This is to certify that the

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RECOVERY OF INTERSPECIFIC VIGNA HYBRIDS
VIA EMBRYO CULTURE

presented by

James Francis Parrot

has been accepted towards fulfillment
of the requirements for

M.S. degree in Horticulture

John F. Kelly
Major professor

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RECOVERY OF INTERSPECIFIC VIGNA HYBRIDS VIA EMBRYO CULTURE

By

James Francis Parrot

A THESIS

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ABSTRACT

RECOVERY OF INTERSPECIFIC *VIGNA* HYBRIDS VIA EMBRYO CULTURE

By

James Francis Parrot

Embryo culture was used for 28 interspecific crosses representing 18 species combinations of various accessions of *Vigna angularis* (Willd.) Ohwi & Ohashi, *V. glabrescens* Maréchal, Mascherpa, & Stainier, *V. mungo* (L.) Hepper, *V. radiata* (L.) Wilcz., and *V. umbellata* (Thunb.) Ohwi & Ohashi. Mature hybrid plants were recovered from ten interspecific combinations including three that are apparently new: *V. glabrescens* x *V. mungo*, *V. glabrescens* x *V. radiata*, and (*V. radiata* x *V. umbellata*) amphidiploid x *V. glabrescens*. Although some hybrids were partially fertile, few produced seed. The ease of propagating and maintaining each hybrid depended upon its indeterminate vegetative habit. Problems with germination, quiescence, callus, deformity, leaf expansion, root development, and acclimation of plants from culture were encountered. A cutting procedure to overcome one type of deformity and an aseptic potting system to facilitate acclimation were developed.

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INTRODUCTION

The *Vigna* species of the subgenus *Ceratotropis* include a number of species of economic importance. Three of the species, *V. radiata*, *V. angularis*, and *V. mungo*, are important pulse crops for human food in Asia and Africa. *V. umbellata* is a minor crop in China and India, and *V. glabrescens* is an amphidiploid of natural occurrence in the Philippines (Maréchal et al., 1978) of potential value for disease resistance (J. M. Poehlman, personal communication). There has been considerable interest in hybridization among these five species both to study their phylogenetic relationships and isolating mechanisms, and to improve the crop species by introducing new characters from one species to another, inducing new characters not present in the parental species, and by increasing the range of variation in each species (Ahn, 1976; Ahn and Hartmann, 1978c; Chen et al., 1978). A number of these hybrid combinations previously attempted either were not successful, were obtained only with considerable difficulty and low return, or needed advancement to later generations (Ahn, 1976; Ahn and Hartmann, 1978a, 1978b, 1978c; Al-Yasiri and Coyne, 1966; Biswas and Dana, 1975a; Chowdhury and Chowdhury, 1977; Dana, 1964, 1965a, 1965b, 1966a, 1966b; De and Krishnan, 1966; Evans, 1975; Krishnan and De, 1968; Sawa, 1973; Sen and Ghosh, 1960; Singh et al., 1964).

This investigation was performed in conjunction with a comprehensive crossability study in *Vigna* (Chen, 1980) and focuses on *in vitro* culture of hybrid embryos which have not, or have only rarely progressed to maturity *in vivo*. It was undertaken to obtain new interspecific *Vigna* hybrid combinations, to advance the amphidiploid derived from a *V. radiata* x *V. umbellata* cross to later generations, and to develop information for the improvement of existing techniques.

REVIEW OF LITERATURE

Numerous successful attempts to obtain mature interspecific F_1 plants among these five species of the Ceratotropis subgenus have been reported (Ahn, 1976; Ahn and Hartmann, 1978a, 1978b, 1978c; Asian Vegetable Research and Development Center, 1974, 1975, 1976a, 1979, Baker et al., 1975; Biswas and Dana, 1975a; Chen, 1980; Chen et al., 1978; Dana, 1964, 1965a, 1965b, 1966a, 1966b, 1968; De and Krishnan, 1966; Gupta and Wagle, 1978; Krishnan and De, 1968; Sawa, 1973; Sen and Ghosh, 1960), but other desired interspecific hybrids attempted were not obtained. Included in this latter group are V. angularis x V. mungo, V. angularis x V. radiata, V. mungo x V. angularis, and V. umbellata x V. mungo, the last of which was attempted by Chowdhury and Chowdhury (1977) and all of which were attempted by Ahn and Hartmann (Ahn, 1976; Ahn and Hartmann, 1978c) and Chen (1980). Other attempted but unsuccessful combinations were V. glabrescens x V. radiata (Dana, 1965b; Krishnan and De, 1968) and V. umbellata x V. glabrescens (Dana, 1964, 1965a). The status of V. mungo x V. radiata is not clear; of the numerous attempts (Ahn, 1976; Ahn and Hartmann, 1978c; Chen, 1980, Dana, 1966a; De and Krishnan, 1966; Luyeye, 1975; Sen and Ghosh, 1960) no one reported mature F_1 plants, but Luyeye apparently obtained seeds sufficient to run an analysis of dipeptides. Gupta and Wagle (1978) reported on the biochemical composition of seeds of Phaseolus mungoreous, an amphidiploid of a cross between V. mungo and V. radiata obtained by pulse breeders at

Haryana Agricultural University, but they did not make clear which species served as the maternal parent.

