pollen grain diameters of P. lunatus var. Fordhook, P. polystachyus and the amphidiploids (categories 1, 2, 4, 10 and 11)

Source	d/f	S.Square	M.Square	F F
Plants	7	9,161.79	1,308.83	16.90**
Buds in plants	32	2,478.26	77.45	2.33**
Pollen in buds	760	25,306.49	33,30	
Total	799			

**Significant at .01 level.

Table 7

"t" test for comparison of mean diameters of pollen grains of P. lunatus and P. polystachyus

$$"t"\ (.01)_{2(n-1)} = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{2\,S_p^2}{n}}} = \frac{41.89 - 38.87}{\sqrt{\frac{2\,S_p^2}{n}}} = \frac{3.02}{0.752} = 4.013^{**}$$

S_p^2 = pooled sum of squares.

**Significant at .01 level.

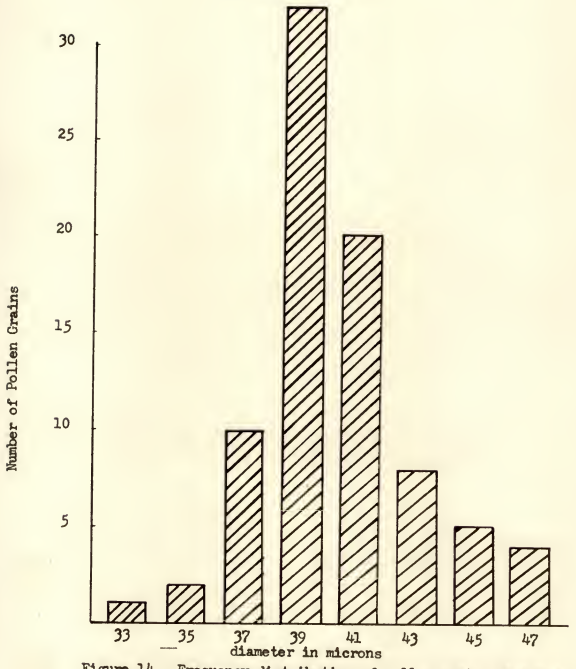

Figure 14.--Frequency distribution of pollen grain diameters of P. polystachyus.

of hybrid vigor as shown by a more vigorous vine development than in the P. polystachyus parent. It has not been possible to determine yet whether it can overwinter by means of a cold hardy rootstock. The seed coat color, unlike either parent, was a uniform purplish black. It was inferred by Lorz (29) that there are interacting factors responsible for seed coat color, because solid or self coloring does not exist in P. polystachyus and the dark pigmentation is not present in P. lunatus var. Fordhook. These characters can be seen in Figures 4, 5 and 6.

Cytology

The present observations basically substantiated the findings of Dhaliwal et al. (10) regarding the meiotic behavior of the F_1. A large number of irregularities of various kinds were observed. Table 8 summarizes these observations.

Table 8

Frequencies of chromosome configurations in PMC's of the F_1 (category 3) at diakinesis and M_I

Configurations	11_{II} 00_I	10_{II} 2_I	9_{II} 4_I	8_{II} 6_I	7_{II} 8_I	6_{II} 10_I	5_{II} 12_I	3_{II} 16_I	2_{II} 18_I	Total
Frequencies	1	1	2	8	5	2	4	2	4	29

There were rare cells in which there was a regular pairing of chromosomes at M_I or diakinesis. The abnormal pollen-mother-cells had 2 or more univalents. The largest number of univalents observed was 18 with only 2_{II}. The most frequent configuration

consisted of 8_{II} and 6_{I} which can be seen in Figure 15. Some of the univalents undoubtedly appeared due to an early disjunction as was also the case in both the parents. A critical examination of several diakineses showed that probably the most frequent configuration was 8_{II} and 6_{I}.

The separation of chromosomes at anaphase I was also frequently irregular. Pollen-mother-cells with 10/12 were quite common. Several cells with one or more chromosomes forming micronuclei were also observed. In one pollen-mother-cell the distribution of chromosomes at anaphase I was 8/9/3 forming 2 macro-nuclei and 1 micro-nucleus. Another commonly observed irregularity was the presence of laggards which were excluded from being incorporated in the principal daughter nuclei after anaphase I (Figure 16). Chromosome bridges and fragments could also be observed.

Figure 17 shows a cell with 2 fragments and a short broken bridge.

In those pollen-mother-cells where the number of univalents is high there was a tendency for all the chromosomes to aggregate in the center of the cell after anaphase I. This condition probably arose by non-attachment of the univalents to the spindle fibers and consequent lack of movement to the poles at anaphase I. Figure 18 shows this condition in a pollen-mother-cell.

It was probable that the preceding irregularity gave rise to still another kind of irregularity observed in an exceptional pollen-mother-cell. Normally, there are two macro-nuclei in

Figure 15.--PMC of F_1 showing 6_{II} and 10_I at M_I (1,700X)

Figure 16.--PMC of F_1 showing 2 lagging chromosomes at A_I (1,700X)

Figure 17.--PMC of F_1 showing a short broken bridge and fragments (1,700X).

Figure 18.--PMC of F_1 showing a non-movement of chromosomes to the poles at A_I (1,700X).

meiotic interphase at the end of the first division. They may be more or less separated within the cell. However, their separate identities are maintained. Unlike the mitotic interphase, the meiotic interphase was characterized by the presence of a nucleolar-like body attached to each condensed chromosome. These nucleolar-like bodies were countable in this stage and gave a fairly accurate indication of the chromosome content of the meiotic interphase nucleus. After the first division each daughter nucleus, during interphase, was seen to possess approximately 11 of these nucleolar-like bodies. The exceptional nucleus shown in Figure 19, however, had approximately 22 of these bodies. There was this single nucleus in the center of the pollen-mother-cell. There appears to be little doubt, therefore, that this was either a restitution nucleus or a nucleus in which the anaphase was abortive. In either case this nucleus seemed to possess the unreduced chromosome number.

Pollen grain studies showed that more than 90 per cent of the pollen was apparently abnormal. The pollen grains were devoid of contents and did not take stain.

The Amphidiploid and Progeny
(Categories 4, 10 and 11)

The fertile F_2 (category 4) and its progeny, F_3 (category 10) and F_4 (category 11) are treated together as being amphidiploids. They are basically alike cytologically as well as morphologically,

Figure 19.--PMC showing an unreduced nucleus following M_I in F_1 (1,700X).

and constitute a group different from the diploids. As some of the individuals in these progenies exhibited segregation of parental characters it was thought desirable to keep track of different individuals. To facilitate this a pedigree has been constructed and is shown in Figure 20.

F_2, fertile, 4n (No. 4)

Morphological characters

In almost all respects this single F_2 plant was essentially like its progenitor, the F_1 hybrid. It combined the characters of both the original parents. Like the F_1, it showed a strong resemblance to P. polystachyus in vine habit, colored flowers, shape of wings, pigmentation of the hypocotyl and the internodes and the near hypogeal germination. The seed coat color, like the F_1 is uniform purplish black. Figure 21 shows some of these characters and Table 1 compares the characteristics of the amphidiploids with those of the parent species. There were, however, a few important respects in which it showed a significant difference from the F_1. A striking difference was observed in the fertility and seed set of the two generations. While the F_1 plant was nearly completely sterile, the F_2 was fairly fertile. There also appeared to be larger flowers in the latter.

Cytology

Cytological examination of the pollen-mother-cells and root tips revealed the 2n chromosome number to be 44. It was

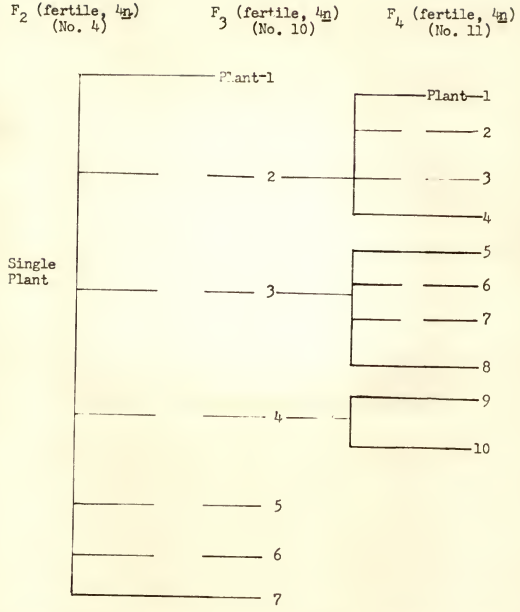

Figure 20.--Pedigree of amphidiploids (F_2, F_3 and F_4) (categories 4, 10 and 11.)

