AN ABSTRACT OF THE THESIS OF

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in Horticulture presented on December 15, 1980

Title: Embryo Development in Relation to Interspecific Hybridization of Phaseolus.

Abstract approved: _____

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Interspecific hybridizations between P. vulgaris and P. acutifolius and between P. vulgaris and P. lunatus were investigated. Prefertilization events were similar in inter- and intra-specific crosses. Fertilization was completed in all crosses and the time of fertilization was dependent on the maternal parent, reflecting differences in the time of maturation of the female gametophyte. There were large reciprocal cross differences in the time of endosperm and embryo division. The time interval between fertilization and division of endosperm and embryo was longer than in selfings when species other than P. vulgaris were used as the female parent. In P. lunatus x P. vulgaris crosses, only 7-12% of the ovules contained dividing endosperms at 72 hours and the embryo ceased to grow at the four-celled stage. Embryos of P. vulgaris x P. lunatus developed up to the pre-heart shaped stage. Hybrid embryos between P. vulgaris and P. acutifolius reached the cotyledon stage, however, no mature hybrid seeds were obtained. The characteristic morphology of the interspecific hybrid embryos, uneven cotyledons, provided a clear distinction from selfed embryos. The growth rate and final size of hybrid embryos were influenced by the parental combinations. As hybrid embryos did not develop to maturity, embryo culture

was used to recover hybrid plantlets. Glutamine was beneficial to the survival of hybrid embryos of P. vulgaris x P. lunatus. However, hybrid plants obtained were slow-growing and developed symptoms of premature senescence after three to four sets of trifoliolate leaves were formed. Embryos obtained from reciprocal crosses of P. vulgaris and P. acutifolius gave mature plants. Meiosis and fertility of interspecific hybrids between P. vulgaris and P. acutifolius were examined. At Metaphase I, the majority of the microsporocytes had 4 to 8 univalents with an average of 6 univalents per microsporocyte. However on the average, only 2 lagging chromosomes per cell were observed at Anaphase I. The most frequent chromosomal distribution at late Anaphase I was 10-12. It is likely that some univalents observed at Metaphase I may have resulted from precocious separation of loosely paired univalents. When these interspecific hybrids were self-pollinated, 26% of the ovules were fertilized; however, no dividing embryos were observed. When interspecific hybrids were used as the female parents in backcrossing to P. acutifolius and P. vulgaris, the frequencies of ovules being fertilized were 31 and 20% respectively, and the frequencies of ovules containing dividing embryos were 13 and 4%. The difference in the survival rate of backcrossed embryos suggested an influence of genomic dosage on embryo viability. Backcrossed-progeny were self-fertile and the seed set was two seeds per five pods. Additional experimental results and other observations provided indirect evidence that the development of interspecific hybrid embryos may be related to differences in the hormonal metabolism, possibly that of cytokinins, of parental species.

EMBRYO DEVELOPMENT IN RELATION TO INTERSPECIFIC HYBRIDIZATION OF PHASEOLUS

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed December 15, 1980

Commencement June 1981

APPROVED:

Associate Professor of Horticulture in charge of major

Head of Horticulture Department

Dean of Graduate School

Date thesis is presented December 15, 1980

Typed by Karla Sorensen for Aimee Rabakoarihanta

Rien n'est impossible a l'horticulteur
qui met a contribution par sa patience et son génie,
le feu du soleil, la candeur de l'eau.
les sucs de la terre et les souffles de l'air.

A. Dumas, Père. La Tulipe Noire

(Nothing is impossible to the grower
who, by patience and genius, lays under contribution
the heat of the sun, the clearness of water,
the juices of the earth and the breath of the wind.

A. Dumas, Père. The Black Tulip
Translated by A. J. O'Connor)

ACKNOWLEDGEMENTS

The author wishes to express her deepest appeciation to Dr. David Mok for his guidance and longstanding patience throughout this study, and without whose help this work would have never been completed.

She also takes pleasure in acknowledging her indebtedness to Dr. Machteld Mok for her assistance in laboratory work and in reviewing the thesis.

To all friends and colleagues who shared in this ordeal she is profoundly indebted.

Finally, she gratefully acknowledges the African-American Institute for the continuing financial support.

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INTRODUCTION

The application of interspecific hybridization as the means for improvement of *Phaseolus* is dependent on the recovery of hybrids and the efficient utilization of these hybrids. At present, many limitations persist in employing this approach for plant improvement. For example, despite many published accounts on the hybridization between *P. vulgaris* and *P. coccineus*, the effective incorporation of the hybrids obtained (*P. vulgaris* x *P. coccineus*) in breeding programs was difficult. As a matter of fact, no successful transfer of *P. coccineus* characters have been successful inspite of the ease of obtaining hybrid seeds. The lack of basic information concerning the hybrids such as meiotic behavior, the basis for reversion to parental phenotype in subsequent generations, and the cytoplasmic-nuclear interactions, hamper the efficient design of methods for their utilization. In addition, the uncertainty concerning the cross in the direction of *P. coccineus* x *P. vulgaris* represents another example of insufficient understanding of the developmental process of the hybrid embryos, which may be of fundamental importance in exploring the potential of interspecific hybrids between these two species. Hybridizations involving other *Phaseolus* species, such as *P. acutifolius* and *P. lunatus* have not been systematically examined.

The relative development of hybrid vs. selfed embryos, the methods of obtaining hybrid plantlets and the possible methods of utilizing hybrids once they are obtained represent major areas demanding attention.

Based on these considerations, the present study was designed to elucidate in detail the hybridizations between P. vulgaris and P. lunatus, P. vulgaris and P. acutifolius and to examine the potential of hybrids obtained. Reasonable effort was also directed to examining the cross P. coccineus x P. vulgaris in order to determine the basis of reciprocal cross differences and to explore the possible mechanisms of reversion to parental types.

LITERATURE REVIEW

Desirable Characteristics of *Phaseolus* Species

The common bean, *Phaseolus vulgaris*, is a self-pollinating crop through cleistogamy. Genetic variability resulting from recombination alone is limited. Therefore, although *Phaseolus* species as a group represent a high degree of genetic diversity (Evans, 1976), the genetic base of individual species is relatively narrow. A study examining the genetic vulnerability of major American crops indicated that popular bean varieties have a common genetic base which disposes them to the risk of epiphytotics. The need to broaden the genetic base of *Phaseolus vulgaris* seems imperative to avoid eventual spread of diseases and subsequent loss of germplasm (Committee on Genetic Vulnerability of Major Crops, 1972). One of the possible methods of increasing variability is through interspecific hybridization. The added advantage of this approach is the transfer of desirable characteristics from other species into *P. vulgaris* (Chavan *et al.*, 1966). The majority of the snap bean and dry bean varieties (*P. vulgaris*) are susceptible to a wide range of diseases which include anthracnose, mosaic, bacterial blights, rust, root rots and powdery mildew (Zaumeyer and Thomas, 1957). Bean diseases are more numerous and more severe in the tropics than in the temperate zone (Bird and Maramorosch, 1975). Common bean blight, caused by bacterium *Xanthomonas phaseoli* is a seedborne disease of the dry bean and is a serious problem in many bean-producing areas in the tropics. Control by chemicals is not satisfactory at present. The use of disease-free seed and crop rotation offers a temporary control. The long term solution may be identifying sources of resistant germplasm and incorpo-

rating these materials into breeding programs. In testing several Phaseolus species for reaction to X. phaseoli it was found that certain P. acutifolius lines showed high tolerance after heavy inoculation whereas all P. vulgaris varieties tested showed disease symptoms (Coyne et al., 1963). The presence of resistance to X. phaseoli among P. acutifolius accessions was confirmed in further testings (Coyne and Schuster, 1973). In a test conducted at CIAT (Centro Internacional de Agricultura Tropical), Cali (Columbia) none of the 4,000 P. vulgaris accessions were free from symptoms of X. phaseoli, whereas the two P. acutifolius lines included in the test were highly tolerant, showing neither foliage nor pod symptoms (CIAT, 1976; Yoshii et al., 1978).

The bean golden mosaic virus, a disease present in several tropical countries in South America and Africa, is known to be transmitted by the whitefly Bemisia tabaci. Resistance to this disease was also found in several P. acutifolius lines tested at CIAT (CIAT, 1973).

The cultivated tepary bean (P. acutifolius), native to the arid southwest of the United States and Northern Mexico, is also tolerant to high temperature and severe drought (Freeman, 1912). Some botanical varieties have been found possessing a narrow-leaf character which may contribute to such tolerance. The transfer of this characteristic to P. vulgaris would improve adaptability in hot semi-arid regions. In addition some varieties of P. acutifolius were shown to have at least four times high yield than P. vulgaris in dry land situations (Freeman, 1912).

The lima beans (Phaseolus lunatus) are known to be less susceptible to attack by pests and diseases than P. vulgaris (Zaumeyer and Thomas, 1957). For example, resistance to anthracnose has been found among lima

bean varieties (Barrus, 1911). Anthracnose caused by Colletotrichum lindemuthianum, is a serious disease of the snap beans and dry beans. The use of disease free seed is suggested as a method of control, but in certain parts of the tropics where the production of disease-free seed is impossible because of weather conditions, resistant varieties are of interest.

The root rot complex which is important in the United States and other parts of the world has two major components; the Rhizoctonia root rot (Rhizoctonia solani) and the Fusarium root rot (Fusarium solani f. phaseoli). No effective resistance in P. vulgaris has yet been developed although preventive measures are taken through the use of proper crop rotation and the use of less susceptible varieties. Lines of P. lunatus showed a high degree of resistance to Fusarium root rot and may be promising species in hybridizing with P. vulgaris (Baggett et al., 1965).

The Mexican bean beetle, Ephilachna varvivestis is a serious pest of snap beans and may cause total defoliation during years of heavy infestation. Although both P. vulgaris and P. lunatus are attacked by this insect, P. lunatus lines were shown to be less susceptible to beetle feeding damage than P. vulgaris (Wolfenbarger and Sleesman, 1961a). In general, lima beans were less preferred by beetles than snap beans (Raina et al., 1978).

Resistance to the potato leafhopper Empoasca fabalis is known among varieties of lima bean. Among the several legume species tested, plant introductions of P. lunatus were reported to be almost immune to insect attack and resistant to nymphal infection which is the most destructive stage (Wolfenbarger and Sleesman, 1961b).

The green cotyledon of the mature seed of lima beans is a character sought by canners and freezers (Magruder and Wester, 1941). Additional values of the lima beans are their wide adaptability, high yielding ability and an excellent nutritional quality (Rachie and Roberts, 1974).

Scarlet Runner, a variety of P. coccineus was shown to contain resistance to root rot (Wallace and Wilkinson, 1965). P. coccineus is also a valuable source for resistance to bean yellow mosaic and bean common mosaic virus. When plants of this species were inoculated with strains of BYMV isolated from a severely infected bean field, no symptoms were observed (Baggett, 1956). Also lines of P. coccineus artificially inoculated with BCMV cultures showed no infection (Baggett and Frazier, 1959). Some lines of P. coccineus tested at CIAT showed resistance to leafhopper due to abundant pubescence (CIAT, 1977).

Phaseolus coccineus is a cross-pollinating species, while P. vulgaris is self-pollinated. The cross-pollinating character of P. coccineus was shown to be due primarily to the morphology of the reproductive parts including the extrorse position of the stigma, the shedding of pollen on stylar hairs below the stigma, and the presence of hairs around the stigma (Ibrahim and Coyne, 1975). Natural hybrids are known (Rutger and Beckman, 1970). The transfer of the cross-pollinating character of P. coccineus into P. vulgaris may facilitate crossing to other germplasm sources.

Germination in P. vulgaris is epigeal while it is hypogeal in P. coccineus. It was suggested that the hypogeal germination in P. coccineus was due to lack of auxin in the hypocotyl (Gates, 1951). In addition hypogeous seedlings were reported to exert a larger lifting

force at emergence than epigeous seedlings (Inouye et al., 1979). The transfer of P. coccineus into P. vulgaris would reduce injury during emergence in hard-crusted soils and increase seedling vigor.

A unique characteristic of the legumes is their symbiosis with certain nitrogen-fixing bacteria. The fact that legumes can add to the nitrogen supply of the soil renders them even more desirable in the agricultural production of less developed countries. The relationship between the host and bacterial strains is specific as indicated by the type and region of nodule formation (Fred et al., 1932). P. vulgaris is known to be very poor nitrogen-fixing species. More recently it has been shown that P. acutifolius and P. lunatus have more efficient nitrogen-fixing capacity and are specifically infected by strains of Rhizobium of the Vigna group (Henzell, 1977). The incorporation of P. lunatus germplasm into P. vulgaris would increase the efficiency of P. vulgaris nitrogen fixation.