The F_1 hybrids of *V. radiata* x *V. umbellata* that have been obtained have all been sterile. Fertile amphidiploids of this hybrid have been derived (Ahn, 1976; Ahn and Hartmann, 1978c; Asian Vegetable Research and Development Center, 1976a, 1976b; Chen, 1980; Dana, 1966c; Sawa, 1974) and it appeared useful to backcross such amphidiploids to *V. radiata* and *V. umbellata*. The success obtained in the effort to backcross to *V. radiata* has been reported by Chen (1980).

One of the barriers to interspecific hybridization is abortion of the hybrid embryos *in vivo*. The technique of embryo culture was first applied to this problem with success by Laibach (1929). The first successful report of embryo culture of an interspecific *Phaseolus* hybrid was that of Honma (1955). Several other attempts to culture embryos of interspecific *Phaseolus* and *Vigna* hybrids have been reported subsequently (Ahn, 1976; Ahn and Hartman, 1978a, 1978b, 1978c; Alvarez et al., 1978; Biswas and Dana, 1975a, 1975b; Braak and Kooistra, 1975; Chen, 1980; Dana, 1964, 1965a, 1966a; Evans, 1975; Honma, 1956; Ibrahim, 1974; International Institute of Tropical Agriculture, 1976; Kroh, 1962; Le Marchand et al., 1976; Le Marchand and Maréchal, 1977; Mok et al., 1978; Sawa, 1973). Although all of these workers except two (Evans, 1975; Ibrahim, 1974) succeeded in rearing at least one interspecific hybrid to maturity, a number reported hybrid combinations that did not respond favorably to this technique (Ahn, 1976; Ahn and Hartmann, 1978a, 1978b, 1978c; Chen, 1980; Dana, 1966a; Evans, 1975; Ibrahim, 1974).

Nutritional and other factors that effect the growth of bean embryos *in vitro* have also been reported (Braak and Kooistra, 1975; Cionini et al., 1976; Honma, 1955; Mok et al., 1978; Parthadev, 1977; Singh and Ahuja, 1974; Skene, 1969; Solacolu and Constantinesco, 1936; Thakur, 1977; Yeung and Sussex, 1979). Honma (1955) studied the effect of sucrose concentration in the medium on embryo growth. He found that 4% sucrose gave the best root and shoot growth. He also devised a liquid culture system for gradually lowering the concentration of sucrose so as to enhance root development and adapt the plants for potting. Skene (1969) studied the effect of gibberellic acid (GA_3) on immature _P. vulgaris_ embryos and found that at 10^{-4}M it hastened germination, increased the length of the radicle, hypocotyl and epicotyl, stimulated the expansion of leaves, and enhanced formation of lateral roots. Both embryo elongation and greening were enhanced by GA_3 at 10^{-7}M in interspecific _Phaseolus_ hybrids, although ultimate survival was not aided (Mok et al., 1978). Removal of the suspensor reduced the development of small _P. coccineus_ embryos but not those larger than 5 mm (Cionini et al., 1976). These investigators enhanced the growth of small, suspensor-deprived embryos (0.5-1.5 mm) but inhibited larger embryos (2-3 mm) with GA_3 at 10^{-8} to 10^{-6}M. At 10^{-5} to 10^{-4}M, GA_3 was generally inhibitory and tended to induce callus. Yeung and Sussex (1979) expanded the suspensor study and found that either GA_3 or kinetin was able to substitute for the suspensor, and that the effective concentration range of kinetin was broader than that of GA_3. For embryos with suspensor intact, the effect of kinetin was minimal. Also for embryos with suspensor intact, there was little effect of GA_3 at concentrations of 5 mg/l or less, but when GA_3 was increased to 10 mg/l inhibition was observed. Yeung and Sussex also found that indole acetic acid (IAA) had a slightly beneficial effect at 0.01 mg/l on suspensor-deprived embryos, but a detrimental effect (callus and abnormal growth) on all embryos when supplied at 0.1 mg/l. Abscisic acid at 0.01 mg/l had no effect, but at 0.1 mg/l or greater it inhibited precocious germination but not overall fresh weight. *V. radiata* embryos devoid of cotyledons had limited leaf expansion unless adenine was added to the medium (Parthadev, 1977). Media containing indolebutyric acid, kinetin, GA_3, and casein hydrolysate (CH) gave the best results in a test of various auxins, kinetin, GA_3, and CH on seven-day-old *V. mungo* embryos (Singh and Ahuja, 1974). The addition of CH (0.1%) accelerated the growth of small *P. vulgaris* x *P. ritensis* embryos (up to 0.7 mm without cotyledons) but retarded growth of embryos larger than 0.7 mm (Braak and Kooistra, 1975). The addition of glutamine at both 10 and 100 mg/l also enhanced the survival of small embryos of some interspecific *Phaseolus* hybrids (Mok et al., 1978). Thakur (1977) used egg white to increase survival, fresh weight, and hypocotyl elongation in embryos of *P. vulgaris*. Smith (1973) analyzed the endosperm of *P. vulgaris* at various stages of development for ions and organic compounds, which may be useful to devise a defined medium for hybrid embryos that have not responded to media currently in use.

Some researchers, successful in embryo culture, experienced difficulty in adapting their plantlets to the soil environment (Braak and Kooistra, 1975; Honma, 1955; Mok et al., 1978). Though these researchers eventually adapted occasional plantlets to soil medium, adaptation was a considerable obstacle.