Figure 21.—The amphidiploid F_2 plant (category 4) showing flower color, and leaf and pod shape.

apparent that a reduplication of the entire chromosome complement of the F_1 plant had occurred, in some way, to give rise to this amphidiploid individual. A detailed analysis of the chromosome configurations at diakinesis and metaphase I showed that bivalent association of chromosomes had much increased as compared to that in F_1. This is probably due to autosyndetic pairing giving rise to 22_{II}. All the pollen-mother-cells, however, did not show regular meiosis. Irregularity was observed in the form of quadrivalents, univalents and an occasional trivalent. Thus it would appear that there were homologies in whole or part of some of the chromosomes thereby giving rise to multivalent associations resulting from the simultaneous occurrence of allo- and autosyndesis. About 50 per cent of the cells showed one or more quadrivalents. As many as 3_{IV} with $\bar{1}4_{II}$, 1_{III} and 1_I were observed. Figure 22 shows a pollen-mother-cell with a chain quadrivalent and 8_I. The origin of most of the univalents appeared to be an early disjunction, which also characterized the parental species and the F_1. Figure 23 shows another metaphase I with 6 univalents and 19 bivalents. Another characteristic feature of metaphase I was the presence of secondary associations between the bivalents, a possible indication of allosyndetic homology. This was more clearly demonstrated in F_3.

A comparison of the mean diameter of this plant's pollen grains (Table 10) and that of either parent (Tables 3 and 5) indicates a big difference in the volume of their pollen grains. If the mean diameter (49.48) of the F_2 and a mean value between the two parents (40.38) be converted into volume the respective

Figure 22.--PMC of F_2 (category 4) showing a chain quadrivalent and 8_I (2,675X).

Figure 23.--PMC of F_2 (category 4)showing 19_{II} and 6_{I} (2,675X).

Table 9

Frequencies of chromosome configurations in the PMC's of F_2 (category 4) at diakinesis and metaphase I

	Configuration Types									Total
I	0	2	4	0	6	2	0	8	1	
II	22	21	20	20	19	19	18	16	14	
III	0	0	0	0	0	0	0	0	1	
IV	0	0	0	1	0	1	2	1	3	
Frequencies	12	4	5	7	1	6	2	1	1	39

values are 62, 993.01μ^3 and 34,237.43μ^3. Thus the pollen grains of F_2 have almost doubled in volume as compared to the intermediate value between the two parents.

Table 11 compares the mean diameter of the pollen grains of F_2 with that of P. lunatus, diploid species. It can be seen that there is a highly significant difference between the two.

The frequency distribution of the pollen grain diameters (Figure 24) shows a greater range of dispersion as compared to the diploid ancestors. This might be an indication of some variation of chromosome contents of the microspores. Approximately 85 per cent of the pollen grains appear to be normal.

Table 10

Pollen grain diameter of F_2 (category 4)

Observation Numbers	Buds				
	1	2	3	4	5
1	60.90	55.10	52.78	56.84	49.30
2	49.88	53.36	44.66	55.10	46.40
3	58.00	55.68	45.24	40.60	51.62
4	53.94	55.10	48.14	49.30	44.66
5	49.30	53.94	46.98	46.98	52.20
6	50.46	49.88	55.10	37.70	51.62
7	49.30	40.60	52.20	56.26	52.20
8	46.98	55.68	53.94	42.92	41.18
9	53.94	63.80	49.30	48.72	52.20
10	40.60	46.40	47.56	48.72	46.98
11	53.94	37.70	50.46	44.66	46.40
12	42.92	55.10	41.76	53.36	46.40
13	50.46	41.18	51.62	44.08	39.44
14	45.24	43.50	48.14	40.02	51.04
15	64.38	52.78	51.62	47.56	46.40
16	59.74	59.16	35.38	44.66	49.88
17	45.24	56.26	41.18	49.30	52.20
18	46.40	59.74	57.42	49.30	40.02
19	49.30	56.84	46.98	42.34	40.02
20	56.84	49.88	55.10	41.18	52.78
Total	1,027.76	1,041.68	975.56	939.61	962.94 = 4,947.55

$\bar{x}$ = 49.48

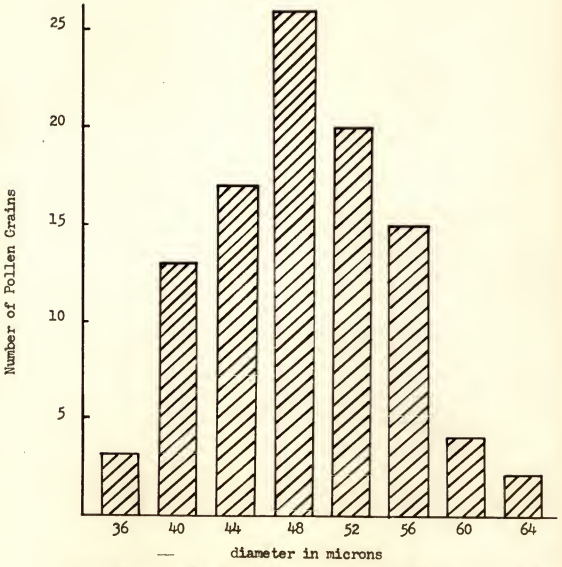

Figure 24.--Frequency distribution of pollen grain diameters of F_2 (category 4).

Table 11

"t" test for comparison of mean diameter of pollen grains of F_2 (category 4) and P. lunatus (category 2)

$$t(.01)_{2(n-1)} = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{2S_p^2}{n}}} = \frac{49.48 - 38.87}{\sqrt{\frac{2S_p^2}{n}}} = \frac{10.61}{0.906} = 11.71^{**}$$

S_p^2 = pooled sum of squares.

**Significant at .01 level.

F_3 fertile, 4n (category 10)

Morphological characters

This progeny of the amphidiploid F_2 plant comprised 7 surviving individuals. Basically all these plants were similar in morphological features in which they also resemble their parents, the F_2. The flower characters of the first four were observed and they resemble the F_2 in this respect. The germination was hypogeal as seen in Figure 25. A comparison of the plants showed that they were not entirely uniform. There appeared to be segregation of some of the parental characters with perceptible variations in vigor and flowering response observed from plant to plant. Plants 2 and 3 were comparatively insensitive in photoperiodic response while plants 1 and 4 to 7 appeared to be responsive to short photoperiod. Plant 4 (Figure 26) was planted in the field in the fall of 1961 and the aerial parts were killed by the frost in December and January, 1962. It regenerated in

Figure 25.--Germinating seedlings of F_3 (category 10) showing near hypogeal germination.

Figure 26.--Plant 4 of F_2 (category 10) showing leaf, pod and flower characters and general habit of growth.

the spring from the cold hardy rootstock, a P. polystachyus derived character. It was not determined whether this property was also possessed by its sibs.

Cytology

Chromosome number was determined to be $2n = 44$ by the examination of the pollen-mother-cells and in several cases, of the root tip squashes. However, one of the plants (No. 4) showed a deviation in chromosome number. The best estimate of the number that could be obtained from the examination of the pollen-mother-cells of this plant was $2n = 42$. Confirmation of this could not be made by the study of root tips as this plant had died. Another attempt was made to obtain verification of the above count. Root tip smears from the seedling of one of its progeny were examined. The number in this plant was estimated to be $2n = 40$.