Current Status of Interspecific Hybridization

Numerous attempts have been made in hybridizing Phaseolus species. Consistent success has been limited to crossing P. vulgaris (female) with P. coccineus (male). The first interspecific hybrid between these two species was reported by Mendel in 1865. The F_1 hybrids resembled the male parent P. coccineus but showed limited fertility. Segregation for plant habit and pod shape occurred in the F_2 generation but no satisfactory ratio was obtained due to the small number of progeny examined. Later studies showed large reciprocal differences in crosses involving these two species (Coyne, 1968). Viable and mature hybrid seeds were obtained only when P. vulgaris was the female parent whereas

the reciprocal cross failed to mature seed. The failure to obtain mature seeds has been attributed to various causes such as the disturbed development of the hybrid embryo and endosperm (Lamprecht, 1941; Kroh, 1962), the retarded growth of P. vulgaris pollen tubes in P. coccineus styles (Thomas, 1964) and plasmon-genome interactions (Smartt, 1970) which may be related to the difference in the breeding system of the two species (Hawkins and Evans, 1973; Miranda and Evans, 1973). However, these suggestions have not been well tested. Rare successes of using P. coccineus as the female parent in crossing with P. vulgaris have been reported. In these studies, either particular genotypic combinations were involved in the production of mature hybrid seed (Lamprecht, 1948; Thomas, 1964; Al Yasiri and Coyne, 1966; Smartt, 1970) or successful crosses occurred at a very low frequency during the screening of large numbers of genotypic combinations (Al Yasiri and Coyne, 1966). In some cases intraspecific hybrids of P. vulgaris (Wall and York, 1960; Bemis and Kedar, 1960) and P. coccineus (Smartt and Haq, 1972) were used. It was also reported that detaching the pods or partially breaking the pedicel enabled the recovery of hybrid seeds (Ibrahim and Coyne, 1975). Such a treatment was suggested to prevent the flow of inhibitors from the maternal plant into the embryo. As the frequency of success was extremely low and no further description of the alleged P. coccineus (female) x P. vulgaris (male) hybrids was reported, it is doubtful if such results are repeatable.

Various degrees of fertility of the hybrids P. vulgaris x P. coccineus were reported ranging from highly sterile (Lamprecht, 1941) to partially fertile (Thomas, 1964; Smartt, 1970), to fertile (Ibrahim and Coyne, 1975). In all cases morphologically abnormal types appeared in

the F_1 generation.

A skewed distribution toward the maternal parent was observed in F_2 and backcross generation for cotyledon position (Wall and York, 1957; Wall, 1970) and other morphological characters of the parents (Lamprecht, 1948; Smartt, 1970). The basis of such reversion to the maternal types was not known.

Hybridization of P. vulgaris with P. acutifolius was first investigated by Honma (1956) in order to incorporate common bean blight resistance into P. vulgaris. Reciprocal cross differences were observed in crosses between P. vulgaris and P. acutifolius. Crosses using P. vulgaris as female resulted in pod development, but all pods collapsed before maturity (Honma, 1956; Al Yasiri and Coyne, 1966; Smartt, 1970). It was suggested that fertilization had occurred but embryos aborted during early stages of development. The reciprocal cross failed to set any pods. The only exception was the use of specific cultivars of P. vulgaris and P. acutifolius which resulted in mature hybrid seeds (Smartt, 1970). However subsequent attempts by various investigators did not result in the recovery of hybrid seeds (Guttenmaher, 1971; Mok et al., 1978).

As hybrid embryos aborted before reaching a reasonable size, it was thought that the use of synthetic growth regulators might result in the production of larger embryos by delaying the time of embryo abortion and pod abscission (Al Yasiri and Coyne, 1964). Enhancement of pod set and delay of embryo abortion were observed following application of the growth regulators naphtalene acetamide and potassium gibberellate. Embryos resulting from such treatments were larger than those from untreated crosses but failed to reach maturity.

Embryo culture was used to recover hybrid plantlets between P. vulgaris and P. acutifolius. However, the frequency of viable hybrids was very low; four mature plants were obtained from several hundreds of embryos cultured (Honma, 1955).

Little information is available concerning hybridization between P. vulgaris and P. lunatus. Previous attempts in crossing these two species were unsuccessful. The cross P. vulgaris (female) x P. lunatus (male) set pods which collapsed in the early stages of development while the reciprocal cross did not form pods (Al Yasiri and Coyne, 1966). In other cases, no pod development occurred from crosses in either direction (Smartt, 1970). The only report of recovering mature hybrid seeds (Honma and Heeckt, 1959) as a result of intercrossing two intra-specific hybrids has never been repeated. The failure of the crosses between P. vulgaris and P. lunatus has been related to their distant phylogenetic relationship (Derbishyre et al., 1976).

MATERIALS AND METHODS

Plant Materials

Two genotypes of the species Phaseolus vulgaris, P. acutifolius and P. lunatus were included in the major studies. Additional genotypes and species were included as indicated. The genotypes and abbreviations are presented in Table 1.

Table 1. Species and genotypes of Phaseolus included in the present study.

Species	Genotype	Code Name
Phaseolus acutifolius	P.I. 310880	AC 1
	P.I. 321637	AC 2
Phaseolus coccineus	Scarlet Runner	SR
Phaseolus lunatus	Kingston	K
	Burpee Hybrid	B
Phaseolus vulgaris	Great Northern Nebraska #1	GN
	Gallatin 50	G 50
	P.I. 181955	-

Plants were grown in the greenhouse. The temperature was maintained at 24°C/18°C (day/night) and supplemental lighting was provided when necessary. Plants were grown in Jiffy Mix Plus (obtained from George Ball Pacific Company, California).

Examination of Pollination, Fertilization and Embryo Development of Interspecific Hybrids

Two genotypes each of P. acutifolius and P. lunatus were crossed reciprocally with each genotype of P. vulgaris. In addition, each of

the six genotypes was artifically self-pollinated. Flowers were emasculated and pollinated one day before opening. Pollinated buds were collected at three-hour intervals for the first 24 hours following pollination, and thereafter at 12 hour-intervals up to 72 hours. Buds and young pods were fixed in fixative (3 volumes of 95% ethanol: 1 volume of glacial acetic acid) for 24 hours and then stored in 70% ethanol. Styles and ovules were excised under stereo dissecting microscope.

1. Pollen tube growth

The styles were softened in 1 N NaOH for 15 min and stained with 1% aniline blue, and examined with a UV-microscope. The length of the style and the number of pollen tubes in each style were recorded. The length of each pollen tube was measured as a fraction of the stylar length. The growth rate of the pollen tube was expressed as percent of the average pollen tube length in relation to the stylar length.

2. Fertilization and early embryo development

Ovules were stained with 45% acetocarmine (approximately 1 g carmine per 100 ml of 45% acetic acid) and covered with a plastic cover slip. The embryo sacs were squeezed out of the ovules by slightly pressing and tapping the cover slip with a needle. Embryo and endosperm development was examined. Based on the descriptions of Weinstein (1926), the pollination and fertilization processes were classified into five stages as presented in Table 2.

Table 2. Classification of pre- and post-fertilization events in Phaseolus.

Stage	Embryo sac
1	presence of the egg cell, the polar nuclei and the synergids
2	degeneration of the synergids
3	penetration of the pollen tube or discharge of sperm nuclei in the embryo sac
4	fusion of the polar nuclei and fertilization of the egg
5	division of the endosperm and the zygote proper

3. Embryo development and embryo culture

Developing pods were collected at three-day intervals for the study of embryo development. At later stages the size of pods, seeds and embryos was recorded for each sample. Pods were surface-sterilized by immersion in 95% ethanol for three minutes and in 1% sodium hypochlorite for 10 minutes, and then rinsed in sterile water. Seeds and embryos were excised with flame-sterilized instruments under a dissecting microscope. All operations related to embryo culture were performed in a laminar flow hood (Baker Instrument).

The culture medium consisted of mineral salts and organic nutrients including sucrose, vitamins and growth factors as necessary. The composition of the inorganic nutrients (Table 3) was that described by Murashige and Skoog (1962). Sucrose and myo-inositol were added at the concentrations of 30 g and 100 mg per liter respectively. The following vitamins were added: thiamine-HCl (1 mg/l), nicotinic acid (5 mg/l) and pyridoxine HCl (0.5 mg/l). The pH of the medium was adjusted to 5.7 and

Table 3. Composition of inorganic nutrients in the embryo culture medium (Murashige and Skoog, 1962).

Major elements				Minor elements		
Salts	mg/l	mg atoms/l		Salts	mg/l	mg atoms/l
NH_4NO_3	1650	N	41.2	H_3BO_3	6.2	100
KNO_3	1900	N,K	18.8	$MnSO_4$ $4H_2O$	22.3	100
$CaCl_2$ $2H_2O$	440	Ca	3.0	$ZnSO_4$ $4H_2O$	8.6	30
$MgSO_4$ $7H_2O$	370	Mg,S	1.5	KI	0.83	5.0
KH_2PO_4	170	P,K	1.25	Na_2MoO_4 $2H_2O$	0.25	1.0
Na_2EDTA	37.3[1]	Na	0.20	$CuSO_4$ $5H_2O$	0.025	0.1
$FeSO_4$ $7H_2O$	27.8	Fe	0.10	$CoCl_2$ $6H_2O$	0.025	0.1

[1] 5 ml/l of a stock solution containing 5.57 g $FeSO_4$ $7H_2O$ and 7.45 g Na_2EDTA per liter of dd H_2O

Difco Bacto Agar was added at 10 g/l to solidify the culture medium. The medium was dispensed into sample bottles (25 ml/bottle), and autoclaved at 120°C for 15 min. The effects of gibberellin and glutamine in supporting the embryo growth were tested by adding appropriate amount of these chemicals dissolved in DMSO (Schmitz and Skoog, 1970).

Cultured embryos were maintained at 28°C under constant light for the initial four weeks. Embryos that survived were transferred to fresh medium in mason jars (75 ml/jar) and then maintained at a photoperiod of 18 hours at 28°C. The resulting plantlets were transferred to Jiffy Mix Plus and placed in a misting chamber in the greenhouse.

Special treatments were given to P. lunatus x P. vulgaris crosses. In order to test the effects of cytokinins on hybrid embryo development, seedlings of P. lunatus cv. Kingston were also grown in hydroponics. The liquid solution consisted of a 25% strength Murashige and Skoog inorganic nutrients. Flower buds were pollinated with P. vulgaris cv. G 50 and N^6-benzyladenine (bzl^6Ade) was applied on the flower peduncle or supplied to the nutrient solution at the concentration levels of 3 μM and 10 μM. Flowers and young pods were collected upon abscission. Fixation and examination procedures were the same as in previous sections.

Examination of the Meiosis and Fertility of Interspecific Hybrids

Meiosis was studied in hybrids obtained from reciprocal crosses of vulgaris and P. acutifolius. Flower buds were fixed in a solution of ethanol-glacial acetic acid (3:1) saturated with iron acetate. Anthers were dissected and squashed in 45% acetocarmine. Critical stages of microsporogenesis were examined.

The male fertility was estimated by pollen stainability with acetocarmine and *in vivo* germination (on stigmatic surface) at 12 and 24 hours after artificial self-pollination. Five flowers from each plant were dissected and 200 pollen grains were examined from each flower.

The frequencies of fertilized ovules and ovules containing dividing embryos were determined in selfing and backcrosses to G 50 and AC 2. Young pods were collected at 24 hour-intervals.

Embryos obtained from backcrosses were dissected from 14 to 26 day-old pods and were cultured on embryo culture medium. The frequency of viable embryos was recorded.

RESULTS

Pollination and Early Development of Interspecific Hybrid Embryos

The pollination of selfed and interspecific crosses was examined. In all genotypic combinations, the majority of the pollen grains germinated on the surface of the stigma by three hours after pollination, but only a few pollen tubes penetrated the stigmatic surface. The number of pollen tubes observed in the style ranged from 7 to 13. The length of the style varied with the species and the genotypes (Table 4). The longest styles were observed in the flowers of G 50 (P. vulgaris) measuring 14 mm, and the shortest were found in P. lunatus and P. acutifolius genotypes (9 mm). Despite the variation in stylar length, the number and growth rate of pollen tubes were not different between selfing and interspecific crosses. The growth rate of pollen tubes is presented in Table 4. On the average, pollen tubes reached 80 to 90% of the length of the style at three hours after pollination. At nine hours all pollen tubes reached the base of the style.

Usually one pollen tube entered each ovule and fertilization began soon after. Cytologically, fertilization was defined by the fusion of a male gamete with the central cell (Figure 1a). The time of fertilization was arbitrarily defined as when fertilization has taken place in 50% or more of the ovules examined. The time intervals to fertilization following pollination in selfings are presented in Table 5. The proportion of ovules self-fertilized at each sampling time was recorded. In P. vulgaris genotypes, at three hours after pollination, 5% and 14% of the ovules examined had been fertilized in GN and in G 50 respectively. Over 50% of the ovules were fertilized between 18 and 21

Table 4. Pollen tube growth (in percent of stylar length) at different time intervals following pollination in intra- and interspecific crosses.

Cross	3 h	6 h	9 h	Stylar length (cm)
Intraspecific				
GN x GN	90	95	100	11
G50 x G50	76	90	100	13.7
K x K	95	100	100	9.4
AC2 x AC2	100	100	100	9.4
Interspecific				
GN x K	98	96	100	12.3
K x GN	88	100	100	10.0
G50 x K	93	95	100	13.2
K x G50	86	89	100	10.0
GN x AC2	87	94	100	12.9
AC2 x GN	100	98	100	9.9
G50 x AC2	93	94	100	13.0
AC2 x G50	89	93	100	9.6

Figure 1. Fertilization and division of endosperm and embryo in *Phaseolus* selfing and interspecific crosses.

a. Post-fertilization embryo sac in GN selfed, 18 hours.

b. Dividing endosperm and embryo in GN x AC 2, 24 hours.

c. Dividing endosperm and embryo in GN x K, 72 hours.

d. Dividing endosperm and 3-celled embryo in K x GN, 72 hours.