MATERIALS AND METHODS

Plant Materials

Cultivars and intraspecific hybrids of *V. angularis*, *V. mungo*, *V. radiata*, *V. umbellata* (all 2n=22), *V. glabrescens* (2n=44), an interspecific F_1 hybrid of *V. umbellata* x *V. angularis*, and amphidiploids (2n=44) of *V. radiata* x *V. umbellata* were used for hybridization (Table 1). Due to the number of parental types and hybrid combinations as well as the complexity of some of them in regard to genomic constitution, a system of abbreviation has been adopted whereby each haploid genome is represented by a capital letter, and each cultivar or accession by an Arabic numeral. Thus, the diploid species, *V. angularis*, *V. mungo*, *V. radiata*, and *V. umbellata*, are represented by AA, MM, RR, and UU, respectively. The amphidiploid, *V. glabrescens*, is designated VVGG. The first accession of *V. angularis* is labeled AA1; a cross of the third accession of *V. umbellata* by the first accession of *V. angularis* is abbreviated as UU3 x AA1, and the resulting F_1 as UA31; and so on.

Cultural Conditions

Plants for crossing were grown in greenhouses heated to maintain 24-27°C day (9hr) and 18-21°C night (15 hr) and with supplemental lighting from high-intensity metal-halide and/or cool white fluorescent lamps to provide a 14 hour photoperiod. Short day conditions were provided as needed by use of blackcloth 12-15hr/day. Plants were grown

Table 1. *Vigna* species and cultivars used as parents for interspecific hybridization.

Species	Common name	'Cultivar name' or (derivation)	Accession number^z	Code
V. angularis	Adzuki bean	'Chien Shien'	5124	AA1
		'KS # 210'	5122	AA2
V. mungo	Black gram	'T-9'	3115	MM1
		--	NI 208	MM3
		(F_1 of MM3 x MM1)	--	MM31
V. radiata	Mungbean	'Tainan # 1'	2013	RR1
		'ML-3'	2773	RR2
		--	1837	RR5
		--	PI 377276	RR6
		--	PI 207504	RR8
		(F_1 of RR1 x RR5)	--	RR15
		(F_1 of RR2 x RR1)	--	RR21
V. umbellata	Rice bean	'HK'	4006	UU1
		'S-91'	4065	UU2
		--	4023	UU3
		--	NI 300	UU5
		--	PI 322571	UU7
		(F_1 of UU2 x UU1)	--	UU21
V. glabrescens		--	PI 207655	VVGG
(*V. radiata* x *V. umbellata*) amphidiploid		(RR6 x UU2)4x	--	RRUU62
		(RR8 x UU7)4x	--	RRUU87
		(RRUU62 x RRUU87)	--	RRUU6827
(*V. umbellata* x *V. angularis*) F_1		(F_1 of UU3 x AA1)	--	UA31

^{z}Accession numbers of the Asian Vegetable Research and Development Center, Shanhua, Taiwan, ROC, except as indicated by PI (U.S. Plant Introduction) or NI (Numéro d'introduction, Belgium).

in 20 cm pots containing either a commercial potting mix (Metro Mix 200 or Redi-Earth, W. R. Grace and Co., Cambridge, Massachusetts) or a mixture of soil:sand:peat (1:1:1). Nutrient levels were maintained with bi-weekly applications of 20N-9P-17K (3g/pot).

Crossing Methods

All the hybrid embryos for this investigation were supplied from a simultaneous study by Chen (1980) who followed the pollination techniques of Boling et al. (1961) and Buishand (1956).

In Vitro Methods

Embryo cultures were prepared from 28 parental combinations representing 18 different interspecific hybrid crosses and backcrosses (Table 2). Pods were collected 7-25 days after pollination either at abscision, at early signs of degeneration (yellowing, bloating, loss of turgor), or at a predetermined number of days after pollination just prior to the anticipated onset of degeneration. Pods were surface disinfected in a laminar-airflow hood by dipping in 95% ethanol and flaming briefly. Ovules were excised and placed on moist sterile filter paper for embryo excision under a dissecting microscope. Much time and effort was spent excising numerous selfed embryos in order to develop, practice, and gain dexterity in the technique so that hybrid embryos could be excised with a minimum of damage.

Embryos were measured using an ocular micrometer. Embryos had one to four cotyledons, so the cotyledon lengths were summed for each embryo and divided by two. Unless noted otherwise, the hypocotyl measurement is the length from the cotyledonary node to, but not including, the suspensor which often remained attached to the hypocotyl. Occasionally,

Table 2. Interspecific *Vigna* hybrids and conditions of their culture *in vitro*.

Interspecific cross	Embryos plated	Initial plating medium^z	Environment^y	Cotyledon treatments^x	Hybrid code
V. radiata x *V. umbellata*					
RR1 x UU1	14	D1, D3, E1, E2, E3	F	7w0, 7w2	RU11
V. umbellata x *V. angularis*					
UU1 x AA1	19	D1, D2	B&M		UA11
UU3 x AA1	8	D1, D2	O		UA31
V. angularis x *V. umbellata*					
AA2 x UU2	15	D4	W		AU22
V. angularis x *V. radiata*					
AA1 x RR1	14	D4	Q		AR11
AA1 x RR5	4	D4	Q		AR15
V. mungo x *V. angularis*					
MM3 x AA1	2	D4	H		MA31
MM1 x AA2	4	D4	I		MA12
V. mungo x *V. radiata*					
MM1 x RR1	2	D4	I		MR11
V. mungo x *V. umbellata*					
MM3 x UU1	4	D4	Q		MU31
MM1 x UU1	1	D4	I		MU11
MM1 x UU2	5	D4	I		MU12
MM31 x UU21	9	D4	H		MU31-21

