Theor. Appl. Genet. 62, 59–64 (1982)

Embryo Development in Reciprocal Crosses of *Phaseolus vulgaris* L. and *P. coccineus* Lam.

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Summary. Embryo development was examined in reciprocal crosses of *Phaseolus vulgaris* cv. 'Great Northern' and *P. coccineus* cv. 'Scarlet Runner'. The formation of abnormal (shrunken and underdeveloped) embryos constituted the primary crossing barrier between the two species when *P. coccineus* was the female parent. Plants of *P. coccineus* × *P. vulgaris* were obtained by embryo culture. Although the *P. vulgaris* × *P. coccineus* cross resulted in normal seed development, the fertility of the resulting hybrids was much lower (27%) than that of the reciprocal hybrids (81%). Three classes of F_2 embryos, normal, shrunken, and underdeveloped were formed on reciprocal F_1s and the frequencies did not differ between reciprocal populations. Thus, the interactions between embryo and endosperm and/or maternal parent rather than cytoplasmic-nuclear effects seem to be important in the determination of the extent of embryo growth. The examination of pollen fertility of F_2 plants and the development of F_2 and F_3 embryos suggests that the formation of abnormal embryos and reduced male fertility are independent events. The *P. vulgaris* – *P. coccineus* crosses may be useful in studying the possible involvement of interspecific differences in hormonal metabolism in the development of hybrid embryos.

Key words: Interspecific hybridization – Embryogeny – Male fertility – *Phaseolus* – Endospermembryointeraction

Introduction

The development of hybrid embryos upon interspecific hybridization of *Phaseolus* has been examined in *P. vulgaris* – *P. acutifolius* and *P. vulgaris* – *P. lunatus* combinations (Mok et al. 1978a; Rabakoarihanta et al. 1979). The primary objectives are to design suitable methods for gene transfer between *Phaseolus* species as well as to assess the possible influence of observed interspecific variations in cytokinin metabolism (Mok et al. 1978b, 1979, 1980a, 1980b) on the development of interspecific hybrid embryos. These studies of embryo development have now been extended to reciprocal crosses between *P. vulgaris* and *P. coccineus*.

Numerous attempts have been made to hybridize *P. vulgaris* and *P. coccineus*. Consistent success has been limited to crosses with *P. vulgaris* as the female parent from which mature seeds are recovered routinely (Lamprecht 1941; Smartt 1970; Thomas 1964). The reciprocal cross is generally unsuccessful although rare successes have been reported (Al-Yasiri and Coyne 1966; Lamprecht 1941; Smartt 1970; Smartt and Haq 1972; Thomas 1964). Thomas (1964) attributed this low seed set partly to a failure to complete fertilization and partly to the slow development of interspecific hybrid embryos and endosperm. However, Hawkins and Evans (1973) have reported that fertilization was normal in these crosses. Interspecific hybrids of *P. vulgaris* × *P. coccineus* had lower pollen fertility than the occasional reciprocal hybrids (Ibrahim and Coyne 1975; Smartt 1970). The reduced fertility was attributed to genic male sterility, insufficient chromosome homologies and cytoplasmic-nuclear interactions (Haq et al. 1980; Smartt 1970; Smartt and Haq 1972). Genetic studies in subsequent generations have centered mainly on populations derived from *P. vulgaris* × *P. coccineus* crosses. Segregation patterns of quantitative and qualitative traits indicated a selective recovery of *P. vulgaris* characteristics (Lamprecht 1941; Smartt 1970; Wall and York 1957).

The present paper deals with embryo development and male fertility in F_1 and F_2 populations of reciprocal crosses between *P. vulgaris* cv. 'Great Northern' and *P. coccineus* cv. 'Scarlet Runner'. The occurrence of two distinct classes of abnormal embryos, the shrunken and underdeveloped, is described. The application of embryo culture techniques has facilitated the recovery of plants from abnormal embryos and enabled the studies of their progeny.

Materials and Methods

Plant materials included the parental genotypes *P. vulgaris* L. cv. 'Great Northern Nebraska No. 1' (GN) and *P. coccineus*

0040-5752/82/0062/0059/$ 1.20

Lam. cv. 'Scarlet Runner' (SR) and their reciprocal F_1 and F_2 progenies. The plants were grown in the greenhouse at approximately 22 °C/18 °C (day/night).