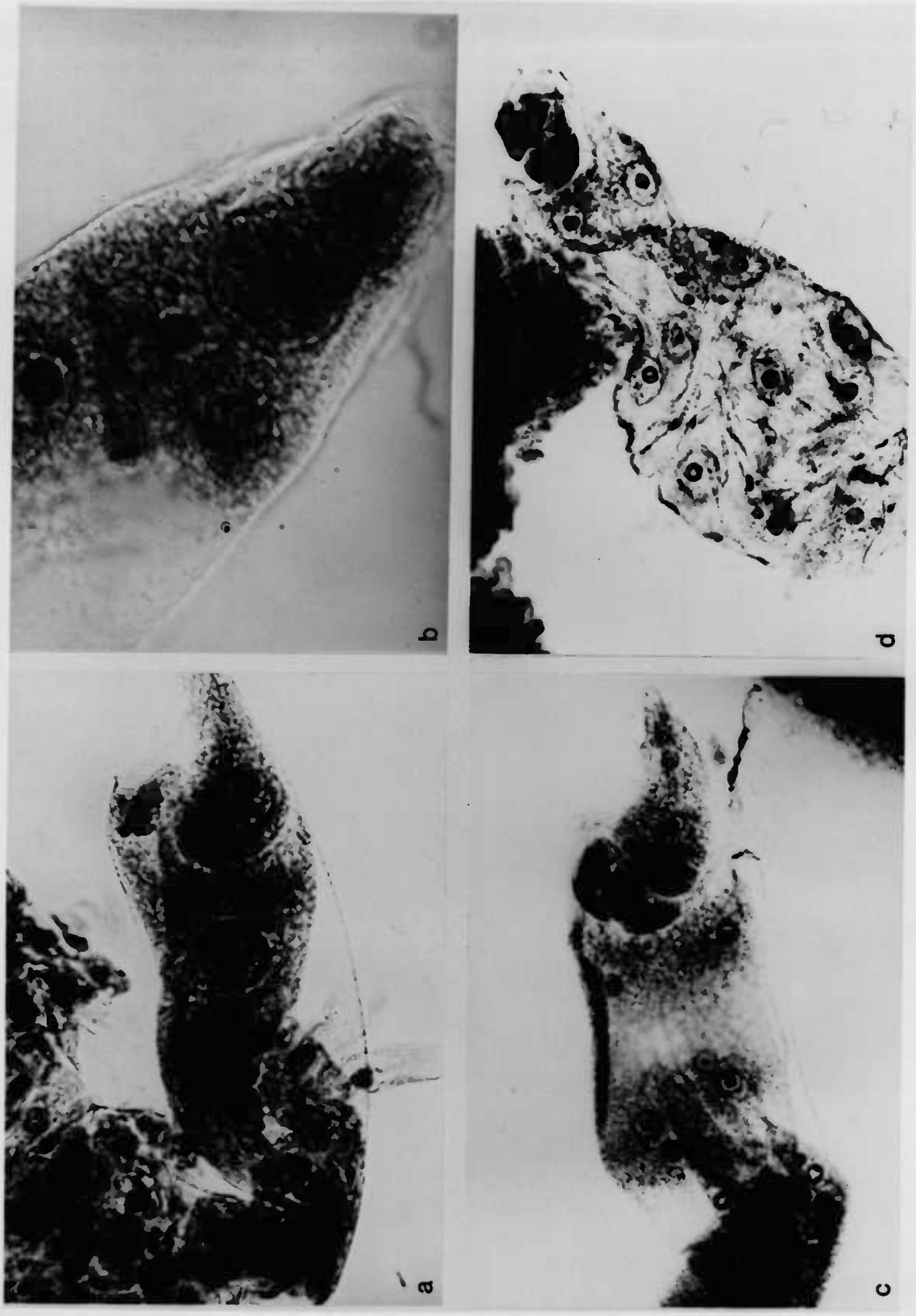
b
d
a
c

Table 5. The intervals (in hours) between pollination and fertilization, and between pollination and endosperm division in selfing.

Species/Cross	Fertilization		Endosperm division		Total no. of ovules examined at all times
	Time interval	No. of ovules examined	Time interval	No. of ovules examined	
P. vulgaris					
GN x GN	18-21	78	24-36	113	595
G50 x G50	6-9	78	21-24	112	568
P. acutifolius					
AC1 x AC1	0-3	34	24-36	45	246
AC2 x AC2	0-3	56	21-24	108	534
P. lunatus					
K x K	9-12	66	36-48	67	386
B x B	3-6	41	48-60	40	257

hours in GN and between 6 and 9 hours in G 50. In both genotypes of P. acutifolius fertilization had taken place in more than 60% of the ovules at the first sampling time (3 hours). In P. lunatus genotypes, the majority of the ovules were fertilized between 9 and 12 hours after pollination in K and between 3 and 6 hours in B.

After fertilization, the central cell which became the primary endosperm cell, underwent division. This stage was defined cytologically when four or more nuclei were observed in place of the central cell (Figure 1b). The fertilized egg divided soon after endosperm division. The time intervals between pollination and endosperm division in selfings are presented in Table 5. Division of the endosperm in GN was initiated at 12 hours after pollination but the majority of the ovules (50%) contained dividing endosperm in the interval between 24 and 36 hours after pollination. In G 50 endosperm division also began at 12 hours after pollination and the majority of the ovules examined between 21 and 24 hours had dividing endosperm. The first embryo division occurred when endosperm division was completed in more than 50% of the ovules examined, which were at 24 (GN) and 21 hours (G 50) after pollination. In P. acutifolius genotypes, despite an early fertilization, endosperm division did not occur until 24 to 36 hours after pollination in AC 1 and 21 to 24 hours in AC 2. The two-celled embryo stage was reached in AC 2 at 21 hours when the endosperm contained approximately 30 nuclei, and in AC 1 at 24 hours. At 72 hours after pollination the embryo consisted of 18 to 30 cells. P. lunatus genotypes were characterized by a long interval between fertilization and endosperm division. In K, endosperm began to divide at 18 hours after pollination, and dividing endosperms were observed in more than 50% of the ovules between 36 and 48

hours. In B, endosperm division occurred at 21 hours and was not observed in the majority of the ovules until 48 to 60 hours after pollination. Embryos started to divide at 48 hours after pollination in K and at 60 hours in B. At 72 hours the most advanced embryos contained 25 to 30 cells.

Fertilization of ovules was observed in all interspecific crosses examined at different sampling times. The time intervals at which fertilization took place are presented in Table 6. In all interspecific crosses, fertilization occurred at the same time as in the female parent when self-pollinated. In crosses between P. vulgaris and P. acutifolius, fertilization always occurred within the first three hours after pollination when P. acutifolius was used as the female parent. The process was completed in the period between 6 and 12 hours with G 50 and between 18 and 24 hours with GN as the female parents in crossing with P. acutifolius or P. lunatus genotypes. In crosses between P. lunatus (female) and P. vulgaris (male) fertilization occurred earlier than in the reciprocal crosses.

The time of endosperm division was influenced by the genotypes of both the male and the female parent. When P. vulgaris genotypes were the female parents, endosperm division was observed at similar time interval after pollination as in the male parent P. acutifolius or P. lunatus. Embryo division took place when the endosperm contained 6 to 12 nuclei. At 72 hours embryos derived from the crosses P. vulgaris (female) x P. acutifolius (male) contained approximately 16 cells and measured 0.2 mm in length. In the reciprocal crosses, endosperm division occurred much later than in the selfed parents. In P. acutifolius (female) x P. vulgaris (male) crosses, endosperm division was observed

Table 6. The intervals (in hours) between pollination and fertilization, and between pollination and endosperm division in interspecific crosses.

Species/Cross	Fertilization		Endosperm division		Total No. of ovules examined at all sampling times
	Time interval	No. of ovules examined	Time interval	No. of ovules examined	
P. vulgaris x *P. acutifolius*					
GN x AC1	18-21	56	24-36	58	369
GN x AC2	18-21	57	24-36	112	566
G50 x AC1	6-9	62	24-36	57	285
G50 x AC2	6-9	107	21-24	96	541
P. acutifolius x *P. vulgaris*					
AC1 x GN	3-6	84	60-72	48	438
AC2 x GN	0-3	56	60-72	100	568
AC1 x G50	0-3	44	60-72	32	340
AC2 x G50	0-3	48	48-60	109	512
P. vulgaris x *P. lunatus*					
GN x K	21-24	85	36-48	112	524
GN x B	21-24	92	48-60	68	530
G50 x K	6-9	103	36-48	98	643
G50 x B	9-12	68	48-60	71	384
P. lunatus x *P. vulgaris*					
K x GN	12-15	67	> 72	53	409
K x G50	9-12	47	> 72	28	312
B x GN	3-6	58	> 72	42	301
B x G50	3-6	53	> 72	25	261

Table 7. Frequencies of ovules in *P. lunatus* x *P. vulgaris* with dividing endosperm at 72 hours after pollination.

Cross	No. of ovules examined	Freq. (%) of ovules with dividing endosperm
K x GN	35	11
K x G50	28	7
B x GN	42	12
B x G50	25	8

between 36 and 60 hours after pollination. When P. lunatus genotypes were used as female, endosperm division was observed in only a small proportion of the ovules examined as late as 72 hours after pollination (Table 7). However, endosperm tissues appeared to be normal and had reached the free nuclei stage (Figure 1d). The hybrid embryos derived from these crosses were found to initiate division at 60 hours after pollination, but the development ceased at the four-celled stage, approximately three days after pollination.

Embryo Development at Later Stages

Embryos obtained from the crosses between P. vulgaris and P. acutifolius and between P. vulgaris and P. lunatus were examined at later stages (3 to 24 days after pollination). Reciprocal crosses were made between genotypes of P. vulgaris and P. acutifolius, however results involving AC 1 as the female parent were not compared due to insufficient flowering of this genotype. The size of pods, seeds and embryos resulting from these crosses were measured (Tables 8, 9 and 10). In P. vulgaris (female) x P. acutifolius (male) crosses, pods developed on G 50 were larger than those on GN, but there was no difference in the seed size. The early growth rate (up to 9 days) of the embryo appeared to be influenced by the maternal genotype. Embryos formed on G 50 plants were initially larger and had a higher initial growth rate. However, after 15 days embryos obtained from either female parent reached the same size and developed equally until 24 days after pollination. The genotype of the male parent did not seem to affect the growth rate or the final size of the embryos (Table 8).

Table 8. The average sizes and standard deviations of pods, seeds, and embryos obtained from P. vulgaris x P. acutifolius crosses as measured at three day intervals.

Cross	GN X AC 1			G50 X AC 1		
Day	Pod (cm)	Seed (mm)	Embryo (mm)	Pod (cm)	Seed (mm)	Embryo (mm)
3	4.5±0.3 (10)[1]	2.8±0.3 (31)	0.2±0.0 (31)	5.7±0.3 (6)	2.1±0.3 (11)	0.2±0.0 (11)
6	5.5±0.4 (11)	3.5±0.5 (28)	0.3±0.1 (27)	6.6±0.5 (6)	2.5±0.5 (10)	0.4±0.0 (10)
9	6.6±0.6 (10)	4.3±0.6 (31)	0.3±0.1 (31)	7.0±0.5 (6)	3.0±0.5 (11)	1.5±0.1 (11)
12	8.5±0.7 (10)	3.8±0.1 (34)	1.1±0.1 (34)	8.2±0.7 (7)	3.5±0.4 (15)	1.8±0.2 (14)
15	8.0±0.8 (10)	5.0±0.2 (25)	2.0±0.2 (25)	8.5±0.6 (10)	4.3±0.8 (20)	2.4±0.1 (18)
18	9.0±0.5 (10)	5.4±0.5 (33)	2.8±0.2 (30)	8.8±0.9 (8)	5.2±0.4 (13)	3.2±0.2 (13)
21	9.2±1.0 (10)	5.3±0.4 (30)	3.2±0.3 (28)	9.2±0.7 (5)	5.5±0.7 (8)	3.6±0.3 (8)
24	9.5±0.8 (9)	5.5±0.4 (24)	4.0±0.4 (24)	10.5±0.5 (6)	5.5±0.6 (9)	3.9±0.4 (9)

Cross	GN X AC 2			G50 X AC 2		
Day	Pod (cm)	Seed (mm)	Embryo (mm)	Pod (cm)	Seed (mm)	Embryo (mm)
3	4.5±0.4 (10)	2.3±0.4 (32)	0.2±0.0 (30)	5.6±0.3 (10)	2.0±0.3 (12)	0.2±0.0 (10)
6	5.7±0.8 (12)	2.8±0.3 (40)	0.3±0.0 (39)	6.5±0.4 (10)	2.5±0.4 (10)	0.4±0.0 (14)
9	7.5±1.2 (11)	3.6±0.7 (42)	0.4±0.1 (41)	7.5±0.8 (10)	3.3±0.5 (12)	0.6±0.1 (12)
12	8.1±1.2 (17)	3.3±0.8 (60)	1.3±0.4 (60)	8.0±0.9 (8)	3.8±0.4 (16)	1.5±0.2 (15)
15	7.5±1.0 (7)	3.8±0.5 (19)	1.4±0.6 (19)	8.8±0.7 (8)	4.5±0.4 (22)	2.1±0.1 (22)
18	9.3±0.8 (10)	4.0±0.8 (41)	2.6±0.6 (40)	8.5±0.4 (10)	5.0±0.7 (20)	2.4±0.2 (20)
21	9.2±0.8 (11)	4.5±0.5 (30)	3.0±0.4 (28)	9.8±0.8 (7)	5.3±0.4 (15)	2.8±0.3 (15)
24	9.5±0.7 (10)	5.0±0.4 (28)	3.5±0.2 (24)	11.0±0.5 (8)	6.0±0.4 (24)	3.5±0.3 (24)

[1]Number of individuals examined.

Table 9. The average sizes and standard deviations of pods, seeds and embryos obtained from P. acutifolius x P. vulgaris crosses as measured at three day intervals.