Table 2 continued

Interspecific cross	Embryos plated	Initial plating medium^z	Environment^y	Cotyledon treatments^x	Hybrid code
V. glabrescens x *V. umbellata*					
VVGG x UU5	7	C	C		VGU5
V. glabrescens x *V. radiata*					
VVGG x RR1	20	C, D4	S&U&V	13w0, 5w1, 2w2	VGR1
VVGG x RR21	7	D4	S	4w0, 3w1	VGR21
V. radiata x *V. glabrescens*					
RR21 x VVGG	10	D4	R		RVG21
V. glabrescens x *V. mungo*					
VVGG x MM1	11	D4	T	8w2, 3w1	VGM1
(*V. radiata* x *V. umbellata*) amphidiploid x *V. radiata*					
RRUU62 x UU1	33	E1, E2, E3, E4	F		RUU621
(*V. radiata* x *V. umbellata*) amphidiploid x *V. radiata*					
RRUU62 x RR1	61	D1, D2, D3	F		RUR621
RRUU62 x RR2	18	D1, D2, D3	B&M		RUR622
RRUU87 x RR1	22	D1, D2, D3	F		RUR871
V. radiata x (*V. radiata* x *V. umbellata*) amphidiploid					
RR1 x RRUU87	9	D1, D2, D3	F		RRU187
(*V. radiata* x *V. umbellata*) amphidiploid x *V. radiata*, BC2					
RUR621 x RR21	2	D4	F		RR61-21+u
RUR621 x RR1	54	C, D1	D&N		RR61-1+u

Table 2 continued

Interspecific cross	Embryos plated	Initial plating medium[z]	Environment[y]	Cotyledon treatments[x]	Hybrid code
(*V. radiata* x *V. umbellata*) amphidiploid x *V. glabrescens* RRUU6827 x VVGG	9	D4	I		RUVG68-27
V. radiata x (*V. umbellata* x *V. angularis*) RR15 x UA31	5	D4	J		R-UA15-31
(*V. umbellata* x *V. angularis*) x *V. mungo* UA31 x MM3	7	D4	H&P		UA-M31-3

[z] See table 3 for composition of the various media. Initial plating medum used from plating until potting except as specifically mentioned in the text.

[y] See table 4 for conditions of the various environments. Except as noted in the results, these environments were in effect from plating until transfer to larger vessels. At the time of transfer to larger vessels, embryos growing in environments B through F and M through O were transferred to environment G, while embryos in the other environments continued in those same environments.

[x] One or both cotyledons were removed from some embryos. Each 3 or 4 character code indicates "number of embryos with number of cotyledons left intact at plating", thus, "7w0" indicates "7 embryos with 0 cotyledons left intact".

a clear delineation betwen hypocotyl and cotyledon could not be made, so total length of each embryo was divided into hypocotyl and cotyledon length according to the hypocotyl:cotyledon ratio of the other embryos of the same cross.

Each embryo was plated onto agar-solidified medium consisting of Murashige and Skoog (1962) mineral salts with supplements (Table 3) prepared in advance and dispensed into Petri dishes. Care was taken to place the radicle in contact with the medium. A channel, gradually increasing in depth, was cut into the agar from the embryo toward an open area of the agar surface so as to prevent beading of excess moisture around the embryo. Petri dishes were sealed with Parafilm to retard moisture loss from the medium during incubation (Figure 1A).

Plates were placed on shelves in a room equipped with unshielded fluorescent tubes (cool white or cool white:warm white as indicated in Tables 2 and 4). Irradiation just above the plates was measured (under cool white regimes only) with a foot-candle meter (Type 214 Light Meter, General Electric, Fairfield, Connecticut). This meter was later calibrated with a Li-Cor Radiometer LI-185A (LI-COR, Inc., Lincoln, Nebraska) over a spectrum of 400 to 700 nm. Because of the spectrum changes characteristic of fluorescent tubes, both measurements are given but should be regarded as approximations. The irradiation changes from daytime (16 hr) to nighttime (8 hr) and vice versa were made abruptly. Several different environments for embryo culture were used during this study (Table 4) and the environments used for each of the particular hybrids are listed in Table 2.

Embryos were transferred to large sterilized jars for further development when the shoot reached an average length of 18 mm and had,

Table 3. Composition of media used for embryo culture I; principal media[z]

Ingredient	Unit	Agar solidifed media used from plating until potting									Liquid media for potting			
		C	D1	D2	D3	D4	E1	E2	E3	E4	L1	L2	L3	L4
mineral salts[y]	-[y]	0.5	1.0	1.0	1.0	1.0	1.1	1.1	1.1	1.1	0.5	0.5[w]	0.5	1.0
myo-inositol	mg	50.0	100.0	100.0	100.0	100.0	111.1	111.1	111.1	111.1	0.0	0.0	50.0	100.0
thiamine HCl	mg	0.5	1.0	1.0	1.0	1.0	1.1	1.1	1.1	1.1	0.0	0.0	0.5	1.0
nicotinic acid	mg	0.0	0.0	1.0	1.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
pyrodoxine HCl	mg	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Kinetin	mg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.3	0.0	0.0	0.0	0.0
glutamine	mg	0.0	0.0	0.0	50.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
sucrose	g	30.0	30.0	30.0	30.0	30.0	33.3	33.3	33.3	33.3	0.0	0.0	30.0	10-30
agar	g	9.0	9.0	9.0	9.0	9.0	10.0	10.0	10.0	10.0	0.0	0.0	0.0	0.0
coconut water[x]	ml	0.0	0.0	0.0	0.0	0.0	0.0	27.8	0.0	27.8	0.0	0.0	0.0	0.0

[z] All components expressed in units per liter. The pH was adjusted to 6.0 for all media with KOH prior to addition of sucrose, agar, and autoclaving at 121°C.