Bud material was available from several plants (Nos. 1, 2, 3 and 4). The examination of the pollen-mother-cells from these plants showed that meiosis was more or less irregular. Table 12 records the chromosome configurations as observed at diakinesis and metaphase I of the pollen-mother-cells. It can be seen that a total of about 50 per cent of the pollen-mother-cells had a regular meiosis with the formation of 22 bivalents. F_3 thus showed comparatively greater regularity of meiosis than F_2. Most of the irregularities here consist of varying numbers of quadrivalent formations as was the case in F_2. Figure 27 shows a diakinesis with 18_{II} and 2_{IV}. As many as 6 quadrivalents

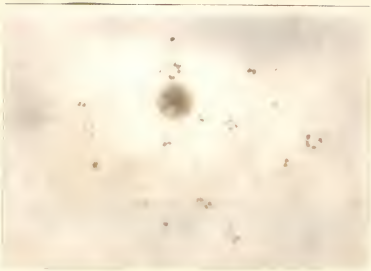

Figure 27.--Photomicrograph of PMC of F_3 (category 10) plant 4 showing 18_{II} and 2_{IV} at diakinesis (1,000X)

Figure 28.--PMC of F_3 (category 10) plant 4 showing 19_{II} and 6_{I} and several secondary associations between the bivalents (2,675X)

Table 12

Frequencies of chromosome configurations in PMC's of F_3 (category 10) at diakinesis and M_I

Configuration																				
	I	0	2	0	1	4	6	0	1	4	2	0	1	2	0	1	4	2	0	Total
	II	22	21	20	20	20	19	18	18	18	17	16	16	15	14	14	14	13	10	
	III	0	0	0	1	0	0	0	1	0	0	0	1	0	0	1	0	0	0	
	IV	0	0	1	0	0	0	2	1	1	2	3	2	3	4	3	3	4	6	
F_3 Plant	2	10		6				4				1								21
	3	7		12								2							1	22
	4	19		6		2	1	9												37
Total		36		24		2	1	13				3							1	80

were observed in rare instances but the most commonly occurring number of quadrivalents was 1 with 20_{II}. Thus the formation of quadrivalents as well as univalents was reduced in F_3 as compared to F_2.

Presence of secondary associations between the bivalents which was noticed for the first time in F_2 was more clearly noticeable in F_3. Figure 28 shows such associations between several of the bivalents at metaphase I in the pollen-mother-cells of one of the F_3 plants (No. 4).

Separation of the chromosomes at anaphase I was mostly regular, 22 chromosomes going to each pole (Figure 29). However, cases of 21/23 and 20/24 were also observed.

Pollen grain measurements of diameter was recorded for 1 of the F_3 plants in Table 13. The pollen grain mean diameters of the other two plants were not significantly different from each other or from the F_2 plant at .01 level. The diameter of the third one was significantly different from the F_2 as shown in Table 14.

This particular F_3 plant (No. 1) was the same which showed an aberrant chromosome number. Apparently an aneuploid loss conditioned a significant change in the volume of the pollen grains as indicated by the reduction in its diameter.

The frequency distribution of the diameters does not appear to yield any significant information. The distribution shown by F_3 plant 1 was, however, very much like that of P. polystachyus (Figure 30).

Figure 29.--Separating chromosomes in 22/22 at A_I in PMC of F_3 (category 10) plant 4 (1,200X).

Table 13

Pollen grain diameters of F_2 (category 10)
plant 1 in microns

Observation Numbers	Buds				
	1	2	3	4	5
1	49.30	45.98	49.30	46.40	49.30
2	37.70	44.66	48.14	46.98	46.40
3	44.66	46.98	44.08	44.08	45.82
4	48.72	49.30	50.36	38.86	49.30
5	44.66	35.38	43.50	44.08	43.50
6	31.90	48.18	50.46	44.08	47.56
7	37.12	47.56	46.98	42.92	38.86
8	40.02	50.46	51.62	40.60	47.56
9	46.40	52.78	46.40	46.40	41.18
10	44.66	42.34	43.50	46.40	52.78
11	41.76	37.70	46.40	41.18	47.56
12	51.62	41.18	41.18	45.82	49.30
13	40.60	40.60	44.66	39.44	39.44
14	52.78	42.34	43.50	47.56	45.24
15	63.22	37.12	46.98	46.98	39.44
16	42.34	44.66	43.50	40.60	43.50
17	43.50	41.18	47.56	43.50	46.40
18	52.20	40.02	43.50	44.08	41.18
19	45.24	48.14	42.34	35.96	42.34
20	44.66	38.86	34.80	51.04	41.18
Total	903.06	876.38	911.76	877.16	897.84 = 4,466.20

$\bar{x} =$ 44.66

Figure 30.--Frequency distribution of diameter of pollen grains of F_3 (category 10) plant 1

Table 14

"t" test for means of diameter of F_2 (category 10) plant 1 and F_2 (category 4)

$$t(.01)_{2(n-1)} \equiv \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{2S_p^2}{n}}\sqrt{\frac{2S_p^2}{n}}} = \frac{49.48 - 44.66}{\sqrt{\frac{2S_p^2}{n}}\sqrt{\frac{2S_p^2}{n}}}$$

$$= \frac{4.82}{.762} = 6.33^{**}$$

S_p^2 = pooled sum of squares.

**Significant at .01 level.

F_4, fertile, 4n (category 11)

Morphological characters

This generation of the amphidiploid showed a continued resemblance to the original amphidiploid, the F_2 in most of the characters. However, it was by no means constant, at least, as far as morphological characters were concerned. One of the F_4 plants (No. 2) showed white flowers (Figure 31) through in other respects it was not much different than its sibs. Figure 31 also shows a marked difference in flower size of a diploid and an amphidiploid.

Cytology

Cytological examination of the root tips and the pollen-mother-cells revealed that the 2n chromosome number was 44 in

Figure 31.--Flowers of a diploid, Fordhook like derivative and an amphidiploid, F_4 (category 11) plant 2.

plants 1 and 2 the only plants available for study. Table 15 shows the chromosome configurations observed at diakinesis and metaphase I. There appeared to be no essential difference between the configurations observed for F_3 and F_4. There were several trivalent and quadrivalent associations in these 2 F_4 plants. Secondary associations were also observed (Figure 32).

Table 15

Frequencies of chromosome configurations in PMC's of F_4 (category 11) at diakinesis and M_I

Configurations								Total
I	0	2	0	4	0	1	1	
II	22	21	20	20	18	18	14	
III	0	0	0	0	0	1	1	
IV	0	0	1	0	2	1	3	
Frequencies								
Plant 1	8	4	2		3		1	18
Plant 2	6	3	—	7	—	6	—	22
Total	14	7	2	7	3	6	1	40

The amount of apparently viable pollen produced by plant 2 is about 90 per cent. Figure 33 shows some of the pollen grains. There were no significant differences in the mean pollen grain diameters of F_4 and F_3 and F_2 (Appendix, Tables 23 and 24), nor are there any significant differences in the frequency distribution of the pollen grain diameters of these progenies.

Figure 32.--PMC of F_1 (category 11) plant 2 showing 20_{II} and 4_I at metaphase I.

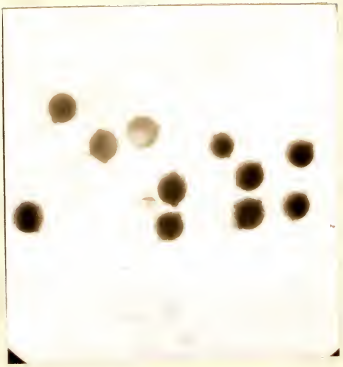

Figure 33.--Pollen grains of F_4 (category 11) plant 2 (168X).

P. lunatus var. Fordhook x P. polystachyus
"sterile," diploid F_2 (category 5)

Morphological characters

The single plant belonging to this category was also like the F_1 in several morphological characters including flower color and seed coat color. The germination of this plant was completely hypogeal. It was weak and was highly sterile. It set only two seeds so far even with manual pollination. The seedlings produced by this plant did not survive.

Cytology

The weak growth of the plant prevented collection of enough bud and root tip material for a thorough cytological examination and whatever buds were available did not fix well. A few pollen-mother-cells were, however, observed at metaphase I. Observation showed that this was a diploid individual with $2n = 22$. Table 16 is a record of the chromosome configurations observed.