Cross	AC 2 X GN			AC 2 X G50		
Day	Pod (cm)	Seed (mm)	Embryo (mm)	Pod (cm)	Seed (mm)	Embryo (mm)
3	3.5±0.4 (5)[1]	2.1±0.3 (8)	0.2±0.0 (8)	3.6±0.4 (4)	2.3±0.3 (5)	0.25±0.0 (5)
6	4.2±0.3 (4)	2.5±0.4 (6)	0.4±0.0 (6)	4.8±0.2 (4)	2.4±0.3 (6)	0.40±0.0 (6)
9	5.2±0.2 (5)	2.8±0.3 (5)	0.8±0.1 (5)	5.7±0.4 (5)	2.9±0.2 (5)	1.00±0.1 (5)
12	6.0±0.5 (4)	3.7±0.2 (3)	1.5±0.2 (3)	6.1±0.4 (5)	4.0±0.3 (5)	1.20±0.1 (5)
15	6.8±0.5 (3)	4.0±0.3 (4)	1.7±0.1 (4)	6.2±0.6 (6)	4.3±0.4 (7)	1.90±0.2 (7)
18	7.0±0.5 (4)	4.5±0.2 (5)	1.6±0.1 (5)	7.5±0.6 (4)	5.0±0.4 (6)	2.00±0.2 (6)
21	7.5±0.4 (5)	4.5±0.3 (6)	2.0±0.1 (6)	8.2±0.5 (4)	5.1±0.5 (4)	2.50±0.4 (4)
24	7.2±0.3 (6)	5.0±0.5 (8)	2.3±0.4 (8)	9.0±1.5 (3)	5.5±0.5 (5)	3.00±0.2 (5)

[1]Number of individuals examined.

Table 10. The average sizes and standard deviations of pods, seeds, and embryos obtained from P. vulgaris x P. lunatus crosses as measured at three day intervals after pollination.

Cross	GN X K			G50 X K		
Day	Pod (cm)	Seed (mm)	Embryo (mm)	Pod (cm)	Seed (mm)	Embryo (mm)
3	3.5±0.7 (14)[1]	0.6 ±0.3 (54)	0.02±0.01 (53)	5.5±0.5 (9)	1.5 ±0.2 (27)	0.05±0.02 (27)
6	4.5±0.4 (15)	0.9 ±0.3 (51)	0.02±0.01 (51)	7.2±0.5 (8)	1.7 ±0.2 (44)	0.10±0.02 (44)
9	4.9±0.8 (14)	1.7 ±0.3 (51)	0.05±0.02 (50)	9.3±0.4 (10)	2.8 ±0.7 (20)	0.23±0.09 (20)
12	6.9±1.2 (21)	2.0 ±0.6 (61)	0.14±0.03 (58)	8.7±1.0 (13)	3.4 ±0.5 (33)	0.24±0.04 (30)
15	6.2±2.1 (27)	2.3 ±0.5 (81)	0.30±0.16 (79)	8.8±0.6 (11)	3.0 ±0.5 (30)	0.35±0.02 (28)
18	6.3±0.5 (21)	2.3 ±0.3 (67)	0.29±0.07 (66)	9.0±0.3 (8)	3.2 ±0.2 (24)	0.40±0.03 (21)
21	6.0±0.8 (12)	2.8 ±0.4 (38)	0.34±0.08 (35)	10.0±0.5 (6)	3.5 ±0.3 (24)	0.40±0.02 (20)
24	6.7±1.2 (5)	3.0 ±0.3 (15)	0.32±0.06 (15)	10.5±0.5 (9)	4.25±0.4 (7)	0.42±0.05 (21)

Cross	GN X B			G50 X B		
Day	Pod (cm)	Seed (mm)	Embryo (mm)	Pod (cm)	Seed (mm)	Embryo (mm)
3	3.5±0.8 (11)	1.0±0.3 (10)	0.02±0.01 (10)	5.8±0.3 (10)	1.5±0.4 (21)	0.05±0.02 (19)
6	4.5±0.4 (11)	1.2±0.3 (10)	0.02±0.02 (10)	7.5±0.4 (10)	2.0±0.4 (20)	0.05±0.03 (18)
9	5.6±0.4 (7)	1.6±0.3 (10)	0.05±0.03 (9)	8.3±1.1 (10)	2.4±0.8 (31)	0.10±0.04 (30)
12	5.4±0.4 (18)	1.5±0.3 (53)	0.12±0.02 (37)	8.0±0.5 (8)	2.5±0.5 (24)	0.15±0.03 (24)
15	6.6±0.6 (9)	1.6±0.4 (22)	0.14±0.04 (22)	8.5±0.7 (8)	3.0±0.4 (19)	0.26±0.02 (19)
18	6.5±0.7 (11)	1.6±0.3 (13)	0.13±0.03 (10)	10.0±0.8 (7)	4.0±0.3 (14)	0.25±0.05 (14)
21	6.6±0.5 (8)	1.8±0.4 (8)	0.15±0.03 (8)	11.0±0.7 (8)	4.5±0.3 (16)	0.27±0.04 (15)

[1]Number of individuals examined.

The sizes of pods, seeds and embryos from the crosses P. acutifolius (female) x P. vulgaris (male) are presented in Table 9. Pods and seeds were in general smaller than in the reciprocal crosses. The later development of the hybrid embryos appeared to be influenced by the genotype of the male parent. Although the growth rates during the first 15 days were similar, the final size attained by the hybrid embryos using G 50 as the male parent were larger than those pollinated with GN pollen (3.0 v.s. 2.3 mm).

The hybrid embryos developed normally until late heart or early cotyledon stage. At this time, the hybrid embryos were characterized by asymmetrical cotyledons (Figure 2a). The asymmetry was accentuated at later developmental stages (Figure 3). The cotyledons were open rather than closely attached as in the selfed embryos. This characteristic morphology of hybrid embryos was observed in all P. vulgaris - P. acutifolius crosses and was used to distinguish hybrid embryos from selfed embryos, which occurred occasionally.

In the crosses between P. vulgaris and P. lunatus, measurements were obtained only from crosses using P. vulgaris as the female parent since pods from the reciprocal crosses abscised within 2 to 3 days after pollination. In all four crosses between genotypes of P. vulgaris (female) and P. lunatus (male) the pods, seeds and embryos were much smaller than those from the crosses with P. acutifolius. The hybrid embryos were initially 0.02 to 0.05 mm long and reached a final size of 0.5 mm at 24 days. Hybrid embryos developed on G 50 exhibited a faster growth rate than those formed on GN. However, after 15 days the growth rate of embryos derived from G 50 or GN as the female parent was similar. Embryos formed on G 50 were initially larger compared with those formed

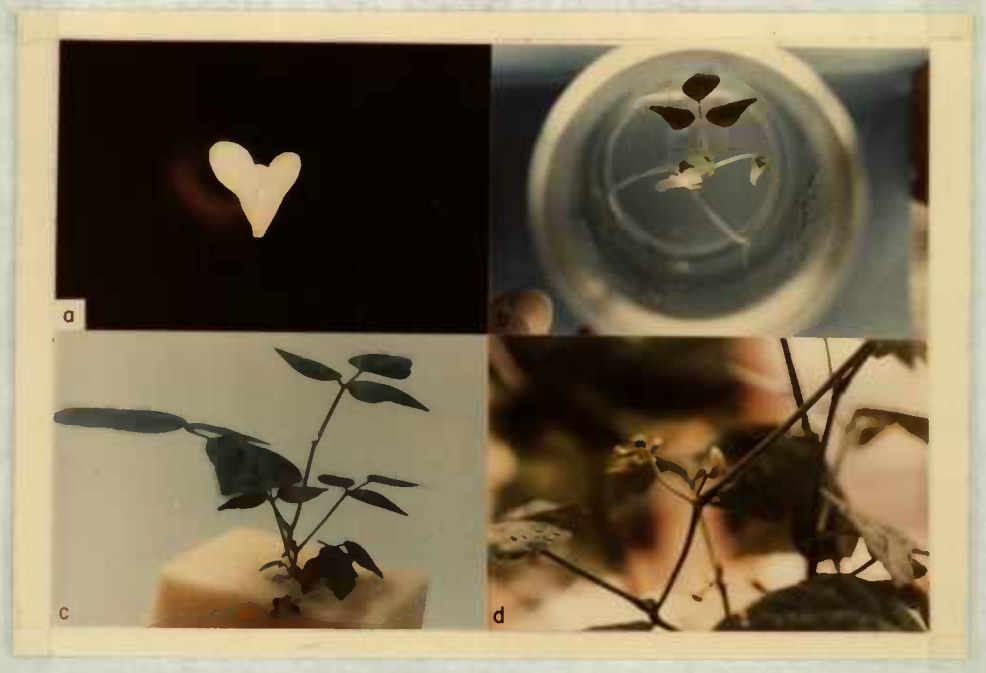

Table 2. Embryo and plantlets obtained from crosses between P. vulgaris and P. acutifolius.

a. Embryo of GN x AC 1, 12 days after pollination.

b. Plantlet derived from cultured embryo of GN x AC 1.

c. Plantlet transferred to hydroponic culture.

d. Inflorescence of hybrid GN x AC 1.

Figure 3. Hybrid embryos of the cross between P. vulgaris (P.I. 181955) and P. acutifolius (AC 2) with asymmetrical cotyledons.

on GN and the final size was larger.

The genotype of the male parent also affected the early growth rate and the final size of hybrid embryos. The growth rate was slower when B was the male parent and the final size (at 21 days after pollination) was smaller although the initial sizes of the hybrid embryos were the same when either K or B was used as the male parent.

P. vulgaris (female) x P. lunatus (male) embryos developed up to the pre-heart shaped stage in a rod shape (Figure 4a). No apparent progress in the development of the embryo was detected although the hybrid embryos increased slightly in size after 21 days. The final size attained by the hybrid embryos was 0.45 to 0.50 mm.

Due to the abnormal development of interspecific hybrid embryos and the failure to obtain mature seeds, culturing of embryos on artificial medium was necessary for the recovery of hybrids. The effects of glutamine and gibberellin were tested as these substances were reported (Raghavan, 1976) to be beneficial for the growth of immature embryos. Hybrid embryos from reciprocal crosses between P. acutifolius and P. vulgaris were classified into two groups according to size: larger or smaller than 1 mm. Hybrid embryos of P. vulgaris (female) x P. lunatus (male) were separated into two groups, smaller or larger than 0.3 mm. The survival rates of embryos after a culture period of four weeks were recorded (Tables 11, 12 and 13).

The addition of glutamine to the culture medium had a beneficial effect on hybrid embryo survival. The survival rate of the smaller embryos (<1 mm) from P. vulgaris (female) x P. acutifolius (male) increased significantly from 7 to 33% (Table 11). For the embryo of the reciprocal cross, 0 to 14% survived on medium devoid of glutamine

Figure 4. Embryo and plantlet derived from crosses between P. vulgaris and P. lunatus.

a. Hybrid embryo of G 50 x K, 15 days after pollination.

b. Plantlet derived from cultured embryo of G 50 x K (45 days).

c. Plantlet transferred tc soil.

d. Plantlet transferred to hydroponic culture.

Table 11. The effects of glutamine and gibberellin on the survival rate of hybrid embryos obtained from *P. vulgaris* x *P. acutifolius* crosses.

Cross	Embryo size (mm)	Survival Rate				
		Control	Glutamine (mg/l)		Gibberellin + Glutamine (mg/l)	
			10	100	10	100
GN X AC1	< 1	14%(22)	30%(23)	33%(21)	30%(23)	28%(25)
	1-4	45%(49)	50%(38)	50%(34)	56%(36)	59%(29)
GN X AC2	< 1	13%(24)	24%(25)	29%(21)	28%(25)	24%(21)
	1-4	47%(15)	43%(51)	50%(40)	44%(52)	58%(33)
G50 X AC1	< 1	7%(14)	27%(11)	27%(11)	25%(12)	25%(12)
	1-4	50%(40)	64%(11)	58%(12)	75%(12)	69%(13)
G50 X AC2	< 1	13% (8)	31%(16)	14%(14)	21%(14)	13%(16)
	1-4	50%(14)	50%(18)	52%(21)	60%(15)	63%(19)

Table 12. The effects of glutamine and gibberellin on the survival rate of hybrid embryos obtained from *P. acutifolius* x *P. vulgaris* crosses.

Crosses	Embryo size (mm)	Survival Rate				
		Control	Glutamine (mg/l)		Gibberellin + Glutamine (mg/l)	
			10	100	10	100
AC2 X GN	< 1	0% (6)[1]	40%(15)	20%(5)	20%(5)	25%(4)
	1-4	50% (8)	43% (7)	50%(6)	33%(6)	43%(7)
AC2 X G50	< 1	14%(17)	25% (4)	25%(4)	25%(4)	25%(4)
	1-4	44% (9)	37% (8)	57%(7)	50%(8)	43%(7)

[1]Number of embryos cultured

Table 13. The effects of glutamine and gibberellin on the survival rate of hybrid embryos obtained from P. vulgaris x P. lunatus crosses.

Cross	Embryo size (mm)	Survival Rate				
		Control	Glutamine (mg/l)		Gibberellin + Glutamine (mg/l)	
			10	100	10	100
GN X K	< 0.3	0%(33)[1]	17%(60)	21%(70)	16%(67)	21%(68)
	0.3-0.5	5%(21)	30%(23)	35%(31)	32%(31)	35%(31)
GN X B	< 0.3	0%(21)	0%(27)	4%(25)	0%(25)	4%(26)
G50 X K	< 0.3	0%(14)	16%(32)	26%(31)	14%(35)	24%(38)
	0.3-0.5	11%(18)	29%(17)	50%(18)	35%(17)	56%(18)
G50 X B	< 0.3	0%(12)	3%(34)	5%(37)	3%(34)	5%(37)

[1]Number of embryos cultured

whereas 25 to 45% survived when glutamine was added to the culture medium. The levels of glutamine (10 and 100 mg/l) did not influence the survival of hybrid embryos. No significant effect of glutamine was observed in the survival of larger embryos (>1 mm). The addition of gibberellin did not enhance the survival of hybrid embryos.