[y] Mineral salts are the same as those of Murashige and Skoog (1962). The number entered for each medium in this table times the quantity indicated by Murashige and Skoog equals the amount of salts incorporated into each medium.

[x] Filter sterilized coconut water (deproteinized) added after autoclaving.

[w] All NH_4NO_3 omitted from L2 to give ammonium-free medium.

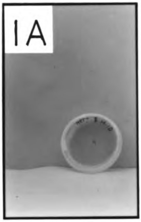
Figure 1. Stages of the embryo culture system.

A. Stage 1 embryo germinating on petri plate.

B. Stage 2 embryo transferred to larger vessel for expansion of roots, stem, and leaves. Note bottom half of Petri dish inside.

C. Stage 3 plantlets. At left is a plantlet recently potted aseptically for gradual acclimatization. Note wire support frame that clamps firmly to the pot. At right is a plantlet nearly ready for transplanting to the greenhouse. Note deep lid and its supports that allow gradual decrease of humidity toward the end of stage 3.

D. Stage 4 plantlet fully acclimated and ready to transplant to the greenhouse.



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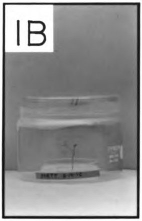

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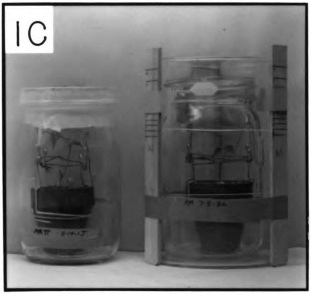

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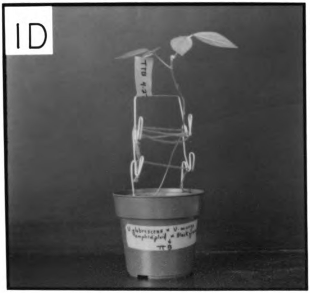


1D

Table 4. Conditions of incubation from initial plating until potting.

Environment designation	Temp °C	Light type[z]	Light quantity and duration			
			Day (16 hours)		Night (8 hours)	
			ft-c	$nEs^{-1}cm^{-2}$	ft-c	$nEs^{-1}cm^{-2}$
A	21±1	0	0	0	0	0
B	21±1	CW	50-150	1.4-2.9	0	0
C	21±1	CW	100-150	2.0-2.9	0	0
D	21±1	CW	70-200	1.7-3.7	0	0
E	21±1	CW	130-200	2.5-3.7	0	0
F	21±1	CW	50-260	1.4-4.5	0	0
G	21±1	CW	160-240	3.0-4.2	0	0
H	25±2	CW	160-240	3.0-4.2	0	0
I[y]	25±2	CW	185-290	3.5-5.0	25-50	1.0-1.4
J[x]	25±2	CW:WW	---	---	0	0
K	25±2	CW	75-225	1.5-4.0	75-225	1.5-4.0
M	environment A until growth commenced, then environment B					
N	environment A until growth commenced, then environment D					
O	environment A until growth commenced, then environment E					
P	environment H for 12 days, then environment I					
Q	environment H for 4 weeks, then environment I					
R	environment H for 1 week, then environment J					
S	environment H for 3 weeks, then environment J					
T	environment J for 4 days, then environment H					
U	environment C for 2-3 weeks, then environment H for 3 weeks, then environment J					
V	environment C for 9 weeks, then environment H for 3 weeks, then environment J					
W	environment H for 4 weeks, then environment I for 3 months, then environment H					

[z] 0 indicates no light, CW indicates cool white fluorescent tubes, CW:WW indicates cool white:warm white fluorescent tubes in 1:1 ratio.

[y] Intensity difference of environment I over environment H was not measured but estimated.

[x] Intensity was not measured for environment J. Environment J was produced by replacing one half of the cool white fluorescent tubes of environment H with warm white tubes.

generally, at least one expanded leaf. This transfer was usually accomplished by removing the Parafilm from the Petri plate, cleaning the bottom half of the plate (including its sides) with a pad saturated with 95% ethanol, discarding the top of the plate, and placing the lower half with the embryo into the larger jar (Figure 1B). A few drops of sterile water had been added to the larger jar to assure adequate humidity at the time of transfer. Occasionally, however, the embryo was transferred to a larger vessel into which fresh medium had been dispensed. In some cases these jars were of non-borosilicate glass and later observation indicated leaching of ions into the medium. These media (Table 5) are identified as "defective" by a "d" prefixed to the medium designation. Once in larger jars, cultures growing in environments B through F and M through O were transferred to environment G, while embryos in other environments continued in those same environments.

This general technique was supplemented in some cases where abnormal growth occurred. Some embryos which callused heavily were transferred to a cytokinin-containing medium (S1, S2, S3, or S4; see Table 5) in an effort to obtain shoots (Skoog and Miller, 1957). Also, shoot tips of embryos which produced multiple shoots or grossly elongated and spindly shoots were excised (about 10 mm in length) and the cut end of each was pushed about 2-4 mm into fresh D4 medium for rooting and possible development into normal plantlets. The rest of the plantlet was either discarded or saved to produce new shoots.