To facilitate crossing, flowers were emasculated one day before opening and pollinated on the same day. In self-pollination, the same procedure was followed but pollen from other flowers of the same plant were used. The methods to examine pollen tube growth, fertilization and endosperm division were the same as reported earlier (Rabakoarihanta et al. 1979). The time periods of fertilization and endosperm division were defined as the time interval at which 50% of the ovules examined has completed the process.

The development of embryos resulting from SR × GN crosses was examined at three-day intervals. Since these embryos failed to reach maturity, embryo culture techniques were employed to obtain mature plants. The procedures and the composition of the medium were as previously described (Mok et al. 1978a). The same techniques were used to recover plants from abnormal embryos in later generations.

Chromosome pairing at Metaphase I of microsporogenesis was examined in reciprocal F_1s. The preparation of anthers has been reported earlier (Rabakoarihanta et al. 1980). Acetocarmine squash technique was used to examine chromosome pairing. Pollen fertility was estimated by the stainability with acetocarmine. At least 10 flowers of each plant were examined and 200 grains were scored per flower.

Results

Pollen tube growth and fertilization were examined in selfings of GN and SR and in reciprocal crosses between the two genotypes (Table 1). The pre-fertilization events did not appear to differ between interspecific crosses and selfing. Pollen tubes reached the base of the style within nine hours after pollination in selfing and crosses. Fertilization was completed within 24 h after pollination and the first endosperm division was observed within 48 h after pollination. The slightly later time of fertilization in SR × GN was followed by a later initiation of the endosperm division.

Embryo development was normal in selfings of GN and SR and in GN × SR crosses (Table 2), and mature seeds were obtained. However, morphologically abnormal embryos were formed upon crossing SR (female) with GN (male). The abnormalities became apparent 15 days after pollination when embryos reached the early cotyledon stage. Two classes of abnormal embryos could clearly be distinguished, the shrunken (Fig. 1a) and the underdeveloped (Fig. 1b). The shrunken embryos were characterized by wrinkled cotyledons at an early stage of growth (18 days) and although the seeds and the embryos continued to enlarge (Table 3), the cotyledons became progressively malformed. The embryonic axes were visibly compressed. Seeds containing shrunken embryos reached the length of approximately 23 mm at 36 days after pollination. (At this time the normal seeds of the reciprocal cross had reached maturity and were on the average 13 mm in length.) The seeds containing underde-

Table. 1. Pollen tubes per style and the intervals (in hours) between pollination and fertilization and between pollination and endosperm division in selfing and interspecific crosses of *P. vulgaris* (GN) and *P. coccineus* (SR)

Cross	No. pollen tubes per style	Time of fertilization[a]	Time of endosperm division[a]	No. of ovules examined
GN × GN	12	18 – 21	24 – 36	128
SR × SR	9	18 – 21	24 – 36	97
GN × SR	8	18 – 21	24 – 36	114
SR × GN	13	21 – 24	36 – 48	109

[a] More than 50% of the samples examined at that particular time had completed the process identified.

Table 2. Frequencies (in percent) of the classes of embryos obtained from selfing of *P. vulgaris* (GN) and *P. coccineus* (SR) and from reciprocal crosses

Cross	Seeds with normal embryos		Days to maturity	Seeds with abnormal embryos		Unfertilized ovules	
GN × GN	95	(51)[a]	35	0		5	(4)[a]
SR × SR	81	(68)	52	0		19	(16)
GN × SR	82	(64)	36	5	(4)[a]	13	(10)
SR × GN	0		–	79	(154)	21	(40)

[a] Number of samples

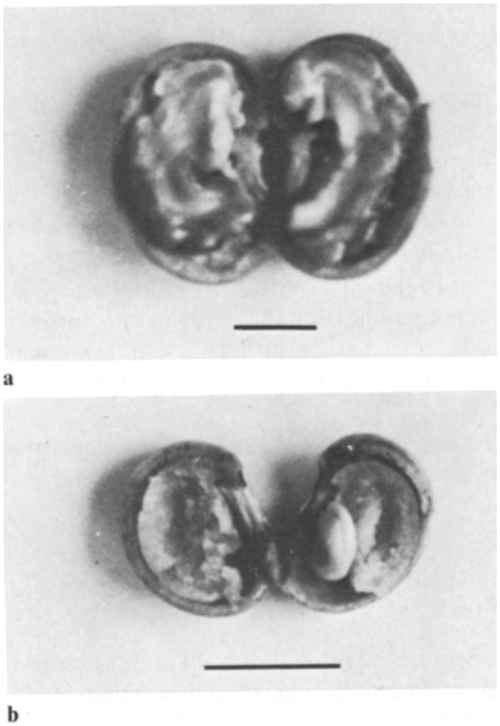

Fig. 1a and b. The two types of abnormal embryos, shrunken (**a**) and underdeveloped (**b**), obtained from *P. coccineus* (SR) × *P. vulgaris* (GN) 35 days after pollination. (Bar represents 10 mm.)