Table 16

Frequencies of chromosome configurations of "sterile," diploid F_2 (category 5)

Configurations	11_{II}	10_{II}	8_{II}	6_{II}	Total
	0_I	2_I	6_{II}	8_I	
Frequencies	0	2	4	1	7

It can be seen that all the cells showed 2 or more univalents. A majority of cells had as many as 8 bivalents and 6 univalents. At anaphase I many fragments were observed. Thus, this plant had a very irregular meiosis. Over 95 per cent of the pollen was apparently abnormal. The irregular meiosis was probably responsible for this condition.

Backcrosses and Progenies

(Categories 6, 7, 8 and 9)

F_1 x P. lunatus var. Fordhook (recurrent), and progeny (categories 6 and 7)

Morphological characters

The first backcross individual (category 6) was a perennial. In many morphological characters it showed a shift to lunatus form while in others it retained the characters of the F_1. In vine habit and seed coat color it was like the F_1. It had, however, white flowers and did not show any response to day length. In these latter respects it was, therefore, like the Fordhook parent. It was relatively infertile.

The progeny of this plant (category 7) consisted of 6 individuals. They showed a segregation in some of the parental characters including vine and bush habit. The germination character was also segregating in this progeny.

Cytology

Cytological examination showed that all these plants were

diploids with $2n = 22$ chromosomes. Figure 34 shows a root tip cell of the first backcross individual. The frequencies of chromosome configurations observed at diakinesis and metaphase I of the pollen-mother-cells of the backcross and 2 of the progeny are recorded in Table 17. One of these two plants (plant 2) was a Fordhook bush type derivative and was interesting from the standpoint of breeding.

Table 17

Frequencies of chromosome configurations in PMC's of first backcross and progeny at diakinesis and metaphase I

Configurations	11_{II} 0_{I}	10_{II} 2_{I}	9_{II} 4_{I}	8_{II} 6_{I}	7_{II} 8_{I}	6_{II} 10_{I}	9_{II} 1_{IV}	Total
BC_1	3	6	8	5	1	0	0	23
Progeny Plant 1	8	6	5	0	0	0	0	19
2	0	4	6	0	1	5	1	17
Total	11	16	19	5	2	5	1	59

It can be seen that meiosis was more or less irregular in all these plants. Only about 1/3 of the total pollen-mother-cells showed a regular formation of 11_{II}. Mostly there are 9_{II} and 4_{I}. An occasional quadrivalent was also observed. Figure 35 shows a typical pollen-mother-cell from one of the first backcross progeny (plant 2).

The amount of apparently good pollen was approximately 80 per cent in these plants.

Figure 34.--A root tip cell from first backcross (category 7) showing 22 chromosomes at metaphase (3,500X).

Figure 35.--A PMC from first backcross (category 7) showing $^{7}_{II}$, $^{8}_{I}$ at M_I (3,500X).

Second Backcross Progenies

(Categories 8 and 9)

Morphological characters

These two progenies of the second backcross were closer to Fordhook in appearance than that of the first backcross. However, they contain several P. polystachyus characters in combination. Both these progenies showed segregation of some of the parental characters which include bush and vine habit. and seed coat color. Figure 36 shows a vine and a bush segregate from the second generation. Individuals of the first generation also exhibited marked variations in fertility. Some were completely sterile while others were fertile and vigorous.

Cytology

An analysis of metaphase I figures of the pollen-mother-cells showed that all these derivatives were diploids with $2n = 22$ number of chromosome and that meiosis was almost as regular as in the parental species. Tables 18 and 19 show the chromosome configurations in first and second generations respectively. Figure 37 shows metaphase I of a typical pollen-mother-cell from the first generation. In most of these plants approximately 90 per cent of the pollen grains were apparently viable (Figure 38).

Figure 36.--Vine and bush segregates from backcross 2 (lunatus recurrent) second generation (category 9).

Figure 37.--A PMC from backcross 2 (lunatus recurrent) first generation plant (category 8) showing 10_{II} and 2_{I} (3,500X).

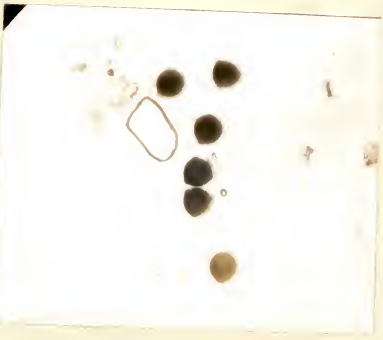

Figure 38.--A photomicrograph showing pollen grains of backcross 2 (lunatus recurrent) second generation plant (category 9) (168X).

Table 18

Frequencies of different chromosome configuration types at diakinesis and M_I of PMC's of 20 plants from first generation of backcross 2 (lunatus recurrent) (category 8)

Chromosome Complement		Configuration Types								Total No. of PMC examined
	I	0	2	4	0	1	6	8	6	
	II	11	10	9	9	9	8	7	5	
	III	0	0	0	0	3	0	0	3	
	IV	0	0	0	1	0	0	0	0	
Plant No.										
1		16	1	0	1	0	0	0	0	18
2		15	1	0	4	0	0	0	0	20
3		18	6	0	2	0	1	0	0	27
4		23	2	0	0	0	0	0	0	25
5		12	3	0	0	0	0	0	0	15
6		21	1	0	0	0	0	0	0	22
7		6	2	0	3	0	0	0	0	11
8		8	5	0	1	0	0	0	0	14
10		16	2	0	0	0	0	0	0	18
12		26	2	0	0	0	0	0	0	28
13		6	4	0	1	0	0	0	0	11
14		10	4	0	0	0	0	0	0	14
15		4	5	4	0	0	0	0	1	14
16		7	0	0	0	0	0	0	0	7
17		24	1	0	0	0	0	0	0	25
19		21	3	0	0	0	0	0	0	24
20		6	2	0	0	0	0	0	0	8
21		5	0	0	0	0	0	0	0	5
25		18	0	0	0	0	0	0	0	18
26		16	5	0	0	0	0	0	0	21
Total	20	278	49	4	12		1		1	345

Table 19

Frequencies of different chromosome configuration types at diakinesis and M_I of PMC's of progeny of 2 first generation plants of backcross 2 (lunatus recurrent) (category 9)

		Configuration Types							
	I	0	2	4	0	1	6	3	Total
Chromosome	II	11	10	9	9	9	8	8	
Complement	III	0	0	0	0	0	0	1	
	IV	0	0	0	1	0	0	0	
Progeny of Plant 3									
Offspring No.									
1		5	12	3	0	0	1	0	21
5		3	14	1	0	0	2	0	20
Total 2		8	26	4	0	0	3	0	41
Progeny of Plant 6									
Offspring No.									
1		20	0	0	1	0	0	0	21
2		18	2	0	0	0	0	0	20
3		19	3	0	0	0	0	0	22
4		17	0	0	0	0	0	0	17
6		20	3	0	0	0	0	0	23
Total 6		94	8	0	1	0	0	0	103
Grand Total		102	34	4	1	0	3	0	144

DISCUSSION

Affinities of Parental Species

Evidence demonstrating the presence of taxonomic affinities between P. lunatus and P. polystachyus has come to light from several different sources during the present investigation. First, there is evidence from the karyotype that not only the general size of the chromosomes was within the same range but also that several pairs of chromosomes of the two species agreed in morphological features to an extent that they may very well be homologous to each other.

Evidence of taxonomic affinity between P. lunatus and P. polystachyus is also derived from meiotic behavior of F_1 hybrid. The pollen-mother-cells of the hybrid showed a variation in the number of bivalents. In rare instances 11 bivalents were observed. According to Stebbins (45) the pairing of chromosomes in species hybrids is affected by environmental conditions. However, the observations described here agree with those of Dhaliwal et al. (10), with respect to the hybrid. It appears, therefore, that the pairing behavior was approximately the same under Gainesville and Utah conditions. There may, however, be genetic or other unknown factors affecting the pairing

relationship in the hybrid. Nevertheless, there were indications that homologies occur in several pairs of chromosomes between the two genomes.