The addition of glutamine to the culture medium had a more pronounced effect on the very small embryos (<0.3 mm) of GN x K and G 50 x K since none of the hybrid embryos survived on medium devoid of glutamine. On glutamine containing medium the frequency of surviving embryos was higher. Higher concentration of glutamine (10 v.s. 100 mg/l) increased slightly the rate of survival. The survival rate of small embryos (0.3 mm) from GN x B and G 50 x B was also enhanced but remained very low (3 to 5%). Enhancement of embryo growth was also observed on larger embryos (>0.3 mm) of GN x K and G 50 x K; with the addition of glutamine the proportion of surviving embryos increased from 5 to 50%. Gibberellin had no apparent effect on the survival of hybrid embryos.

Hybrid plants obtained from embryo culture (Figures 2b and 4b) were transferred to soil (Figure 4c) or to hydroponic culture (Figures 2c and 4d). Reciprocal crosses between P. acutifolius and P. vulgaris resulted in hybrid plants with a climbing habit similar to P. acutifolius. The shape of the leaflets was more elongated than those of P. vulgaris and broader than the P. acutifolius type (Figure 5). The flower size and shape were intermediate between those of the parents. The flower color was lightly tinged with pink (whereas both P. vulgaris and P. acutifolius parents had white flowers). Pods produced on the F_1 plants were of the P. vulgaris type. The F_1 hybrids developed vigorously and flowered abundantly (Figure 2d). Hybrids between P. vulgaris

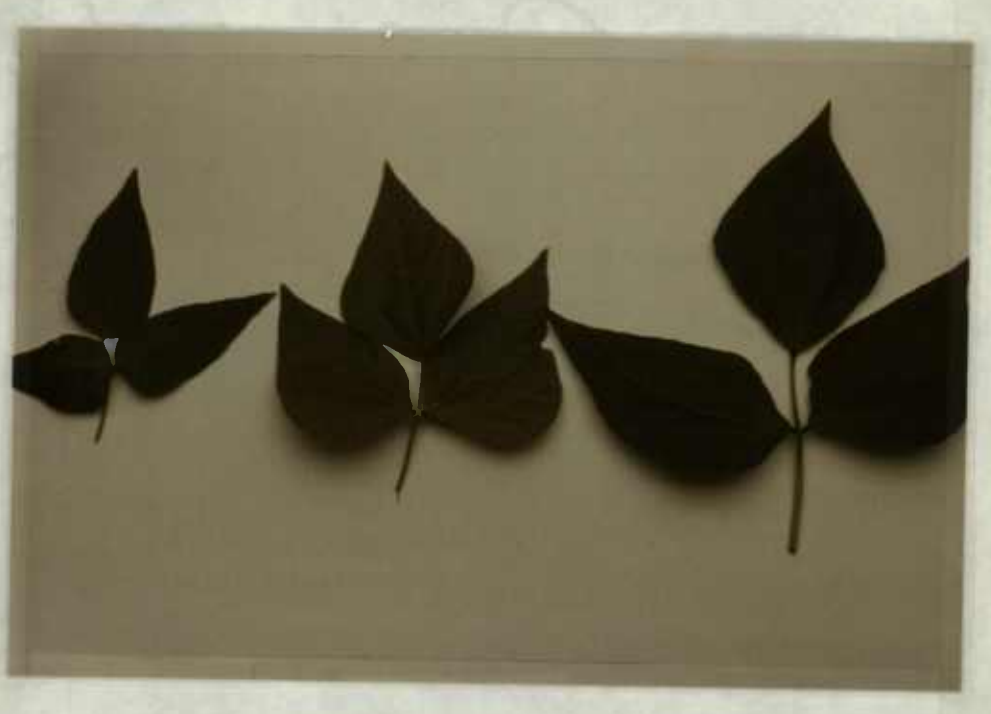

Figure 5. Leaf morphology of P. acutifolius (AC 2, left), P. vulgaris (G 50, right) and interspecific hybrid (center).

(female) and P. lunatus (male) were also obtained, however the growth was very slow and the plants began to senesce after three of four sets of trifoliolate leaves were formed.

Meiosis and Fertility of Interspecific Hybrids

As the utilization of interspecific hybrids is dependent to a large extent on the chromosome compatibility between species, the meiosis and fertility of interspecific hybrids were examined. The plant materials were reciprocal hybrids of G 50 (P. vulgaris) and AC 2 (P. acutifolius) and hybrids of AC 2 x P.I. 181955 (P. vulgaris).

Microsporogenesis was studied and particular attention was centered on Metaphase I and Anaphase I. Chromosome pairing and the frequency of univalents were determined (Tables 14 and 15). The number of univalents ranged from 0 to 14 (Figures 6a and b). The majority of the cells had four to eight univalents. The average number of univalents per microsporocytes was 6.0 in AC 2 x G 50, 6.6 in G 50 x AC 2 and 6.2 in AC 2 x P.I. 181955. These results indicated no reciprocal cross difference in the pattern of chromosome pairing, and a different genotype of P. vulgaris did not change the pattern substantially.

The number of laggards and chromosome distribution at Anaphase I are presented in Tables 16 and 17. All cells at Anaphase I contained 1 to 5 laggards with the majority of cells having 2 laggards present. The average numbers of lagging chromosomes per cell were 2.3 and 1.8 respectively for hybrids of G 50 - AC 2 and AC 2 - P.I. 181955. Although 1 or more lagging chromosomes per cell were observed at early Anaphase I, a high percentage (17% for G 50 - AC 2 hybrids and 20% for AC 2 x P.I. 181955) of cells were found to have equal distribution of

Table 14. Chromosome pairing at Metaphase I of interspecific hybrids between P. vulgaris (G 50) and P. acutifolius (AC 2).

No. of univalents	No. and percent of cells			
	G 50 X AC 2		AC 2 X G 50	
0	1	1.1	3	2.7
2	6	6.7	5	4.5
4	14	15.6	21	18.9
6	35	38.9	49	44.1
8	17	18.9	25	22.5
10	11	12.2	7	6.3
12	5	5.6	1	0.9
14	1	1.1	0	0
Total No. of cells examined	90		111	
Average No. of univalent per cell	6.6		6.0	

Table 15. Chromosome pairing at Metaphase I of interspecific hybrids between *P. acutifolius* (AC 2) and *P. vulgaris* (P.I. 181955).

No. of univalents	No. and percent of cells	
0	1	1.2
2	14	16.5
4	23	27.1
6	12	14.1
8	19	22.3
10	11	12.9
12	2	2.3
14	2	2.3
16	1	1.2
Total No. of cells examined	85	
Average No. of univalents per cell	6.2	

Figure 6. Meiosis of interspecific hybrids of P. vulgaris and P. acutifolius.

a. Metaphase I showing 10 bivalents and 2 univalents.

b. Metaphase I showing 4 bivalents and 14 univalents.

c. Anaphase I with 11-11 disjunction.

d. Anaphase I with 8-11 disjunction and with 3 chromosomes remaining in the center.

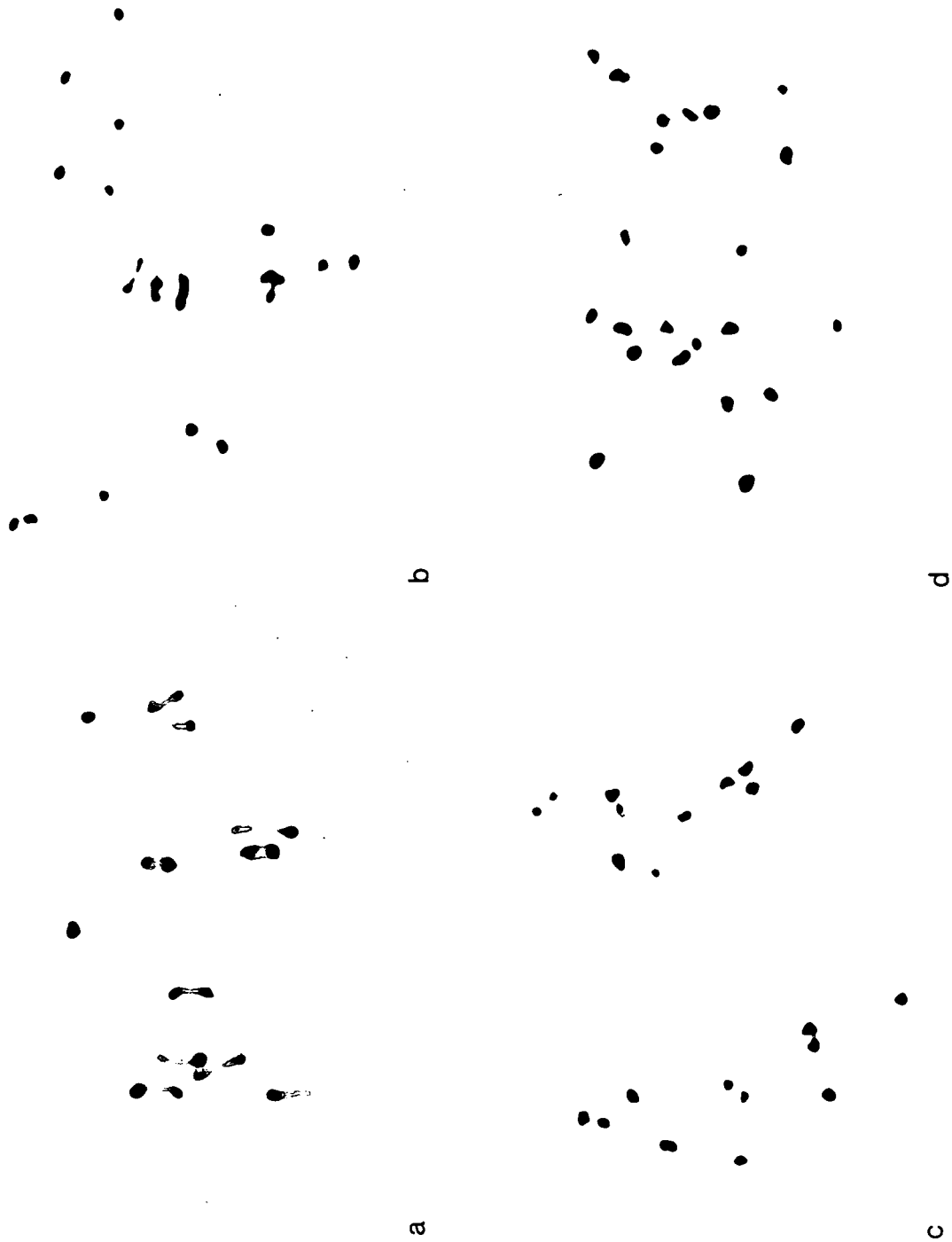
b
d
a
c

Table 16. Anaphase I disjunction of chromosomes of interspecific hybrids of *P. vulgaris* (G 50) and *P. acutifolius* (AC 2).

Early Anaphase I			Late Anaphase I		
No. of lagging chromosomes	No. and percent of cells		Chromosome distribution at Ana. I poles	No. and percent of cells	
0	0	0	11-11	7	17
1	8	21	10-12	18	45
2	21	54	9-13	12	30
3	6	15	8-14	3	8
4	2	5	7-15	0	0
5	2	5	6-16	0	0
Total No. of cells examined	39			40	
Average No. of lagging chromosomes per cell	2.3		Average distribution of chromosomes at Ana. I poles	9.7-12.3	

Table 17. Anaphase I disjunction of chromosomes of interspecific hybrids of *P. acutifolius* (AC 2) and *P. vulgaris* (P.I. 181955).

Early Anaphase I			Late Anaphase I		
No. of lagging chromosomes	No. and percent of cells		Chromosome distribution at Ana. I poles	No. and percent of cells	
0	3	8.1	11-11	6	20
1	8	21.6	10-12	14	47
2	21	56.8	9-13	8	27
3	3	8.1	8-14	1	3
4	2	5.4	7-15	1	3
Total No. of cells examined	37			30	
Average No. of lagging chromosomes per cell	1.8		Average distribution of chromosomes at Ana. I poles	9.5-12.5	

chromosomes (11 at each pole) at late Anaphase I (Figure 6c). The remaining cells had unequal distribution of chromosomes (Figure 6d). The most frequent disjunction (in 45 and 47% of the cells examined) was 10-12.

The male fertility of interspecific hybrids was estimated by pollen stainability with acetocarmine and by the frequency of germinated pollen on the stigma. The percentage of stainable pollen ranged from 10 to 25% with an average of 17% for all hybrids derived from the three parental combinations. Pollen germination was low; only 0.5 to 3.5% respectively germinated at 12 and 24 hours after pollination. No difference in stainability or germination rate was detected between reciprocal hybrids.

The female fertility of the interspecific crosses was evaluated by backcrossing to the parents. The frequencies of fertilized ovules and ovules with dividing embryos in selfing and backcrosses are presented in Table 18. At 12 hours after pollination, fertilization had occurred in 7% of the selfed ovules of interspecific hybrids. In backcrosses to G 50 and AC 2 the same proportion of ovules had been fertilized. At the last sampling time, fertilization was completed in 26% of the ovules in selfing and 20 and 31% in backcrossing to G 50 and AC 2 respectively.