Table 3. Size (mm) of F_1 seeds and embryos in the two abnormal classes (shrunken and underdeveloped) obtained from the cross *P. coccineus* (SR) × *P. vulgaris* (GN)

Days after pollination	Class of embryos							
	Shrunken				Underdeveloped			
	No. of seeds examined	Seed size	Embryo size	Embryo/ seed ratio	No. of seeds examined	Seed size	Embryo size	Embryo/ seed ratio
18	36	12±1	4.3±0.5	0.36	46	10±1	3.5±0.5	0.36
22	44	11±2	6.1±0.2	0.55	42	14±1	5.5±0.4	0.40
26	57	16±1	13.5±0.3	0.84	47	14±2	5.2±0.3	0.37
30	49	17±1	14.5±0.2	0.85	44	14±1	5.8±0.3	0.42
34	22	23±1	22.0±0.3	0.95	15	13±1	5.5±0.3	0.42

Table 4. Chromosome pairing at metaphase I of microsporogenesis in reciprocal hybrids of *P. vulgaris* (GN) and *P. coccineus* (SR)

Number of bivalents	Number of cells	
	GN × SR F_1	SR × GN F_1
11	46	84
10	53	32
9	29	5
8	1	6
7	1	0
Total number of cells	130	127
Average number of bivalents	10.1	10.5

Table 5. Frequency (in percent) of stainable pollen of *P. vulgaris* (GN), *P. coccineus* (SR) and their reciprocal F_1 hybrids

Plants	Genotypes			
	GN	SR	GN × SR F_1	SR × GN F_1
1	93	99	26	83[a]
2	94	96	31	82[a]
3	93	95	27	84[b]
4	94	96	24	79[a]
5	94	96	28	79[b]
Average	94	96	27	81

[a] Derived from shrunken embryo; [b] Derived from underdeveloped embryo

veloped embryos increased in size until 29 days after pollination; thereafter little change in size occurred. The embryos within these seeds were much smaller than the seeds and were at the cotyledon stage (Fig. 1b). The final length of the embryos was approximately 5.5 mm (Table 3). The underdeveloped embryos were surrounded by a thick layer of endosperm tissue, which was not observed in normal seeds.

Table 6. Frequencies (in percent) and numbers of F_2 seeds with normal, shrunken and underdeveloped embryos obtained from reciprocal F_1's of *P. vulgaris* (GN) and *P. coccineus* (SR)

Genotype of F_1's	Classes of F_2 embryos embryos	% and (number) of seeds	
GN × SR	normal	30	(102)
	shrunken	46	(160)
	underdeveloped	24	(81)
Total			(343)
SR × GN	normal	26	(64)
	shrunken	44	(105)
	underdeveloped	30	(71)
Total			(240)

Immature embryos (26 to 29 days after pollination) resulting from SR×GN crosses were cultured on nutrient medium (Mok et al. 1978a) and hybrid plants were obtained. The frequency of success was over 80% (with 75 embryos cultured in one experiment). Twenty hybrid plantlets derived from shrunken and underdeveloped embryos were transferred to soil in the greenhouse. Of the ten plantlets obtained from underdeveloped embryos, only two survived after three weeks. (The other eight plantlets had narrow leaves and thin stems and perished within 21 days after transplanting.) All ten plantlets originating from shrunken embryos developed normally and reached the flowering stage.