Additional evidence is presented in the pairing relationship of the chromosomes during the meiosis in the amphidiploid. All the amphidiploid generations observed so far show the occurrence of quadrivalent associations at metaphase I. This indicates that homologies of sufficient strength exist in these chromosomes to allow a strong attraction between interspecific homologs to occur simultaneously with attractive forces between intraspecific homologs. The presence of secondary associations is an extension of the same phenomenon but indicates a lesser degree of attraction between interspecific chromosomes apparently only partly homologous. According to Lawrence (25) the phenomenon of secondary association is intimately connected with polyploidy and implies a certain relationship between the species.

Genetic evidence of relationship was provided by the occurrence of segregation of parental characters in the amphidiploid generations. The appearance of a white-flowered plant in the F_3 amphidiploid can only be explained in either of two ways: (1) the occurrence of crossing over at one or more loci between segments of chromosomes from the two species, (2) the separation, without crossing over, from the quadrivalent of pairs of homologs derived from the same parental source.

Nevertheless, the differentiation of the genomes during the process of speciation has proceeded to such an extent that a

hybrid between these two species was completely sterile. The presence of chromosome bridges and fragments at the meiotic anaphase I of the hybrid, indicates that part of the genomic differentiation has developed through inversions and translocations. According to Darlington (9) the sterility of F_1 hybrids between closely related species is often due to these small chromosomal rearrangements. As the result of these rearrangements small chromosomal deficiencies and duplications result in non-viable gametes. Dobzhansky (11) also describes a similar situation in connection with the differentiation of races of Drosophila pseudo-obscura.

Origin of the Amphidiploid

The origin of the amphidiploid takes place as a result of natural or artificial hybridization followed by doubling of the chromosomes at some stage during the life cycle of the organism. There are several possible ways in which this can happen. The mode of origin can be basically classified in two types:

1. The union of unreduced gametes

During this process some gametes may have the combined haploid chromosomal complements of the two species as a result of meiotic irregularities in the species hybrid. The meiotic irregularity may be accompanied by a failure of an inadequately developed spindle to cause normal dipolar separation of the chromosomes at anaphase I. Consequently the chromosomes remain in a single group and coalesce to form a restitution nucleus

instead of two nuclei. This condition has been found in Nicotiana tabaccum x N. sylvestris (37), Crepis rubra x C. foetida (36) and in several other species crosses, and is supposed to be responsible for the origin of their amphidiploids. Non-disjunction of chromosomes can also be brought about by the reunion of anaphase complements into a restitution nucleus. This kind of non-reduction has been reported by Tanaka and Kamemoto (50) in Vanda species.

2. Somatic doubling

The other basic mode of origin of amphidiploid is due to the doubling of chromosome number in the somatic tissues of the hybrid. This doubling could occur at almost any stage in the life cycle of the plant. If it occurs in the hybrid zygote the entire F_1 is polyploid. This kind of doubling or a doubling soon after fertilization probably accounts for formation of amphidiploids in Brassica napus x B. campestris (13), Nicotiana glutinosa x N. tabaccum (6) and in other cases. Doubling may also occur in vegetative parts of an F_1 hybrid. This would result in the formation of a chromosomal chimera with the tetraploid number in an otherwise diploid hybrid. Thus the hybrid may show both the 2n and 4n number in the pollen-mother-cells depending on the location of the buds. Such F_1 hybrid would contain sterile and fertile sectors. This kind of origin of amphidiploids has been reported in Primula kewensis (34). Similar conditions were also suspected to have occurred following colchicine treatment of the tips of the vine cuttings of the original P. lunatus x P. polystachyus hybrid reported by Lorz (29). Amphidiploids may also arise through

the doubling of chromosomes in somatic tissues at a late stage of development. During this process an archesporial nucleus begins to divide but the division is arrested after the chromosomes are split; thus a tetraploid pollen-mother-cell originates. Unlike the rest of the pollen-mother-cells of the interspecific hybrid, this type of cell undergoes normal meiosis and produces diploid microspores. This has been reported for the amphidiploid of Fragaria bracteata x F. helleri (19). Whether diploid megaspores can be produced in this way remains to be determined.

Two additional modes of origin which are based on doubling of chromosomes in somatic cells, or production of unreduced gametes involve slightly different processes (14). An amphidiploid can originate by hybridization of two autopolyploids. Another indirect way of origin when there is non-reduction in only one type of gamete, concerns backcrossing in two successive generations.

According to Goodspeed (14) the fertility of a newly discovered amphidiploid may point to the type of its origin especially in hybrids of distantly related species. In most of these cases the amphidiploids have been discovered as F_2 plants produced by a rarely developed seed from the otherwise highly sterile F_1 plant as in Raphanobrassica (22). Thus, the amphidiploid in F_2 of the hybrid P. lunatus x P. polystachyus as found in the present investigation probably originated in this way. Somatic doubling as a means of origin is not supported by circumstantial evidence. It has been observed cytologically that in rare cases all the chromosomes aggregate in the center of the pollen-mother-cell

at anaphase I. This condition results from a failure of the chromosomes to move to the poles following metaphase I. A pollen-mother-cell has been observed with a nucleus containing an unreduced chromosome number following metaphase I. Such a pollen-mother-cell would eventually produce two microspores each with the diploid chromosome content. Assuming that a similar condition exists in megasporogenesis, chance fertilization of an unreduced egg by an unreduced sperm would give rise to a perfectly viable seed carrying the amphidiploid embryo. In the investigation unreduced pollen-mother-cells have been observed prior to the first division. Therefore, somatic duplication of chromosomes in the archesporial tissues is unlikely.

Amphidiploidy with Respect to Speciation and Breeding

The production of an amphidiploid in the genus Phaseolus is not only of academic importance with respect to speciation but may have valuable practical implications. According to Senn (40) Leguminosae is deficient in examples of polyploid species as compared to other plant families. In the literature concerning amphidiploidy in the Leguminosae only one other similar case has been reported. Dana (8) has obtained evidence of spontaneous amphidiploid in the hybrid Phaseolus aureus x P. trilobus both of which are oriental species.

According to the classification proposed by Stebbins (46) the P. lunatus x P. polystachyus amphidiploid falls in the category of segmental allopolyploids. The criterion for this classification

is the presence of partial intergenomal pairing of chromosomes. As a result of this condition a segregation of interspecific differences occurs so that in the later generations the amphidiploids produce segregates resembling more or less closely one or the other of the original parents. Clausen et al. in 1945 (5) advanced the hypothesis that segmental allopolyploids are likely to be less successful and, therefore, infrequent in nature. Two possible reasons are mentioned for this condition by the above authors. First, it is presumed that the incompatibility and sterility barriers which had developed during the differentiation of the diploid species would result in the appearance of weak and sterile types in the progeny. This condition has been noticed in a number of segmental polyploids, for example, Allium cepa x A. fistulosum (20) and Tradescantia canaliculata x T. humilis (43) which are either themselves weak or completely sterile, or they produce sterile offspring. The second reason advanced by Clausen et al. (5), for the failure of the segmental allopolyploids is the occurrence of segregation and hence the lack of true breeding quality. These individuals may be thrown out of proper physiological balance with the environment. This condition has also been met within the above mentioned amphidiploids.

The amphidiploids of Primula kewensis (34) and Triticum durum x T. Timopheevi (54) have also shown segregation in later generations in spite of their high degree of fertility and regular pairing of chromosomes at the start. The authors have, however, recognized the possibility that such types might be successful if they become

stable in later generations through the elimination of the weaker types.

There are, however, some examples of segmental allopolyploids which have been carried through several generations and from which highly fertile and constant lines have been obtained. Kostoff (23) has shown that the fertility and constancy of the amphidiploid Nicotiana glauca x N. langsdorffii increased in the advanced generations. In this connection Stebbins (46) has made the following significant statement, "There is good reason to believe that . . . the segregating allopolyploids are a valuable source of new variants for the plant breeder and well worth his attention, although the production of useful types from them obviously requires considerable time." The occurrence of the white flowered F_4 amphidiploid (Plant No. 2, Figure 20) lends support to this contention. Another valuable feature of segmental allopolyploidy from the plant breeders point of view is the possibility that they can be crossed to autopolyploids of their parental species and thus make possible the transfer of genes from one species to another on the tetraploid level when it is impossible on diploid level.