Plantlets were potted after adequate development (avg. of 51 mm, but minimum of 8 mm shoot length with at least one expanded leaf). The first few hybrid plantlets (all RR x UU and one RRUU x RR plantlets) were potted in perlite or a commercial potting mixture and gradually acclimated

Table 5. Composition of media used for embryo culture II; supplemental media^{z}

Ingredient	Unit	Agar solidifed media used after germination^{y}								Shoot induction media			
		D5	dC	dD1	dD4	dF1	dF2	dF3	dF4	S1	S2	S3	S4
mineral salts^{x}	-^{y}	1.0	0.5	1.0	1.0	1.0	0.5	1.0	0.5	1.0	1.0	1.0	1.0
myo-inositol	mg	100.0	50.0	100.0	100.0	100.0	50.0	100.0	50.0	100.0	100.0	100.0	100.0
thiamine HCl	mg	1.0	0.5	1.0	1.0	1.0	0.5	1.0	0.5	1.0	1.0	1.0	1.0
nicotinic acid	mg	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.5
pyrodoxine HCl	mg	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.5
indole-3-acetic acid	mg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.3	0.2	0.2
benzyladenine	mg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.0	0.0
isopentenyl adenine	mg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.0	10.0	0.0	0.0
zeatin	mg	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.0
sucrose	g	20.0	30.0	30.0	30.0	20.0	20.0	10.0	10.0	20.0	30.0	30.0	30.0
agar	g	9.0	9.0	9.0	9.0	8.0	8.0	8.0	8.0	9.0	9.0	9.0	9.0

[z] All components expressed in units per liter. The pH was adjusted to 6.0 for all media with KOH prior to addition of sucrose, agar, and autoclaving at 121°C.

[y] Media designations beginning with "d" (defective) indicate media that were dispensed into culture vessels made of other than boro-silicate glass. There was evidence that leaching from the glass into the media had occurred during the culture period.

[x] Mineral salts are the same as those of Murashige and Skoog (1962). The number entered for each medium in this table times the quantity indicated by Murashige and Skoog equals the amount of salts incorporated into each medium.

to greenhouse conditions by use of high humidity and/or misting chambers. An aseptic potting procedure was developed due to the high mortality of this preliminary method. A clay pot (6 cm in diameter) was fitted with an aluminum wire frame to support the plantlet and facilitate subsequent aseptic manipulation of the pot. The pot with its frame was placed inside a 946 ml glass jar and autoclaved. A potting medium of either pure sand or a mixture (ca. 1:1 v/v) of perlite and commercial potting mixture was put into another container, moistened, autoclaved, then flushed with an autoclaved nutrient solution. Four nutrient solutions (L1, L2, L3, and L4; see Table 3) were used at the beginning of this study, but L1 was soon adopted as the preferred solution. The plantlet was loosened from the agar in the culture vessel and placed into sterile water to wash away any agar still clinging to the roots. The water that clung to the plantlet after this soaking afforded the plantlet some protection from dessication during the actual potting procedure, but as an added precaution, a small area of the laminar air flow was blocked to provide a guarded area. The flushed, aseptic potting medium was spooned into the pot around the hybrid plantlet. The potted plantlet was then put into an autoclaved 946 ml jar with a glass lid, sealed with Parafilm, and incubated with irradiance of 5.6 $nEs^{-1}cm^{-2}$ (330 ft-c) 16 hr/day at 21 ± 1°C for acclimation (Figure 1C). The plantlet was allowed to acclimate over 10-79 days based on expansion of a new leaf as a signal of adequate acclimation. For prolonged adaptation periods, the pot was flushed occasionally with fresh nutrient solution and transferred to a new jar. Near the end of the adaptation phase, the pot was flushed and transferred to an autoclaved jar with a deep lid (Figure 1C) which was gradually opened over a five to ten day period. After the plant had adapted to ambient laboratory conditions (Figure 1D), it was moved to the greenhouse on a cloudy day, and transplanted to a larger pot inoculated with a mixed Rhizobium preparation (courtesy of J. C. Burton, Nitragin Co., Milwaukee, Wisconsin) primarily to provide a non-pathogenic microbial population around the young plant.

A four-point evaluation system to score the development of each embryo was based on the main stages of the culture procedure. Thus, the various points of the scoring system indicate, respectively, 1) that the plated embryo had initiated growth, 2) that the embryo had, or had nearly, developed a basic root-stem-leaf structure and was transferred to a larger culture vessel, 3) that the plantlet had developed enough photosynthetic area to move to a pot, and 4) that the plantlet had fully acclimated and was ready to be transplanted to the greenhouse.

Hybrid Characteristics

The hybrid plants, or hybrid plants grown from cuttings, were compared with the parental species in greenhouses for morphological traits with emphasis on the patroclinous characters of the hybrid. Pollen fertility was estimated by use of a saturated I_2-KI solution, counting plump, evenly stained pollen grains as fertile.

Statistical Analysis

When several hybrid embryos were obtained from the same parental combination (cultivar by cultivar) they were sometimes given different treatments with respect to cotyledon removal, medium, and/or incubation environment (Table 2). Means of different treatment effects within each hybrid combination were compared by use of the t-test, but because the data often did not meet all the assumptions for this test, observed differences are termed "apparent" rather than "significant".