Chromosome pairing at Metaphase I of microsporogenesis was examined in the reciprocal hybrids. The majority of PMCs had 10 to 11 bivalents and the average number of bivalents was 10.1 and 10.5 respec-

Table 7. Numbers of F_3 seeds with normal, shrunken and underdeveloped embryos obtained from reciprocal F_2's of *P. vulgaris* (GN) and *P. coccineus* (SR)

Genotype of F_1	F_2 Plant number	Phenotype of F_2 seed	Pollen stainability (%) of F_2	Numberber of F_3 seeds obtained		
				Normal	Shrunken	Underdeveloped
GN × SR	VC – 1	normal	33	9	9	7
	VC – 2	normal	52	5	3	0
	VC – 3	normal	67	6	0	6
	VC – 4	normal	83	0	2	1
	VC – 5	normal	31	3	12	5
	VC – 6	normal	42	4	5	0
	VC – 7	shrunken	33	7	7	0
	VC – 8	shrunken	26	7	14	0
	VC – 9	shrunken	96	5	0	0
	VC – 10	shrunken	43	0	7	1
	VC – 11	underdeveloped	71	5	9	1
SR × GN	CV – 1	normal	79	5	6	0
	CV – 2	normal	82	12	19	0
	CV – 3	normal	90	2	1	1
	CV – 4	normal	33	15	0	0
	CV – 5	normal	42	2	1	0
	CV – 6	shrunken	80	3	2	1
	CV – 7	shrunken	89	8	20	2
	CV – 8	underdeveloped	73	5	3	3

tively for GN×SR and SR×GN F_1s (Table 4). The pollen fertility as estimated by the stainability is presented in Table 5. Hybrids of GN×SR had significantly lower pollen fertility (27%) than those of SR×GN which produced a high proportion (81%) of stainable pollen.

F_2 embryo development was examined after artificially self-pollinating F_1 plants. Ten plants of GN×SR and 12 plants of SR×GN obtained by embryo culture (ten from shrunken and two from underdeveloped embryos) were used. The frequencies and classes of F_2 embryos were determined 35 days after pollination. Three types of embryos, normal, shrunken and underdeveloped were recovered (Table 6). The degree of abnormality of the shrunken embryos varied from those with extremely wrinkled cotyledons to those with only rough edges. However, little variation occurred within the normal and underdeveloped classes of embryos. There was no detectable difference in the frequencies of the different types of F_2 embryos obtained from SR×GN and GN×SR F_1s (X^2 values were not significant at the 5% level).

Nineteen F_2 plants obtained from the different classes of seeds were grown in the greenhouse. The pollen stainability of individual F_2 plants and frequencies of F_3 embryos were determined (Table 7). The pollen stainability varied widely between individual F_2 plants and appeared to be independent of the phenotype of the F_2 seed. Some of the F_2 plants gave rise to only normal (VC-9 and CV-4) or only abnormal (VC-10) seeds. It is of interest to note that the plants VC-9 and CV-4 had all normal progeny but differed in seed type origin (shrunken and normal respectively) and pollen stainability (96% and 33%). Although the number of F_3 embryos obtained per F_2 plant were too small to be compared statistically, all three classes of embryos occurred on reciprocal F_2 populations.

Discussion

The results obtained from the present study extend previous observations on interspecific hybridizations between *P. vulgaris* and *P. coccineus*, particularly when the latter species is used as the seed parent. The apparently normal pollen tube growth in initial reciprocal crosses confirm the report of Hawkins and Evans (1973). In addition, fertilization was found to be completed. The observation of Thomas (1964) that *P. coccineus*×*P. vulgaris* F_1 embryos developed at a slower rate (measured as nuclei number) than *P. vulgaris*×*P. coccineus* F_1 embryos at early stages is in agreement with the present finding of developmentally abnormal embryos which become apparent at later stages (18 days after pollination). Therefore, the primary crossing barrier of the initial hybridization when *P. coccineus* is used as the seed parent appears to be

the irregular development of F_1 embryos. This crossing barrier can be overcome with the aid of embryo culture and F_1 plants of *P. coccineus* × *P. vulgaris* can be recovered with relative ease. However, the eventual viability of the plantlets seems to be related to the extent of the embryo development, i.e. plantlets derived from underdeveloped embryos tend to have a much lower survival rate.

The examination of embryo development beyond the F_1 generation provided information which was previously not available. The large reciprocal cross difference in embryo development at the F_1 generation was not observed in the F_2 and F_3 generations; abnormal and normal embryos were found at the same ratios in reciprocal F_2 populations. Thus it appears that the interactions between embryo and endosperm and/or maternal parent may determine the extent of embryo development. Both types of interaction would account for the absence of reciprocal cross differences beyond the F_1 and the recurrent occurrence of different classes of embryos in later generations. The latter explanation (embryo-maternal parent interaction) is more likely in the case of shrunken embryos since the abnormalities occur at a late stage of development, after the degeneration of endosperm.