From the standpoint of natural selection also it seems that the P. lunatus x P. polystachyus amphidiploids stand a better chance of survival as compared to amphidiploids which are annual in habit. It has already been demonstrated that this amphidiploid is a perennial and possesses efficient means of vegetative propagation in the form of a cold hardy rootstock. It is also capable of producing adventitious roots on vine branches in

contact with the ground and some of these branches subsequently became established as independent segments of the clone. Such perennating amphidiploids may hybridize with a parental autotetraploid, which may exist in nature, thereby losing their well-known handicaps and increasing their variability and adaptability to new habitats. Thus, such amphidiploids can play an important role in speciation and evolution.

Another possible use to which this amphidiploid can be experimentally employed is the derivation of aneuploid forms with the parental genom of either parent plus aneuploid numbers from the other. In a backcrossing program, for example, if the amphidiploid is backcrossed with P. lunatus, the resulting offspring will possess three genomes, two of which will be from the P. lunatus species and the third one from P. polystachyus. During the meiosis of the backcross, 11 lunatus bivalents and 11 polystachyus univalents would be ideally present at metaphase I. The separation of bivalents at anaphase I is expected to be normal with 11 chromosomes going to each pole but the univalents will become distributed at random resulting in the formation of aneuploid gametes with a full haploid complement of the lunatus chromosomes with one or more chromosomes of polystachyus. A chance union with similar gamete will give rise to an aneuploid series consisting of 2n lunatus plus 1 to 10 pairs of polystachyus chromosomes. Theoretically, a large number of these combinations are possible. A plant breeder can thus have a large number of variants from which a selection of favorable segregates can be made.

Under natural conditions, though, the aneuploids which possess only favorable combination of genes will survive. The above conditions are possible on the behavior of whole chromosomes. The combinations are further augmented if crossing over occurs. In nature it is conceivable that crosses could occur between the amphidiploid and P. polystachyus with lunatus genes and/or chromosomes.

SUMMARY

Cytological investigations of the backcross, F_1, "fertile" and "sterile" F_2, and amphidiploid derivatives of the interspecific cross Phaseolus lunatus x P. polystachyus were conducted in the Department of Vegetable Crops, University of Florida as a supplement to the interspecific hybridization program in the genus Phaseolus.

The plant material consisted of P. lunatus var. Fordhook, P. polystachyus, their F_1 hybrids and backcross (P. lunatus recurrent) and amphidiploid derivatives.

Acetocarmine squashes of the pollen-mother-cells were prepared according to a modified schedule suggested by Hyde and Cardella (18), using $Fe(OH)_3$ as a mordant. Root tips were obtained from germinating seedlings and indolebutyric acid treated cuttings. They were then prepared according to Fuelgen technique following pretreatment with 8-hydroxiquiniline as suggested by Sagawa (38) and employing pectinase after staining to facilitate dispersion of cells and spreading of the chromosomes.

Karyotypes of the species P. lunatus and P. polystachyus were studied and idiograms prepared. The two species appear to agree in morphological characters of several chromosome pairs while others are distinctly different. There is a significant

difference in the pollen grain diameter of the two species, suggesting a possible correlation of a greater total length of the polystachyus chromosomes with increased pollen grain size. The chromosome number of $2n = 22$, as previously reported, for both species, was confirmed.

Dhaliwal's (10) observations regarding the irregularity of meiosis in the F_1 hybrid was also confirmed by observance of univalents at metaphase I, and of laggards, chromosome bridges and apparent fragments.

Stages interpreted as concerned with the formation of restitution nuclei were observed immediately following metaphase I in the almost completely sterile hybrid.

The chromosome number was established as $2n = 44$ for the single fertile amphidiploid F_2 plant and most of its derived F_3 and F_4 progenies with the exception of a single aneuploid plant. One F_3 individual apparently had $2n = 42$ chromosomes, but in view of the comparatively large number of chromosomes involved, this number is not positively established. Some meiotic irregularity in the amphidiploids was associated with the formation of quadrivalents which involves allosyndetic homologies between chromosomes as suggested by a comparison of the karyotype of the two species.

The occurrence of one essentially amphidiploid F_4 individual with white flowers is genetic evidence that amphidiploidy is not absolute and that the recombination of genes from the two species is possible for at least some homologous units in the

respective lunatus and polystachyus genomes.

Individual characteristics of both species and their amphidiploid derivatives are compared.

All changes in the chromosome numbers were accompanied by a corresponding change in the size of pollen grains.

All backcross derivatives studied had $2n = 22$ number of chromosomes. Segregation of characters was observed with respect to vine habit and seed coat color. Some of the plants were fertile while the others were sterile. Essentially P. lunatus types have been obtained but have some P. polystachyus derived characters concerned principally with seed coat color and plant habit determination.

The fertility of the amphidiploids was found to be high enough to permit easy maintenance of several lines and it would appear likely that later generations could result in the development of fully fertile lines capable of competing in the wild with the native P. polystachyus.

Frequency distribution studies of pollen grain diameters indicated a fairly constant pollen size in P. polystachyus, nearly the same degree of uniformity in P. lunatus but exhibited a greater increase in the range of variability in the amphidiploids.

LITERATURE CITED

1. Allard, H. A. and H. F. Allard. 1940. The wild bean Phaseolus polystachyus (L.) B. S. P. Its chromosome number. J. Wash. Acad. Sci. 30:335-337.

2. Belling, J. 1926. The iron aceto-carmine method of fixing and staining chromosomes. Biol. Bull. 50:160-162.

3. Bhaduri, P. N. and P. N. Ghosh. 1954. Chromosome squashes in cereals. Stain Technology 29:269-276.

4. Buxton, B. H. and W. C. F. Newton. 1928. Hybrids of Digitalis purpurea, their fertility and cytology. Jour. Genetics 19:269-277.

5. Clausen, J., D. D. Keck and W. M. Hiesey. 1945. Experimental studies on the nature of species II. Plant evolution through amphidiploidy and autoploidy with examples from Madinae. Publ. Carnagie Instn. No. 564.

6. Clausen, R. E. and T. H. Goodspeed. 1925. Interspecific hybridization in Nicotiana. II A tetraploid glutinosa-Tabaccum hybrid, an experimental varification of Winge's hypothesis. Genetics 10:279-284.

7. Conger, A. D. and L. Fairchild. 1953. A quick-freezing method for making smear slides permanent. Stain Technology 28:281-283.

8. Dana, S. 1961-62 Personal communication.

9. Darlington, C. D. 1937. Recent Advances in Cytology. 2nd. ed. Blakiston, Philadelphia.

10. Dhaliwal, A. S., L. H. Pollard and A. P. Lorz. 1961. Cytological behavior of an F_1 species cross (Phaseolus lunatus L. var. Fordhook Phaseolus polystachyus L.) In press.

11. Dobzhansky, T. 1941. Genetics and the Origin of Species, Columbia University Press, New York.

12. Florida State Marketing Bureau, Annual Agricultural Statistics Summary. Nov. 1959. Annual Report No. 41, pp. 70-71.

13. Frandsen, H. N. and O. Winge. 1932. Brassica napocampestris, a new constant amphidiploid species hybrid. Hereditas 16:212-218.

14. Goodspeed, T. H. and M. V. Bradley. 1942. Amphidiploidy. Bot. Rev. 8:272-316.

15. Honma, S. 1956. A bean interspecific hybrid. Jour. Heredity 47:217-220.

16. _____ and O. Heeckt. 1958. Bean interspecific hybrid involving Phaseolus coccineus x P. lunatus. Proc. Amer. Soc. Hort. Sci. 72:360-364.

17. _____. 1959. Interspecific hybrid between Phaseolus vulgaris and P. lunatus. Jour. Heredity 50:233-237.

18. Hyde, B. B. and C. A. Cardella. 1953. A mordanting fixation for intense staining of small chromosomes. Stain Technology 28:305-308.