Differences in the embryos that were beyond experimental control (ovule condition, presence of suspensor, embryo size, etc.) were also noted for their effects and analyzed by t-tests or linear or curvilinear regression for many, but not all, of the crosses. Again, as above, interpretation of the results is qualified.

Some hybrid characteristics (*e.g.*, leaf measurements) were also compared by t-test to those of the maternal parent.

The chi-square test was used to determine goodness of fit of observed phenotypic ratios to a proposed genetic model.

RESULTS AND DISCUSSION

The 28 crosses representing 18 species combinations responded to embryo culture with different degrees of success (Tables 6, 7, 8, 9). Compositing the 28 crosses into their 18 species combinations, in only one case was there a total failure of embryos to grow (RR x RRUU). In seven of the crosses growth commenced, but mature (flowering) plants were not obtained (AA x RR, MM x AA, MM x RR, MM x UU, RRUU x UU, RR x UA, and UA x MM). Mature hybrid plants were recovered from ten different species crosses; viz.: three diploid x diploid crosses (RR x UU, UU x AA, AA x UU), one amphidiploid x amphidiploid cross (RRUU x VVGG), one diploid x amphidiploid cross (RR x VVGG), four amphidiploid x diploid crosses (RRUU x RR, VVGG x UU, VVGG x RR, VVGG x MM), and one allotriploid x diploid backcross (RUR x RR).

The initial culture medium did not seem to have much effect on the final degree of success. Only one cross (RRUU62 x UU1) gave any indication of an apparent difference. Medium E1 appeared to be better than either E2 or E4 for these embryos, but this was confounded with age in that all of the embryos at the extremes of the age spectrum (11 and 17 days after pollination) were plated on E2 and E4. If the youngest and oldest embryos were dropped from consideration, there was no longer a detectable difference between these media effects.

Different incubation environments that were compared for some hybrid combinations (Table 2) seemed different in their effect on final embryo

Table 6. Embryo culture of interspecific *Vigna* hybrids involving pairs of diploid species.

Interspecific cross	Embryos or (shoots)^z plated	Embryo age at plating^y	Mean size (mm) at excision: Ovule	Mean size (mm) at excision: Cotyledon	Mean size (mm) at excision: Hypocotyl	Degree of success^x: 1 Growth	Degree of success^x: 2 To jar	Degree of success^x: 3 To pot	Degree of success^x: 4 Adapted	Hybrid code
V. radiata x *V. umbellata*										
RR1 x UU1	14	7-25	4.8	2.8	1.7	13/14	8/14	5^w/13	1^w/12	RU11
V. umbellata x *V. angularis*										
UU1 x AA1	19	12-16	3.8	1.0	0.1	6/19	2/19	2/19	2/19	UA11
UU3 x AA1	8	20	4.8	---	---	8/8	6/8	6/8	4/8	UA31
V. angularis x *V. umbellata*										
AA2 x UU2	15	10-11	5.6	1.2	0.4	8/15	1/15	1/15	1/15	AU22
" x " secondary^z	(8)					---	0/8	0/8	0/8	
V. angularis x *V. radiata*										
AA1 x RR1	14	13-22	5.2	0.7	0.4	8/14	2/14	2/14	0/14	AR11
AA1 x RR5	4	14	6.2	1.1	0.5	2/4	0/4	0/4	0/4	AR15
V. mungo x *V. angularis*										
MM3 x AA1	2	20	2.3	0.6	0.4	0/2	0/2	0/2	0/2	MA31
MM1 x AA2	4	14,17	2.8	0.9	0.6	4/4	3/4	0/3	0/3	MA12
V. mungo x *V. radiata*										
MM1 x RR1	2	12	2.6	0.7	0.3	1/2	0/2	0/2	0/2	MR11
V. mungo x *V. umbellata*										
MM3 x UU1	4	10	3.3	1.3	0.4	3/4	0/4	0/4	0/4	MU31
MM1 x UU1	1	10	2.2	0.2	0.3	1/1	0/1	0/1	0/1	MU11
MM1 x UU2	5	10,14	4.4	0.5	0.4	5/5	0/4	0/4	0/4	MU12
MM31 x UU21	9	19-20	4.8	4.1	1.2	9/9	2/9	0/9	0/9	MU31-21

^{z}Secondary shoot tips obtained from an embryo that produced multiple shoots *in vitro*.

^{y}Days after pollination.

^{x}Number of embryos reaching stated stage divided by number of embryos plated; corrected for losses due to contamination or mechanical error.

^{w}Plantlets potted prior to development of aseptic potting technique, so recovery is probably low.

Table 7. Embryo culture of interspecific *Vigna* hybrids involving *V. glabrescens*.

Interspecific cross	Embryos or (shoots)[z] plated	Embryo age at plating[y]	Mean size (mm) at excision			Degree of success[x]				Hybrid code
			Ovule	Cotyledon	Hypocotyl	1 Growth	2 To jar	3 To pot	4 Adapted	
V. glabrescens x *V. umbellata*										
VVGG x UU5	7	14	3.3	1.4	0.5	7/7	7/7	6/7	5/7	VGU5
V. glabrescens x *V. radiata*										
VVGG x RR1	20	11-15	4.7	3.5[v]	1.1	20/20	20/20	12/20	8/20	VGR1
" x " secondary[z]	(4)					---	2/4	2/4	1/4	
VVGG x RR21	7	14	4.8	4.2	1.1	7/7	6/7	5/6	5/6	VGR21
" x " secondary[z]	(1)					---	0/1	0/1	0/1	
V. radiata x *V. glabrescens*										
RR21 x VVGG	10	14	3.4	1.3	0.9	8/10	2/10	2/10	2/10	RVG21
" x " secondary[z]	(9)					---	8/9	8/9	5/8	
V. glabrescens x *V. mungo*										
VVGG x MM1 total[w]	11	15	4.2	3.7	0.7	11/11	10/11	7/11	5/11	VGM1
" x " normal[w]								6/9	5/9	
" x " defective[w]								1/2	0/2	
(*V. radiata* x *V. umbellata*) amphidiploid x *V. glabrescens*		See Table 9.								