After self-pollination, no embryo division was observed at all sampling times. When backcrossed to G 50, 3% of the ovules at 24 hours contained dividing embryos. The frequency increased only slightly in later sampling times and then remained constant at 4%. In backcrossing to AC 2, the frequency of dividing embryos at 24 hours after pollination was 4%, and increased to 13% at the last sampling time

Table 18. Frequencies (in percent) of (a) fertilized ovules and (b) ovules with dividing embryos, in selfing and backcrosses of interspecific hybrids to P. vulgaris (G50) and P. acutifolius (AC2) at different time intervals after pollination.

Cross	Time intervals after pollination				
	12 hours	24 hours	2 days	3 days	4 days
(G50-AC2) self	a. 7 (94)[1]	15 (144)	21 (57)	21 (66)	26 (89)
	b. 0	0	0	0	0
(G50-AC2) x G50	a. 6 (61)	11 (46)	19 (54)	20 (60)	20 (64)
	b. 0	3	4	4	4
(G50-AC2) x AC2	a. 8 (65)	17 (58)	19 (88)	25 (84)	31 (70)
	b. 0	4	7	13	13

[1]Number of ovules examined at each sampling time.

(4 days).

Pods resulting from backcrosses which were retained on the plants for a period of 14 to 26 days were collected and dissected. Immature embryos with sizes ranging from 0.15 to 1.5 mm were excised and cultured. A higher frequency of embryos was recovered from backcrosses to AC 2 (14%) than from backcrosses to G 50 (4%) (Table 19). Backcrossed progeny were again recovered with the aid of embryo culture (Figure 7).

The chromosome pairing in microsporogenesis and the male fertility of the backcross progeny were examined. The number of univalents at Metaphase I is presented in Table 20. The majority of the microsporocytes had 2 to 4 univalents. The average number of univalents per cell were 3.9 and 3.4 respectively for the two classes of backcross progeny. The lower number of univalents as compared with that of the F_1 hybrids were correlated with a high frequency of stainable pollen which was 55%. The chromosomal distribution at late Anaphase I was generally normal (Table 21). A high proportion of cells with 11-11 disjunction was observed. The average distribution at Anaphase I poles was 10.3-11.7 for (G 50 x AC 2) x AC 2 10.6-11.4 for (AC 2 x G 50) x AC 2. The backcross progeny were self-fertile and produced seeds with a frequency of 2 seeds per 5 pods (Figure 8).

Table 19. Number and frequency (in percent) of viable backcrossed embryos obtained at 14 to 26 days after pollination.

Cross	Number of ovules	Number	Percent of embryos
(G50-AC2) x G50	90	4	4
(G50-AC2) x AC2	129	18	14

Figure 7. Plants obtained from backcrossing the F_1 (G 50 x AC 2) to AC 2.

Table 20. Chromosome pairing at Metaphase I of backcrossed progeny of interspecific hybrids between P. vulgaris (G 50) and P. acutifolius (AC 2).

No. of univalents	No. and percent of cells			
	(G 50 x AC 2) x AC 2		(AC 2 x G 50) x AC 2	
0	0	0	3	4.8
2	25	55.6	27	43.5
4	12	26.7	23	37.1
6	2	4.4	5	8.1
8	2	4.4	4	6.5
10	3	6.7	0	0
12	1	2.2	0	0
Total No. of cells examined	45		62	
Average No. of univalents per cell	3.9		3.4	

Table 21. Late Anaphase I disjunction of chromosomes of the backcrossed progeny of interspecific hybrids between *P. vulgaris* (G 50) and *P. acutifolius* (AC 2).

Chromosome distribution at Ana. I poles	(G 50 x AC 2) x AC 2		(AC 2 x G 50) x AC 2	
	No. of cells	Percent of cells	No. of cells	Percent of cells
11-11	17	74	22	71
10-12	1	4	7	23
9-13	2	9	1	3
8-14	2	9	0	0
7-15	1	4	1	3
Total No. of cells examined	23		31	
Average distribution of chromosomes at Ana. I Poles	10.3-11.7		10.6-11.4	

Figure 8. Seed samples of P. vulgaris (G 50, left), P. acutifolius (AC 2, right) and the selfed seeds of backcrossed progeny (center).

DISCUSSION

Previous Suggestions on the Causes of Embryo Abortion in Interspecific Hybridization

The failure to produce viable seeds from interspecific hybridization was found to occur in many plant genera such as Triticum (Boyes and Thompson, 1937), Petunia and Nicotiana (Cooper and Brink, 1940), Lycopersicon (Cooper and Brink, 1945), Solanum (Lee and Cooper, 1958), Gossypium (Weaver, 1957 and 1958), Datura (Rietsema, 1959; Rietsema and Satina, 1959). Various hypotheses were suggested resulting from morphological and histological studies. In interspecific crosses of Datura (D. metel x D. discolor, D. stramonium x D. metel, and D. stramonium x D. innoxia) many empty seeds occurred and it was assumed that fertilization was not completed because of the failure of pollen tubes to reach the ovary (Sanders, 1948). Since pollen germination and pollen tube growth in intraspecific crosses are generally good in Datura, the cause of lack of fertilization was attributed to an inhibition of the growth of pollen tubes in a foreign style, the mechanism of which was not known (Sanz, 1945). Likewise, in Solanum, unfertilized seeds from 2x x 2x crosses were reported (Dionne, 1961; Sams et al., 1977). Lack of fertilization in this case was related to germination of pollen or germination followed by defective pollen tube growth (Sams et al., 1977). Pollen tube growth was found to occur at a rate slower than normal in crosses such as Nicotiana rustica x N. glutinosa (Cooper and Brink, 1940). Nevertheless, pollen germination and pollen tube growth were not considered effective barriers to crossability since fertilization occurred normally. Hybrid seeds were

formed which contained embryos at various stages of development and collapsed before maturity. Some hybrid plantlets were produced which died at an early stage. Only shrunken seeds were recovered from interspecific crosses of Triticum (Boyes and Thompson, 1937) and these seeds failed to germinate. Thus pre-fertilization events were not considered a limiting factor in most interspecific hybridizations.

Embryo abortion was often accompanied by endosperm abnormalities such as reduced rate of growth (Cooper and Brink, 1940; Cooper and Brink, 1945 and Boyes and Thompson, 1937) disintegration of endosperm cell walls in the vicinity of the embryo (Cooper and Brink, 1945) and abnormal cell structures (Boyes and Thompson, 1937). These observations suggested that the endosperm may play a central role in the development of the hybrid seed (Brink and Cooper, 1947).

Moreover, the maternal tissues adjacent to the endosperm were thought to influence the development of the hybrid seed since they provide nutrients to the endosperm which in turn serves as a nurse tissue to the developing embryo. An over-development of the maternal tissues was observed in interspecific crosses of Nicotiana rustica x N. glutinosa (Brink and Cooper, 1941), Lycopersicon pimpinellifolium x L. peruvianum (Cooper and Brink, 1945) and Solanum crosses (Lee and Cooper, 1958). The suggested mechanism for embryo abortion was that of maternal tissues drawing an excessive amount of nutrients from the endosperm which therefore became incapable of nourishing the embryo. Such disturbance in the relationship between the three types of tissues may have resulted from the combination of two species with distinct phases of seed development. Thus, the embryo ceased to grow and subsequently the seed collapsed following a disharmonious development

of the embryo and the endosperm.

The idea of a balance between the three types of tissue was expressed in terms of chromosome number. It was suggested that a 2:2:3 ratio was required for the normal development of the hybrid seed (Watkins, 1932; Muntzing, 1933). This assumption was used to explain the failure encountered in crosses between species with different degrees of ploidy, such as in Triticum crosses (Boyes and Thompson, 1937). However, many diploid x diploid crosses failed and reciprocal cross differences were usually observed. It was thought that the endosperm represented a barrier to hybrid embryo development, since the genetic constitution of the embryo and the maternal tissues were identical in reciprocal crosses but that of the endosperm differed. A certain genetic balance or some chromosomal factors were assumed to be involved in the development of the endosperm (Johnston et al., 1980). However, a normal endosperm development did not always ensure normal embryo growth. In Gossypium and Melilotus, embryo abortion occurred despite healthy endosperm and maternal tissues and a "physiological unbalance" between embryo and endosperm was suggested. Such unbalance was thought to be the result of competition between the two tissues (Greenshields, 1954), or the embryo producing certain substances which caused growth-cessation of the hybrid endosperm (Weaver, 1958).

Chemical factors were also invoked as possible causes of embryo abortion. In certain incompatible crosses of Datura the abortion of the hybrid embryo was explained on the basis of some changes in the growth regulating substances or enzyme systems (Sachet, 1948). It was suggested that IAA-like substances found in the ovary inhibited the growth of the hybrid embryo (Satina et al., 1950; Rietsema et al.,

1954). Also unbalance of auxins or other growth regulators were believed to have caused embryo and endosperm abnormalities encountered in the crosses Melilotus officinalis and M. suaveolens and M. alba x M. messanensis where endosperm was absent and the embryo lost its polarity (Greenshields, 1954).

Hypothesis for Embryo Abortion in Interspecific Crosses of *Phaseolus*

Interspecific hybridization in *Phaseolus* usually resulted in premature pod abscission. The results obtained in the present study suggest that the developmental barriers reside primarily in post-fertilization events, as pollen germination and pollen tube growth were essentially identical in both intra- and interspecific crosses and fertilization was completed in all species combinations examined. The difference in time of fertilization between selfing and interspecific crossing is most likely a reflection of the differential development of the female reproductive apparatus, including the time of maturation of the female gametophyte and the receptive period of the stigma (Webster *et al.*, 1977, 1979). Differences in post-fertilization events were first observed in the delayed initiation of embryo and endosperm division. The extent of the delay was determined by the species combinations and the direction of the crosses. Embryo and endosperm division were slower in crosses of *P. vulgaris* with *P. lunatus* than with *P. acutifolius*. Secondly, interspecific hybrid embryos did not reach maturity. Embryos resulting from *P. vulgaris* - *P. acutifolius* crosses were capable of developing to the cotyledon stage, while those derived from *P. vulgaris* - *P. lunatus* crosses ceased to develop at the pre-heart or four-celled stage depending on the direction of the cross. Considering that the essential steps to maturity of the normal embryo include the growth by cell division and cell elongation and the differentiation of embryonic parts by differential cell division (Raghavan, 1976), it appears that certain steps were not completed in interspecific hybrid embryos. The development of *P. vulgaris* - *P. acutifolius* and *P. vulgaris* - *P. lunatus* embryos probably represents two different

aspects of embryo failure. Hence, when conducting an investigation of the possible bases of interspecific crossing barriers, it seems necessary to deal with these combinations separately.

In P. vulgaris x P. acutifolius crosses, the failure of hybrid embryos (at cotyledon stage) to reach maturity might be related to inadequate nutritional supply, as culturing immature embryos resulted in hybrid plantlets. It is generally believed that the embryo is dependent to a large extent on the endosperm for its nutrition and an abnormality of the endosperm leads to embryo abortion (Brink and Cooper, 1947). More recent observations suggest that the suspensor of Phaseolus may supply the required nutrients to the embryo and perform other functions related to the development of the embryo proper during early embryogeny. These functions may include the regulation of proper levels of plant hormones at critical stages of development (Alpi et al., 1975). It was demonstrated that the presence of the suspensor on P. coccineus embryos was required for in vitro development of embryos at early stage, and that gibberellin supplied to the culture medium could partially replace the suspensor (Cionini et al., 1976; Yeung and Sussex, 1979). These reports suggest the regulatory role of the suspensor in the development of the embryo possibly through the control of proper levels of plant hormones such as gibberellin. Furthermore, the involvement of appropriate levels of cytokinins in the development of hybrid embryos was suggested by the work of Nesling and Morris (1979). They found that cytokinin content of P. vulgaris x P. acutifolius embryos was lower than that of the parents and concluded that this may have contributed to the abortion of hybrid embryos. It is difficult to ascertain whether the lower level of cytokinins was a direct cause or

the result of slower embryo growth. Nevertheless, the relationship between appropriate hormonal levels and mobilization of nutrients, particularly carbohydrates, renders the involvement of growth regulators in regulating embryo development an attractive consideration. Our results thus far did not indicate the suspensor as having an essential regulatory role in embryo development, as suspensors examined cytologically were identical in both intra- and interspecific crosses involving P. vulgaris and P. acutifolius. There are however observations which suggest that the differences in hormonal function and metabolism between the parental genotypes may be related to the development of the embryos. For example, the growth rate of hybrid embryos (Tables 8 and 10) was higher when P. vulgaris genotype G 50 was used as the female parent in comparison with the genotype GN, and tissues of G 50 were characterized by their tendency to become cytokinin-autonomous in culture (Mok et al., 1980).

It may be speculated that lower levels of cytokinins in the hybrid embryos as a result of combining genomes of the two species may have affected the amount of nutrients available for embryo growth. The less than optimal levels of cytokinins was however not evident until later in the development. Genotypes of the female parent which are prone to become cytokinin-autonomous may be able to maintain a higher level of cytokinins as compared with genotypes which are cytokinin-dependent, thus accounting for the higher growth rate when G 50 was used as the female parent. These observations by themselves do not substantiate the involvement of hormonal regulation, particularly that of cytokinins in controlling embryo development, but examination of results on P. vulgaris - P. lunatus crosses (cf. following section)

and further experiments presently in progress (Appendix II) indeed suggest their functions are most likely important and worthy of consideration.