19. Ichijima, K. 1926. Cytological and genetic studies on Fragaria. Genetics 11:590-604.

20. Jones, H. A. and A. E. Clarke. 1942. Natural amphidiploid from an onion hybrid Allium cepa L. x Allium fistulosa L. Jour. Heredity 33:25-32.

21. Karpechenko, G. D. 1925. On the chromosomes of Phaseolinae. Bull. Appl. Bot. and Pl. Breeding 14(2):143-148.

22. Karpechenko, G. D. 1928. Polyploid hybrid of Raphanus sativus L. x Brassica oleracea L. Bull. Appl. Botany 17:305-408.

23. Kostoff, D. 1939. Studies on polyploid plants. XXI Cytogetic behavior of the allopolyploid hybrid Nicotiana glauca Grah. x Nicotiana Langsdorffii Weinm. and their evolutionary significance. Jour. Genetics 37:129-209.

24. Lamprecht, H. 1941. Die Artgrenze zwischen Phaseolus vulgaris L. und multiflorus Lam. Hereditas 27:51-175.

25. Lawrence, W. J. C. 1931. The secondary association of chromosomes. Cytologia 2:352-384.

26. Lorz, A. P. 1952. Interspecific cross in Phaseolus. Science 115:702-703.

27. Lorz, A. P. 1953. Preservation of Phaseolus polystachyus germ plasm in crosses with P. lunatus. Unpublished report.

28. _____. 1957. Interspecific hybridization in Phaseolus at the University of Florida. Ann. Rept. Bean Improvement Cooperative 1957 (1):16-17.

29. _____. 1962 Personal communication.

30. MacDonald, M. D. 1961. Barley pachytene chromosome and the localisation of Erectoides translocation point on chromosome 5. Canadian J. Genetics and Cytology 3:13-17.

31. Mackie, W. W. 1943. Origin, dispersal and variability of the lima bean, Phaseolus lunatus Hilgardia 15(1).

32. Metzger, R. J. and E. R. Leng. 1955. A smear technique for some species of Leguminosae. Stain Technology 30:41-42.

33. Meyers, J. R. 1945. Prefixing with para-dichlorobenzene to facilitate chromosome study. Stain Technology 2-:121-125.

34. Newton, W. C. F. and C. Pellew. 1929. Primula kewensis and its derivatives. Jour. Genetics 20:405-467.

35. O'Mara, J. G. 1958. Acetic acid method for chromosome studies in prophase and metaphase in meristems. Stain Technology 201-204.

36. Poole, C. F. 1931. The interspecific hybrid Crenis rubra x C. foetida and some of its derivatives I. Univ. Calif. Publ.

37. Rybin, V. A. 1929. Cytological features of the allotetraploid Nicotiana Tabacum x Nicotiana sylvestris. Proc. USSR Congr. Genet. Plant and Animal Breed. 2:437-445.

38. Sagawa, Y. 1961. Personal communication.

39. Sen, N. K. and J. G. Bhowal. 1960. Cytotaxonomic studies in Vigna. Cytologia 25:195-207.

40. Senn, H. A. 1938. Chromosome number relationship in the Leguminosae. Bibliographica Genetica XII.

41. Setterfield, G., R. Schrieber and J. Woodward. 1954. Meiotic frequency determinations and microphotometric fuelgen dye measurement in root tips. Stain Technology 29:113-120.

42. Singh, D. N. 1961. Use of growth induced rooting in cytological studies. Nature (London) 189:420-421.

43. Skirm, G. W. 1942. Bivalent pairing in an induced tetraploid of Trandescantia. Genetics 27:635-639.

44. Small, J. K. 1933. Manual of the Southeastern Flora. The Science Press Printing Co., Lancaster, Pennsylvania (1st. ed.) 723-725.

45. Stebbins, G. L., Jr. 1945. The cytological analysis of species hybrids II. Bot. Rev. 11:463-486.

46. _____. 1947. Types of polyploids: their classification and significance. Adv. Genet. I 403-429.

47. _____. 1957. Variation and Evolution in Plants. Columbia University Press, New York.

48. Strand, A. B. 1943. Species crosses in genus Phaseolus. Proc. Amer. Soc. Hort. Sci. 42:569-573.

49. Swaminathan, M. S., M. L. Magoon and K. L. Mehra. 1954. A simple propiono-carmine PMC smear method for plants with small chromosomes. Indian Jour. Genet. Plant Breeding 14:87-88.

50. Tanaka, R. and H. Kamemoto. 1960. Meiotic chromosome behavior in diploid and polyploid Vanda Orchid hybrids. Cytologia 25:405-418.

51. Thompson, H. C. and W. C. Kelly, Vegetable Crops. McGraw Hill Book Co., Inc., New York.

52. Vosa, C. G. 1961. A modified aceto-orcein method for pollen mother cells. Caryologia 14:107-110.

53. Yarnell, S. H. 1931. A study of certain polyploid and amphiploid forms in Fragaria. Genetics 16:455-489.

54. Zhebrak, A. R. 1946. New amphiploid series of wheat and their significance for selection and evolution. Amer. Nat. 80:271-279.

APPENDIX

Table 20

Diameter of pollen grains of F_3 (category 10) plant 3 in microns

Observation Numbers	1	2	3	4	5	
1	51.04	43.50	48.16	41.18	45.24	
2	51.62	47.56	53.36	36.54	48.70	
3	51.62	41.76	44.66	52.20	46.98	
4	51.62	52.20	37.70	42.92	44.08	
5	51.04	44.08	36.54	53.36	47.56	
6	57.42	50.46	49.30	45.82	47.56	
7	46.40	44.66	43.50	53.94	43.50	
8	51.62	42.34	52.20	36.54	52.78	
9	56.84	38.86	52.78	54.52	42.39	
10	49.88	41.76	49.88	44.08	49.88	
11	51.04	41.18	49.88	47.56	55.10	
12	50.46	39.44	40.60	45.82	50.46	
13	51.62	49.30	55.10	51.62	50.46	
14	52.20	50.46	40.60	53.94	46.40	
15	45.82	49.30	37.70	37.70	56.84	
16	44.08	45.24	48.14	37.70	44.08	
17	51.62	45.24	52.78	50.46	52.78	
18	53.36	44.08	55.10	53.94	46.98	
19	47.56	43.50	52.78	46.98	48.14	
20	37.10	45.82	46.40	55.68	46.40	
Sum	1,003.98	900.74	947.14	942.50	966.26	= 4,760.62

$\bar{x}$ = 47.61

Table 21

Diameter of pollen grains of F_3 (category 10) plant 4 in microns

Observation Numbers	1	2	3	4	5
1	52.78	39.44	40.60	41.18	52.20
2	44.08	56.84	48.72	38.28	46.40
3	43.50	52.78	53.94	46.98	40.02
4	62.64	40.60	40.60	43.50	50.46
5	51.04	62.64	38.28	42.92	51.04
6	54.52	49.30	37.70	47.56	41.18
7	40.60	52.20	31.90	68.72	51.04
8	40.46	53.36	34.22	47.56	46.98
9	50.46	52.20	51.04	43.50	48.72
10	42.34	53.36	49.30	43.50	46.40
11	53.94	54.52	51.62	49.30	47.56
12	44.08	53.94	48.72	39.44	49.88
13	42.34	59.16	44.08	45.82	51.04
14	58.00	52.20	52.20	52.20	48.14
15	42.92	55.10	42.34	49.30	38.86
16	55.10	44.66	49.88	53.36	40.02
17	54.52	44.66	41.18	48.72	40.46
18	52.20	51.04	42.92	36.34	45.24
19	34.96	47.56	47.56	51.62	49.88
20	44.66	55.10	49.88	49.88	38.28
Sum	976.14	1,030.66	896.68	917.68	933.80 = 4,754.96