Haq et al. (1980) and Smartt and Haq (1972) have suggested that the mechanisms underlying pollen sterility are likely to be complex. Our present findings support this contention. However, it appears that structural differences between chromosomes of the two species, if they exist, are of minor importance in the reduction of pollen fertility. Insufficient chromosome homology would have resulted in reduced fertility of the *P. vulgaris* × *P. coccineus* hybrids as well as the reciprocal hybrids, while the actual frequencies of bivalent formation and pollen stainability of the *P. coccineus* × *P. vulgaris* hybrids were high. Thus the abnormalities in microsporogenesis of the *P. vulgaris* × *P. coccineus* hybrids may have been caused mainly by factors other than a lack of chromosome homology.

Pollen sterility and restricted embryo development appear to be under separate genetic controls since there is no correlation between the extent of embryo development and the degree of pollen fertility in later generations. The selective recovery of certain embryos and different pollen fertility may both have contributed to the skewed distribution of quantitative characters in subsequent generations following *P. vulgaris* – *P. coccineus* crosses (Smartt 1970; Wall and York 1957).

Previous studies using *P. vulgaris* as the common species in cross with *P. acutifolius* and *P. lunatus* have discovered hybrid embryos with distinct developmental potentials (Mok et al. 1978a; Rabakoarihanta et al. 1979). *P. vulgaris* × *P. acutifolius* (and reciprocal crosses) resulted in embryos which developed up to the late cotyledon stage. *P. vulgaris* × *P. lunatus* crosses gave rise to pre-heart embryos whereas *P. lunatus* × *P. vulgaris* embryos ceased to divide at the four-cell stage. The inclusion of *P. coccineus* thus extended the scope to interspecific hybrid embryos with developmental potentials ranging from four-cell stage to mature seeds.

The significant influence of plant hormones on embryo development has been demonstrated in embryo culture studies as well as somatic embryogeny (for review, see Raghavan 1976). In *Phaseolus,* there are indications that the genetic variations in hormonal metabolism may be important in interspecific embryo development. The limited development of *P. vulgaris* – *P. lunatus* embryos can be extended by the addition of N^6-benzyladenine through hydroponic culture solutions to the seed parent (Rabakoarihanta 1980). Furthermore, the amount of extractable cytokinins in *P. vulgaris* – *P. acutifolius* embryos was found to be lower than in selfed embryos of either species (Nesling and Morris 1979). *P. vulgaris* – *P. coccineus* embryos may be particularly useful in attempting to explore the relationship between interspecific variations in hormonal metabolism and developmental controls of embryo growth, since distinct classes of embryos occur and the hybrids are sufficiently fertile to allow genetic manipulation. In addition, previous studies on embryogeny in *P. coccineus* have established the important relationship between gibberellin and cytokinin levels and the suspensor which is crucial to normal embryo development (Alpi et al. 1975; Bennici and Cionini 1979; Cionini et al. 1976; Yeung and Sussex 1979).

Acknowledgement

Research was supported by a grant (PCM-80-10768) from the National Science Foundation, by the Science and Education Administration of the US Department of Agriculture under grant 78-59-2411-0-1-028-1 from the Competitive Research Grants Office, and by the Research Council of Oregon State University (NIH Biomedical Research Support Grant RR07079). Dr. C. T. Shii was supported by a fellowship from the National Science Council of the Republic of China. Dr. A. Rabakoarihanta was supported by a fellowship from the African-American Institute. This is technical paper No. 6152 of the Oregon Agricultural Experiment Station.

Literature

Alpi, A.; Tognoni, F.; D'Amato, F. (1975): Growth regulator levels in embryo and suspensor of *Phaseolus coccineus* at two stages of development. Planta **127,** 153–162

Al-Yasiri, S.A.; Coyne, D.P. (1966): Interspecific hybridization in the genus *Phaseolus.* Crop Sci. **6,** 59–60

Bennici, A.; Cionini, P.G. (1979): Cytokinins and in vitro development of *Phaseolus coccineus* embryos. Planta **147,** 27–29

Cionini, P.G.; Bennici, A.; Alpi, A.; D'Amato, F. (1976): Suspensor, gibberellin and in vitro development of *Phaseolus coccineus* embryos. Planta **131,** 115–117