Embryos from P. vulgaris (female) x P. lunatus (male) crosses developed to the heart shaped stage, and in the reciprocal crosses embryos ceased to divide at the four-celled stage. The failure of endosperm development alone cannot explain embryo abortion since endosperm tissues divided in apparently normal fashion and reached the free nuclear stage in reciprocal crosses. The influence of the suspensor could not be determined, however, since the suspensor is derived from the embryo proper through differentiation, it can only be inferred that its normal development was also affected by the abnormal embryo growth. The recovery of hybrid plantlets by culturing hybrid embryos would indicate insufficient nutrient supply as one of the causes of embryo abortion. However, the inability of hybrid plants to reach flowering and the accompanying symptoms of plants which resemble early senescence suggest other intrinsic factors of the two species which preclude normal plant development even after failure of embryo development was overcome by embryo culture techniques. Again, there are indications that plant hormones, especially cytokinins may play an important role in conditioning the hybrid embryo development. As presented in Table 10, hybrid embryos on cytokinin-autonomous genotypes of P. vulgaris, such as G 50, usually were able to grow much faster than embryos on less cytokinin-autonomous genotypes such as GN. Recently we have compared the growth rate of hybrid embryos by using an extremely cytokinin-autonomous genotype (P.I. 286303) and a strictly cytokinin-dependent genotype (P.I. 200960). The results (Table 22)

Table 22. The average size of embryos obtained from the crosses between cytokinin-independent (P.I. 286303) and cytokinin-dependent (P.I. 200960) genotypes of P. vulgaris and P. lunatus genotype, cv. Kingston.

Days after Pollination	Crosses			
	286303 x K		200960 x K	
	Number of embryos	Size of embryo(mm)	Number of embryos	Size of embryo(mm)
6	14	0.15	16	0.11
9	16	0.21	27	0.14
12	19	0.20	22	0.16
15	42	0.28	33	0.21
18	39	0.31	34	0.22
21	33	0.48	55	0.25
24	38	0.53	34	0.24

appear to indicate a correlation between faster embryo growth with the ability of the tissues of the female parent to become cytokinin-autonomous. Another indication of the possible involvement of cytokinin biosynthesis (or metabolism) in conditioning interspecific hybridization was obtained by pollinating P. lunatus, grown in hydroponic cultures, with P. vulgaris pollen. When the cytokinin N^6-benzyladenine was added to the hydroponic solution, the hybrid embryos which normally abort at the four-celled stage (three days after pollination) were able to continue cell division (Appendix II). Embryos with more than 100 cells could be obtained and pod abscission was also substantially delayed.

It has been previously reported that P. lunatus and P. vulgaris tissues differ dramatically in their ability to grow on cytokinins with an unsaturated N^6-isoprenoid side chain (Mok et al., 1978). One of the possible explanations may be the high levels of cytokinin oxidase activity (Whitty and Hall, 1974) in P. vulgaris tissues. Such an enzyme was first identified in maize (Whitty and Hall, 1974). Cytokinins with unsaturated side chains such as zeatin and N^6-(Δ^2-isopentenyladenine) were sensitive to the enzyme whereas cytokinins with a saturated N^6-side chain (such as dihydrozeatin and N^6-isopentyladenine) are resistant to the enzymatic attack. In P. vulgaris, the major endogenous cytokinins were reported to be dihydrozeatin and its derivatives (Krasnuk et al., 1971; Wang et al., 1977) which are resistant to enzymatic attack (Whitty and Hall, 1974). The endogenous cytokinins of P. lunatus have not been identified, however it is likely that P. lunatus has a low level of cytokinin oxidase as well as produces cytokinins which are less sensitive to enzymatic degradation. The

combination of such qualitatively diverse genomes may have resulted in lower level of cytokinins necessary for normal embryo development.

It is premature to suggest genetic difference in hormonal function and/or metabolism being the basis in conditioning interspecific crossability. However, preliminary results obtained thus far indicate these substances may have significant influence on the development of hybrid embryos especially at early stages. Further examination of hybrid embryo development involving other Phaseolus species and biochemical characterization of cytokinin metabolism should provide additional information on the relationship between hormonal metabolism and species crossability in this genus.

Implications on Plant Breeding

One of the ultimate objectives of interspecific hybridization is to combine the desirable traits of both species. As the value and further utilization of interspecific hybrids depend largely on their fertility, it is necessary to evaluate the factors controlling the fertility of interspecific hybrids. In addition, the rational design of methods to utilize such hybrids will result from an understanding of the processes of gametogenesis and post-meiotic events influencing embryo and seed formation in subsequent generations.

In the present study, the only materials amenable to these investigations thus far are the hybrids between P. vulgaris and P. acutifolius. Thus the following discussion will center on hybrids obtained from these two species. At Metaphase I and Diakinesis both bivalents and univalents were observed. On the average there were six univalents per microsporocyte. The univalents observed at Metaphase I may have originated from failure of the chromosomes to pair or from precocious separation due to lack of chiasmata. An Anaphase I, a higher than expected frequency of normal chromosome distribution seemed to indicate that some of the univalents observed at Metaphase I may be the result of precocious separation of bivalents. Laggards at Anaphase I may represent true univalents. These lagging chromosomes did not have any apparent effect on the formation of pollen tetrads as the majority of microsporocytes (95%) did not contain micronuclei. However, the low pollen stainability (17%) and germinability (3%) seem to indicate that not all pollen contained a complete set of haploid chromosomes. As the chromosome number of microspores have not been

determined, it is not certain at this point if the low viability was caused by aneuploidy or by genetic factors. The determination of the actual fertility of the interspecific hybrids is more complicated. Pollen stainability was apparently an overestimate. The absence of dividing embryos in selfing also obscured the value of pollen germinability. Presently, we are in the process of determining the degree of male fertility by pollinating the parents with hybrid pollen. A definitive assessment of the female fertility was also difficult, since the completion of fertilization (in backcrosses and selfing) did not always lead to embryo division which was influenced by the male parent. Thus the measurement of the fertility of the interspecific hybrids would be somewhat arbitrary with regard to the utilization of interspecific hybrids. However, the number of viable embryos in relation to the total number of ovules seems to be the measurement of practical value. In predicting the potential of hybrids, the frequencies of dividing embryos may be a useful criterion, as they corresponded quite well with the frequencies of viable embryos in both selfing and backcrosses.

As mentioned above, the influence of chromosome pairing on fertility (relative to genetic factors) cannot be determined independently. At present, the possibility of improving fertility of the hybrid by choosing specific parental combinations is difficult. Nevertheless, by comparing the chromosome pairing between F_1 hybrids and backcrossed progeny (6 vs. 3 univalents) and corresponding fertility (0 vs. 2 seeds/5 pods in selfing), it seems possible to select genotypic combinations with limited improvement in chromosome pairing but with substantial increase in fertility.

The difference in the developmental potentials of backcross embryos obtained from crossing to G 50 and AC 2 constitutes an interesting phenomenon. As the contribution of the female gamete of the hybrid should be the same in both crosses, the much higher survival rate of embryos obtained from backcrossing to AC 2 suggests that the relative dosages of genetic material of the two species may be involved in the regulation of embryo development.

The backcrossed progeny were fertile. However the genotypes used in the original crosses were not selected on the basis of fertility or other useful characters such as disease resistance. The screening of a wider collection of genotypes may result in better combinations.

The hybridization between P. vulgaris and P. lunatus represents a substantially different challenge. Hybrid plantlets can be obtained with relative ease, at least when P. vulgaris is used as the female parent. However the potential utilization of such hybrids for plant improvement is dependent on the solution of more fundamental questions, namely the instrinsic differences between these two species in the genetic regulation of development. Based on indirect evidence, we suspect that the hormonal metabolism may be involved in the development of the hybrid embryo, and the immediate approach should be the manipulation of growth regulators in vitro to promote growth and inhibit premature senescence.

SUMMARY AND CONCLUSION

1. There was no apparent difference between intra-and inter-specific crosses in pre-fertilization events. Embryo and endosperm development processes of these two types of hybridization were distinct. Therefore, the limitations of interspecific hybridization in Phaseolus reside in post-fertilization events.
2. Interspecific hybrid embryos did not reach maturity. Embryos of P. vulgaris - P. acutifolius crosses attained the cotyledon stage. Embryos of P. vulgaris x P. lunatus developed only to the pre-heart shaped stage whereas embryos of the reciprocal cross ceased to divide at the four-celled stage. The recovery of P. vulgaris - P. acutifolius hybrids via embryo culture suggested that the failure of normal development may be related to the unavailability of nutrients possibly as the result of lower levels of cytokinins. The limited embryo development and early senescence of P. vulgaris x P. lunatus plants indicated a more severe "incompatibility" of the two genomes.
3. The development of hybrid embryos was dependent on the genotypic combinations and the direction of the cross. Genotypes of P. vulgaris prone to become cytokinin-independent in tissue culture gave faster hybrid embryo growth when used as the female parent in interspecific crosses. This observation, together with the observed effects of cytokinins in promoting the development of P. vulgaris x P. lunatus embryo suggested a relationship between cytokinin metabolism and embryo development.

4. Meiosis of interspecific hybrids of P. vulgaris and P. acutifolius showed pairing between approximately eight pairs of chromosomes. Precocious separation of bivalents may have given rise to some of the univalents observed. The fertility of the interspecific hybrids, as estimated by the frequencies of backcrossed embryos ranged from 4 to 13 percent. The increase in fertility of the backcrossed progeny suggests the possibility of obtaining genotypic combinations with higher fertility.

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APPENDICES

APPENDIX I

Preliminary Results On The Hybridization Between *P. vulgaris* And *P. coccineus*

While the cross *P. vulgaris* (female) x *P. coccineus* (male) produces mature hybrid seed, the reciprocal cross often failed or resulted only in seeds which were not germinable. The cause of such reciprocal cross difference is not known. Only in exceptional cases mature seed from the cross *P. coccineus* (female) x *P. vulgaris* (male) were reported with the use of specific genotypes (Thomas, 1964; Smartt, 1970) or the storing of abscised pods in plastic bags (Ibrahim and Coyne, 1975). However, there are no consistent accounts concerning the behavior of the subsequent generations, and it is doubtful that true hybrids were obtained from such crosses. It was also reported that subsequent generations of F_1 hybrids of *P. vulgaris* x *P. coccineus* reverted back to the *P. vulgaris* parent with respect to cotyledon position (Lamprecht, 1941; Wall and York, 1957) and other morphological characteristics (Smartt, 1970). The basis of such reversion to parental types was again unknown. Because of difficulties in obtaining the hybrid from the cross *P. coccineus* (female) x *P. vulgaris* (male), the development of hybrid embryos of this cross received little attention and many questions relating to the hybridization between these two species remain unanswered.

In an attempt to investigate the above mentioned phenomena, a systematic examination of the embryo development in F_1 and F_2 populations was initiated. Reciprocal hybrids were generated between *P. vulgaris* cv. Great Northern (GN) and *P. coccineus* cv. Scarlet Runner

Table I-1. Frequency (in percent) and class of seeds obtained from selfing of P. vulgaris (GN), P. coccineus (SR) and their reciprocal crosses.

cv/crosses	Unfertilized ovules	Seeds with underdeveloped embryos	Seeds with normal embryos	Days of maturity
GN	5 (4)*	0	95 (51)	35
SR	19 (16)	0	81 (68)	52
GN x SR	13 (10)	5 (4)	82 (64)	36
SR x GN	21 (40)	79 (154)	0	-

* Number of ovules

(SR). Mature hybrid seeds were obtained from the cross GN (female) x SR (male). The proportion of mature F_1 seeds (82%) was similar to selfing of parents (Table I-1). The germination of F_1 seeds was normal. Hybrid plants had pink flowers (whereas GN has white flowers and SR has scarlet flowers) and the cotyledon position was intermediate between the two species.

The reciprocal cross, P. coccineus (female) x P. vulgaris (male) produced seeds which ceased to develop at different stages before maturity. Pods were collected from 18 to 37 days after pollination in order to compare the development of the hybrid embryos. The size of seeds and embryos are presented in Table I-2. Seed size varied from 9.6 to 14 mm. Maximum seed size was attained in the period between 26 and 33 days. The embryos contained in these seeds were all at the cotyledon stage varying from 3.5 to 6 mm in length and with various degrees of abnormalities. Approximately 21% of the ovules examined

Table I-2. Size of F_1 seeds and embryos obtained from the cross between *P. coccineus* (SR) and *P. vulgaris* (GN).

Days after pollination	Number of ovules examined	Average size of seeds(mm)	Average size of embryos(mm)	Ratio embryo:seed
18-21	46	9.6	3.5	0.36
22-25	42	13.6	5.5	0.40
26-29	47	14.0	5.2	0.37
30-33	44	13.9	5.8	0.42
34-37	15	12.4	4.5	0.36

were not fertilized, 22% of the ovules were fertilized but with no developing embryos and the remaining seeds (57%) contained underdeveloped embryos or embryos with shrunken cotyledons. The unorganized tissues observed in seeds without embryos seem to indicate that the pro-embryo may have begun to develop but later degenerated. The underdeveloped embryos did not fill up the seed cavity although they were at early cotyledon stage. Some hybrid embryos reached the late cotyledon stage, but the coytledons were extremely shrunken and twisted around the axis (epicotyls and radicles).

Hybrid plants from the cross SR x GN were obtained from embryo culture. A comparison with hybrids obtained from the reciprocal cross revealed no difference in the general morphology of the plants. Also, to the contrary of the results obtained by Ibrahim and Coyne (1975) we observed no reciprocal cross difference in flower color of the F_1 hybrids. A distinct reciprocal cross difference in pollen stainability was detected. The hybrids of GN x SR had 27% stainable pollen whereas

Table I-3. Chromosome pairing at metaphase I of microsporogenesis in reciprocal hybrids of P. coccineus (SR) and P. vulgaris (GN).