$\bar{x} = 47.55$

Table 22

Pollen grain diameters of F_4 (category 11) plant 1 in microns

Observation Number					
1	53.94	44.08	58.00	46.30	53.90
2	49.88	54.52	40.60	49.88	55.68
3	55.10	34.80	42.34	47.56	52.20
4	51.04	45.24	41.18	58.58	37.70
5	51.04	46.40	48.72	52.78	53.36
6	49.88	58.58	52.78	54.52	52.20
7	56.84	54.52	40.60	47.56	49.30
8	46.26	51.62	47.56	41.76	52.20
9	46.40	40.60	46.40	43.50	52.20
10	44.66	34.80	48.14	34.22	44.66
11	54.52	50.46	49.88	46.40	52.20
2	40.02	44.08	45.24	48.72	52.78
13	42.92	40.60	42.92	37.12	50.46
14	44.08	51.04	40.60	55.68	46.98
15	44.08	55.10	46.40	48.72	44.08
16	40.60	52.20	40.02	47.50	52.20
17	44.66	47.56	51.04	39.44	46.98
18	46.40	45.82	51.04	34.22	44.66
19	51.62	51.62	52.94	45.24	45.24
20	48.72	49.88	40.60	43.50	37.12
Sum	972.66	953.52	927.00	923.20	976.10 = 4,752.48

$\bar{x}$ = 47.52

Table 23

Pollen grain diameter of F_4 (category 11) in plant 2 in microns

Observation Numbers					
1	49.88	42.92	46.40	53.36	48.72
2	38.28	42.92	51.04	56.84	41.76
3	48.72	40.60	51.04	54.52	49.88
4	47.56	38.28	44.08	51.04	46.40
5	59.16	42.92	44.08	56.84	52.20
6	42.92	37.12	39.44	53.36	46.40
7	38.28	45.24	38.28	44.08	48.72
8	48.72	51.04	54.52	52.20	41.76
9	46.40	44.08	42.92	48.72	54.52
10	53.36	52.20	48.72	44.08	53.36
11	49.88	49.88	41.76	27.12	49.88
12	47.56	47.56	44.08	35.96	48.72
13	45.24	42.92	32.48	41.76	48.72
14	63.80	46.40	48.72	54.52	47.56
15	44.08	60.32	52.20	47.56	44.08
16	52.20	61.48	49.88	39.44	54.52
17	59.16	59.16	51.04	53.36	52.20
18	58.00	58.00	45.24	49.88	54.52
19	48.72	37.12	54.52	39.44	46.40
20	55.68	40.60	46.40	53.36	53.36
Sum	987.60	930.76	926.84	957.42	983.68 = 4,786.30

$\bar{X}$ = 47.86

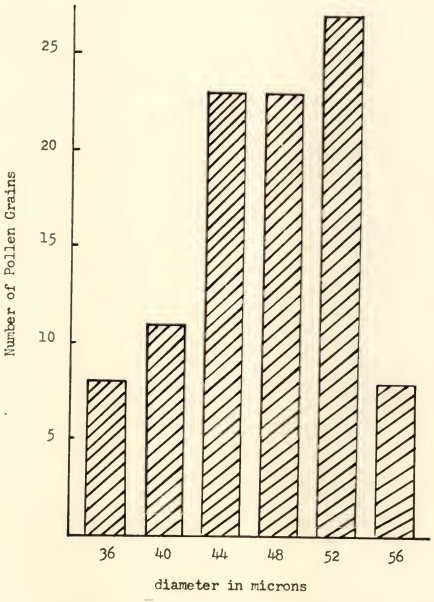

Figure 39.--Frequency distribution of pollen grain diameters of F_3 (category 10) plant 3.

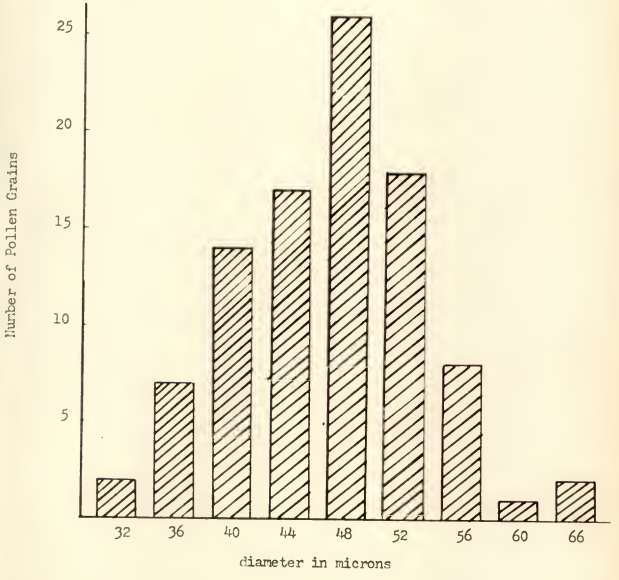

Figure 40.--Frequency distribution of pollen grain diameters of F_3 (category 10) plant 4.

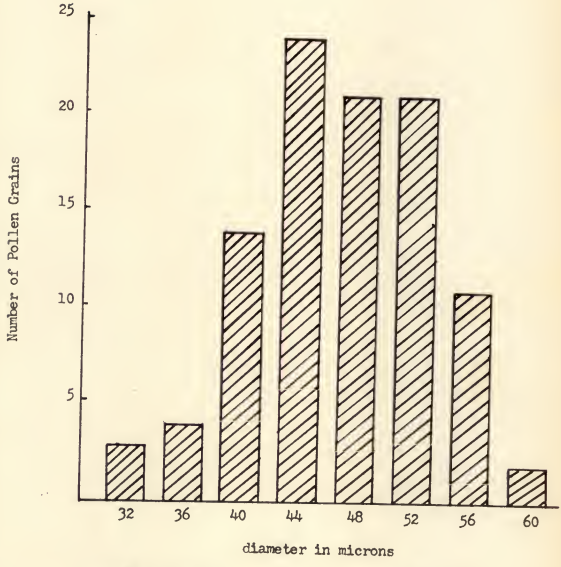

Figure 41.--Frequency distribution of pollen grain diameters of F_4 (category 11) plant 1.

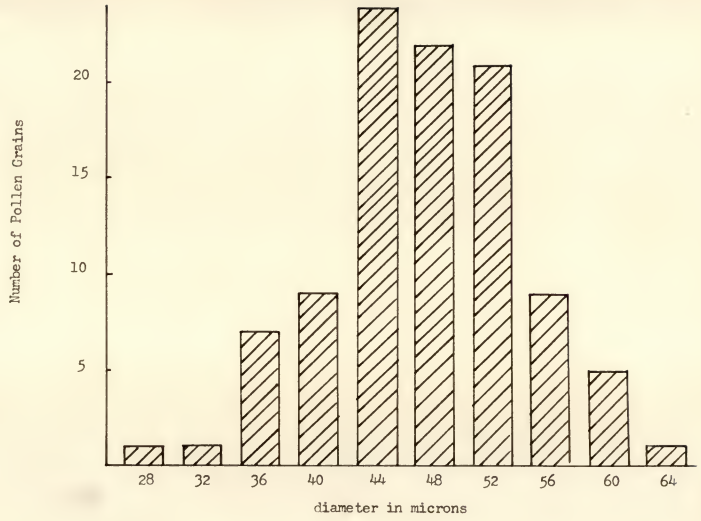

Figure 42.—Frequency distribution of pollen grain diameters of F_4 (category 11) plant 2.

BIOGRAPHICAL SKETCH

Birendra Singh Fozdar was born July 6, 1919, at Hasanpur, India. In 1934, he graduated from Government Intermediate College, Etawah. In 1938, he received the degree of Bachelor of Science in Agriculture from Agra University and the degree of Master of Science in Botany from the same University in 1940.

From 1942 to 1953, he served in the Department of Agriculture, Government of Uttar Prasdesh, as Lecturer in Botany at the Agricultural College, Kanpur. At the same time he also served in the National Cadet Corps of India. From 1953 to August, 1959, he served as Lecturer in the Central College of Agriculture, Indian Agricultural Research Institute, New Delhi. In September, 1959, he enrolled in the Graduate School of the University of Florida. He worked as a graduate assistant in the Department of Vegetable Crops while pursuing his studies towards the degree of Doctor of Philosophy.

Birendra Singh Fozdar is married to the former Vidya Wati Devi and is the father of two children. He is a member of the Indian Society of Genetics and Plant Breeding, Indian Horticultural Society, American Society for Horticultural Science and Gamma Sigma Delta.

This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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