Hawkins, C.F.; Evans, A.M. (1973): Elucidating the behaviour of pollen tubes in intra- and interspecific pollinations of *Phaseolus vulgaris* L. and *Phaseolus coccineus* Lam. Euphytica **22,** 378–385

Haq, M.N.; Lane, G.R.; Smartt, J. (1980): The cytogenetics of *Phaseolus vulgaris* L., *Phaseolus coccineus* L., their interspecific hybrids, derived amphidiploid and backcross progeny in relation to their potential exploitation in breeding. Cytologia **45,** 791–798

Ibrahim, A.M.; Coyne, D.P. (1975): Genetics of stigma shape, cotyledon position, and flower color in reciprocal crosses between *Phaseolus vulgaris* L. and *Phaseolus coccineus* Lam. and implication in breeding. J. Am. Soc. Hortic. Sci. **100,** 622–626

Lamprecht, H. (1941): Die Artgrenze zwischen *Phaseolus vulgaris* L. und *P. multiflorus* Lam. Hereditas **27,** 51–175

Mok, D.W.S.; Mok, M.C.; Rabakoarihanta, A. (1978a): Interspecific hybridization of *Phaseolus vulgaris* with *P. lunatus* and *P. acutifolius.* Theor. Appl. Genet. **52,** 209–215

Mok, M.C.; Armstrong, D.J.; Mok, D.W.S. (1980a): Interspecific differences in the metabolism of (8-^{14}C)-N^6-(Δ^2-isopentenyl)adenosine in the genus *Phaseolus.* Plant Physiol. **65,** 24

Mok, M.C.; Kim, S.G.; Armstrong, D.J.; Mok, D.W.S. (1979): Induction of cytokinin autonomy by N,N′-diphenylurea in tissue cultures of *Phaseolus lunatus* L. Proc. Nat. Acad. Sci. (USA) **76,** 3880–3884

Mok, M.C.; Mok, D.W.S.; Armstrong, D.J. (1978b): Differential cytokinin structure-activity relationships in *Phaseolus.* Plant Physiol. **61,** 72–75

Mok, M.C.; Mok, D.W.S.; Armstrong, D.J.; Rabakoarihanta, A.; Kim, S.G. (1980b): Cytokinin autonomy in tissue cultures of *Phaseolus:* A genotype-specific and heritable trait. Genetics **94,** 675–686

Nesling, F.A.V.; Morris, D.A. (1979): Cytokinin levels and embryo abortion in interspecific *Phaseolus* crosses. Z. Pflanzenphysiol. **91,** 345–358

Rabakoarihanta, A.; Mok, D.W.S.; Mok, M.C. (1979): Fertilization and early embryo development in reciprocal interspecific crosses of *Phaseolus.* Theor. Appl. Genet. **54,** 55–59

Rabakoarihanta, A.; Shii, C.T.; Mok, M.C.; Mok, D.W.S. (1980): Meiosis and fertility of interspecific hybrids between *Phaseolus vulgaris* L. and *P. acutifolius* A. Gray. Theor. Appl. Genet. **57,** 57–64

Rabakoarihanta, A. (1980): Embryo development in relation to interspecific hybridization of *Phaseolus.* 92 pp. Doctoral Diss. Oregon State University

Raghavan, V. (1976): Experimental Embryogenesis in Vascular Plants. London, New York, San Francisco: Acad. Press

Smartt, J. (1970): Interspecific hybridization between cultivated American species of the genus *Phaseolus.* Euphytica **19,** 480–489

Smartt, J.; Haq, N. (1972): The behavior of *Phaseolus coccineus* Lam. as seed parent in interspecific crosses with *Phaseolus vulgaris* L. Ann. Rep. Bean Improvement Coop. **15,** 88–90

Thomas, H. (1964): Investigations into the inter-relationships of *Phaseolus vulgaris* L. and *P. coccineus* Lam. Genetica **35,** 59–74

Wall, R.J.; York, T.L. (1957): Inheritance of seedling cotyledon position in *Phaseolus* species. J. Hered. **48,** 71–74

Yeung, E.C.; Sussex, I.M. (1979): Embryogeny of *Phaseolus coccineus:* The suspensor and the growth of the embryo-proper in vitro. Z. Pflanzenphysiol. **91,** 423–433

Received Nov. 29, 1981
Accepted January 5, 1982
Communicated by H. F. Linskens

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