Number of bivalents	Number of cells	
	SR x GN	GN x SR
11	16	25
10	6	29
9	1	16
8	1	0
7	0	1
Total number of cells	24	71
Average number of bivalents	10.5	10.1

81% of the pollen of SR x GN hybrids were stainable. The basis of such a difference probably did not reside in gamete formation as no reciprocal cross difference was observed in the meiosis of the hybrids. There was good pairing between chromosomes of the two species, with at least 10 bivalents observed at metaphase I (Table I-3). Two univalents were observed in many microsporocytes. They may have resulted from precocious separation of loosely paired univalents as later stages of microsporogenesis were normal.

All F_1 plants were artificially self-pollinated in order to obtain the F_2 generation. Seeds were examined and the classes and frequencies of F_2 embryos were examined (Table I-4).

It appears that although three classes of embryos were obtained from reciprocal F_1 plants, the frequencies of such classes differed

Table I-4. Number and frequency (in percent) of F_2 seeds with three classes of embryos obtained from reciprocal F_1s between P. vulgaris (GN) and P. coccineus (SR)*.

Crosses	Class of embryo	Number and (%) of seeds
GN x SR (F_2)	normal	96 (38)
	shrunken	80 (31)
	underdeveloped	80 (31)
SR x GN (F_2)	normal	34 (52)
	shrunken	24 (37)
	underdeveloped	7 (11)

* Pods were collected from 18-45 days after pollination.

appreciably. These preliminary results suggested that the previously reported phenomena of failure to obtain P. coccineus x P. vulgaris hybrids and the reversion of hybrids to parental types may be related to the differential development of hybrid embryos. It would be reasonable to assume that the occurrence of abnormal embryos would have exerted selection pressure in favor of certain genetic combinations. Although we have obtained preliminary indications in support of this speculation, extensive studies are required to substantiate this hypothesis.

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APPENDIX II

The Effects Of N^6-benzyladenine On The Development Of *P. lunatus* x *P. vulgaris* Embryos

The hybrids of *P. lunatus* (female) x *P. vulgaris* (male) were not obtained because of premature pod abscission. Hybrid pods from this cross abscised before or at three days following pollination and the embryos ceased to divide at the four-celled stage (Rabakoarihanta *et al.*, 1979). The reciprocal cross *P. vulgaris* (female) x *P. lunatus* (male) yielded embryos at the pre-heart shaped stage and the rate of growth of the hybrid embryo was influenced by the maternal genotype (Mok *et al.*, 1978). Larger embryos were obtained on cytokinin-independent genotypes. Hybrid plantlets were obtained from the cross *P. vulgaris* x *P. lunatus*, however full development was not attained due to premature senescence. It appears that nutritional factors as well as intrinsic differences in cytokinin metabolism between the two species may be responsible for the failure of development of hybrid embryos between these two species. The reports of Nesling and Morris (1979) demonstrating lower levels of cytokinin in interspecific hybrid embryos also lend some support to this speculation. These observations led us to the testing of the effect of exogenously supplied cytokinins on hybrid embryo development.

The growth regulator N^6-benzyladenine (bzl^6Ade) was supplied by two means: through hydroponic culture and application to the peduncles. The cross was *P. lunatus* cv. Kingston (female) x *P. vulgaris* cv. Great Northern (male). Plants of cv. Kingston were grown in hydroponic culture solution containing 1/4 strength of inorganic

nutrients as described by Murashige and Skoog (1962). The concentrations of N^6-benzyladenine added were 3 and 10 μM.

With the application of the cytokinin on the peduncle (Table II-1), the period of pod retention was extended. A substantial proportion of developing pods were retained up to 12 days and more pods were retained at the concentration of 10 μM than 3 μM. The final size attained by the embryo was also larger at 10 μM (22 cells vs. 13 cells).

The effect of bzl^{6}Ade was more pronounced when it was supplied through the roots (Table II-2). Although a large proportion of the pods dropped early (3 days after pollination), the few pods which were retained beyond 12 days contained embryos at the globular stage measuring approximately 0.25 mm (Figure 9). Increasing the concentration of bzl^{6}Ade did not seem to affect pod retention as no significant difference was observed in the proportion of pods retained at 12 days at different concentrations. However, the increase in concentration was accompanied by an increase in the proportion of dividing embryos and in the average number of cells in the embryos.

Thus, the role of N^6-benzyladenine was evident in two instances; delay of abscission and promotion of cell division. It appeared that pod retention was enhanced when the growth regulator was supplied through the peduncle whereas cell division was stimulated when it was supplied through the roots. The ability of bzl^{6}Ade in enhancing pod retention may be related to the mobilization of more carbohydrates to the embryo. Abscission of young fruit may occur because of a competition for limited metabolites, especially carbohydrates (Addicott and Lynch, 1955). It was shown that a detached leaf treated with kinetin attracted metabolites from untreated parts of the blade (Mothes, 1961).

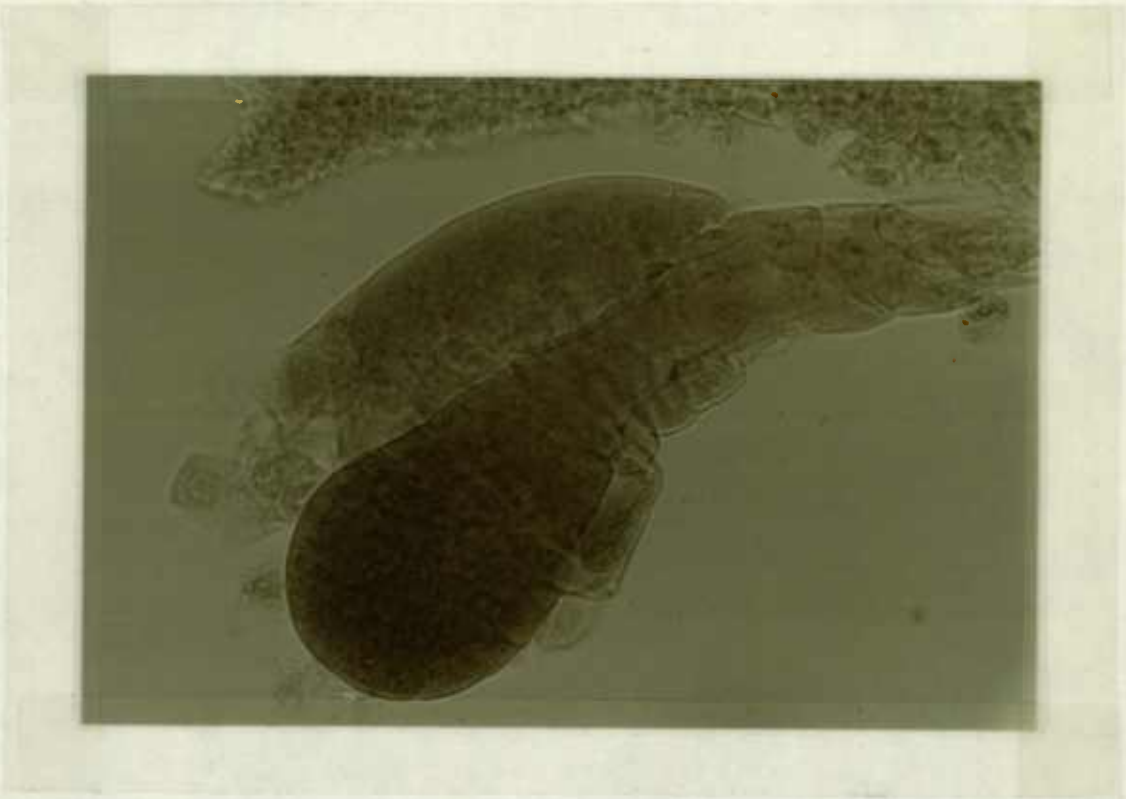

Figure 9. Embryo obtained from P. lunatus (K) x P. vulgaris (GN) with K grown in hydroponic culture supplied with 10 μM of bzl^{6}Ade (14 days after pollination).

Table II-1. Effect of pedicellar application of bzl^6Ade on pod retention and embryo development of *P. lunatus* (cv. K) x *P. vulgaris* (cv. GN).

Days after pollination	Concentrations of bzl^6Ade							
	3 μM				10 μM			
	(1)		(2)	(3)	(4)	(1)	(2)	(3)	(4)
3	44	59.4	1 0.6	6	170	65 50.4	3 1.2	9	252
6	18	24.3	6 10.0	8	60	33 25.6	9 8.6	10	104
9	8	10.8	4 14.8	8	27	21 16.3	5 7.2	8	69
12	4	5.4	3 25.0	13	12	10 7.7	8 25.8	22	31
Total	74		14 5.2		269	129	25 5.5		456

(1) Number and percent of pods retained

(2) Number and percent of ovules with dividing embryos

(3) Average number of cells in the embryos

(4) Number of ovules examined

Table II-2. Effect of bzl^{6}Ade supplied via hydroponic culture on pod retention and embryo development of P. lunatus (cv. K) x P. vulgaris (cv. GN).

Days after pollination	Concentrations of bzl^{6}Ade							
	3 μM				10 μM			
	(1)		(2)	(3)	(4)	(1)	(2)	(3)	(4)
3	37	75.5	6 4.2	4	141	68 80.0	6 2.3	7	261
6	7	14.3	4 18.2	15	22	6 7.1	4 22.2	15	18
9	3	6.1	2 20	17	10	7 8.2	8 36.4	25	22
12	2	4.1	1 14.3	16	7	4 4.7	6 66.7	100	9
Total	49		13 7.2		180	85	24 7.7		310

(1) Number and percent of pods retained

(2) Number and percent of ovules with dividing embryos

(3) Average number of cells in the embryos

(4) Number of ovules examined

Similarly, the application of cytokinin to the peduncle may have caused translocation of metabolites from untreated regions of the plant to the fruit, thereby delaying the abscission of the fruit. The increased rate of cell division is probably a direct effect of the cytokinin. This observation further demonstrates the important role of cytokinins on early development of the hybrid embryo.

Although these results seem to substantiate a relation between cytokinin levels and hybrid embryo development, further experiments are needed to obtain more direct evidence in support of the proposed hypothesis.

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APPENDIX III

Embryo Development In Relation To Ovule Position

Due to the elongated morphology of the ovary of legumes, ovules in the same ovary are not expected to develop equally after pollination and fertilization. It was noted by Harris (1915) that in Phaseolus vulgaris the proportion of ovules which developed into seeds was higher at the stigmatic end than at the base of the pod, indicating that the development of ovules was influenced by their position in the pod. Embryos became smaller toward the proximal end (Williams, 1962). Similar observations were made on the field bean Vicia faba where more seed abortion occurred near the proximal end than near the distal (stigmatic) end of the pod (Kambal, 1969).

In examining the development of intra- and interspecific hybrid embryos of Phaseolus, we recorded the developmental stages according to the position of the ovule in the ovary. Individual ovule positions were identified by numbering system starting from the stigmatic end. The proportions of fertilized ovules plus ovules with dividing embryos over all sampling times (3-72 hours after pollination) are presented in Table III-1.

Fertilization and embryo division began in the first three ovules at the stigmatic end, then, the frequency of developing ovules gradually decreased toward the pedicellar end. It appears that fertilization of the ovules occurs in a sequential manner.

The position of the ovules in relation to the time of fertilization was further examined by pollinating P. vulgaris cv. GN and P. lunatus cv. Kingston. The plants were emasculated one day before

Table III-1. The proportion (%) of ovules with dividing embryos at all sampling times (3 to 72 hours after pollination) in relation to the ovule position in the ovary starting from the stigmatic end.

crosses	ovule position					
	1	2	3	4	5	6
GN x GN	10.1(111)*	9.0(111)	10.7(111)	8.7(109)	5.7(101)	1.2(52)
G50xG50	11.8(105)	12.8(108)	12.5(105)	12.3(100)	10.0(100)	3.7(51)
GN x K	9.4(108)	9.9(93)	9.9(93)	7.6(92)	4.3(81)	1.7(48)
G50x K	11.6(120)	12.3(120)	13.6(120)	11.3(119)	10.8(107)	4.2(57)

*Total number of ovules examined.

Table III-2. The distribution of hybrid and selfed embryos in pods of P. vulgaris (GN) following sequential pollination with P. lunatus (K) and P. vulgaris (GN) pollen.

ovule position						number of
1	2	3	4	5	6	pods
+	+	+	-	0	0	16
+	+	-	-	0	0	24
+	+	-	-	-	0	8
+	-	-	-	-	0	16
-	+	-	-	-	0	8
-	-	-	+	-	0	2
% +	86	76	22	3		Total:
% -	14	24	78	97		74

+ : hybrid

- : P. vulgaris selfed

0 : ovules not fertilized

flowering and pollinated with K pollen. On the following day the same flowers were self-pollinated again with pollen of cv. GN. All pods were collected at 12-15 days after pollination. As the hybrid embryos of P. vulgaris and P. lunatus have a distinct morphology (Mok et al., 1978) it was possible to distinguish hybrid embryos from selfed embryos. The proportions of hybrid and selfed embryos according to the ovule position in the pod are presented in Table III-2. More hybrid seeds were found at the stigmatic end than at the pedicellar end. Conversely, more selfed seeds developed near the pedicellar end.

Again the sequence of fertilization appeared to begin at the stigmatic end. The predominant occurrence of hybrid embryos at positions 1, 2 and 3 as the result of prior pollination substantiate this suggestion. Such observations would explain the report of mature selfed seeds of P. vulgaris concentrating at the stigmatic end (Harris, 1915). A possible basis for such difference between the stigmatic end and the pedicellar end may be the differential time of maturity of ovules. The fact that more non-fertilized ovules are found near the pedicellar end, which is closer to the source of nutrients, suggests that the competitive advantage of embryos may depend primarily on the early accomplishment of fertilization and the initiation of embryo division. The supply of nutrients may still influence embryo development at later stage, but the initial advantage resides with ovules fertilized at an earlier time.

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