[z] Secondary shoot tips obtained from an embryo that produced multiple shoots *in vitro*. The highest scoring shoot-tip cutting of any one embryo is entered in the pool of embryos proper, with only the multiples assigned to the "secondary" category.

[y] Days after pollination.

[x] Number of embryos reaching stated stage divided by number of embryos plated; corrected for losses due to contamination or mechanical error.

[w] The defective medium used for some embryos did not entirely prevent embryo recovery, but did appear to be disadvantageous. Therefore, in addition to the running total for each hybrid, the two classes making up that total (embryos carried through culture only on normal medium and those transferred to defective medium) are listed. Classes are separated at that point in the table after which transfer to defective medium was made.

[v] This value is a mean of only 15 of the 20 embryos because of cotyldeon deterioration of five of the embryos.

Table 8. Embryo culture of interspecific *Vigna* hybrids involving amphidiploids of (*V. radiata* x *V. umbellata*).

Interspecific cross	Embryos plated	Embryo age at plating[z]	Mean size (mm) at excision			Degree of success[y]				Hybrid code
			Ovule	Cotyledon	Hypocotyl	1 Growth	2 To jar	3 To pot	4 Adapted	
amphidiploid x *V. umbellata*										
RRUU62 x UU1	33	11-17	2.9	0.2	0.2	21/33	0/33	0/33	0/33	RUU621
amphidiploid x *V. radiata*										
RRUU62 x RR1 total	61	11-16	3.2	0.5	0.2	44/61	12/61	4/60	3/60	RUR621
" x " normal[x]							12/57	3[w]/49	2[w]/49	
" x " defective[x]							0/4	0/4	0/4	
" x " defective[x]								1/7	1/7	
RRUU62 x RR2 total	18	12-13	2.8	0.4	0.1	18/18	2/18	2/18	2/18	RUR622
" x " normal[x]							2/14	2/14	2/14	
" x " defective[x]							0/4	0/4	0/4	
RRUU87 x RR1	22	8-14	2.9	0.5	0.1	19/22	0/22	0/22	0/22	RUR871
V. radiata x amphidiploid										
RR1 x RRUU87	9	14	3.6	0.6	0.6[v]	0/8	0/8	0/8	0/8	RRU187
amphidiploid x *V. radiata*, BC2										
RUR621 x RR21	2	19	2.3	0.6	0.2	0/2	0/2	0/2	0/2	RR61-21+u
RUR621 x RR1 total	54	9-14	2.5	0.6	0.3	26/54	7/54	4/54	1/54	RR61-1+u
" x " normal[x]								2/52	1/52	
" x " defective[x]								2/2	0/2	
amphidiploid x *V. glabrescens*	See Table 9.									

[z] Days after pollination.

[y] Number of embryos reaching stated stage divided by number of embryos plated; corrected for losses due to contamination or mechanical error.

[x] The defective medium used for some embryos did not entirely prevent embryo recovery, but did appear to be disadvantageous. Therefore, in addition to the running total for each hybrid, the two classes making up that total (embryos carried through culture only on normal medium and those transferred to defective medium) are listed. Classes are separated at that point in the table after which transfer to defective medium was made.

[w] One of the three embryos was potted prior to development of aseptic potting technique. The two that survived were both potted aseptically.

[v] This hypocotyl value may not be accurate because a clear delineation between hypocotyl and the attached suspensor could not be made.

Table 9. Embryo culture of interspecific *Vigna* hybrids involving three species.

Interspecific cross	Embryos plated	Embryo age at plating^{z}	Mean size (mm) at excision			Degree of success^{y}				Hybrid code
			Ovule	Cotyledon	Hypocotyl	1 Growth	2 To jar	3 To pot	4 Adapted	
(*V. radiata* x *V. umbellata*) amphidiploid x *V. glabrescens* RRUU6827 x VVGG	9	13	4.0	1.2	0.5	9/9	7/9	5/9	5/9	RUVG68-27
V. radiata x (*V. umbellata* x *V. angularis*) RR15 x UA31	5	16-17	3.4	1.4	1.1	4/5	3/5	1/5	0/5	R-UA15-31
(*V. umbellata* x *V. angularis*) x *V. mungo* UA31 x MM3	7	14-15	3.9	1.1	0.3	4/7	0/7	0/7	0/7	UA-M31-3

^{z}Days after pollination.

^{y}Number of embryos reaching stated stage divided by number of embryos plated.

score, but were also confounded. In one cross (UU1 x AA1) embryos incubated in the light did not perform as well as those initially incubated in darkness. (None of these light-incubated embryos grew.) However, this possible light effect was confounded with age in that all of the embryos initially incubated in the light were older (15-16 days) than those incubated in the dark (12-14 days). The germination of other interspecific hybrids in the present investigation, including a cross of the same two species but a different cultivar as the maternal parent, was not inhibited by light. Probably, the older embryos were simply excised after irreversible degeneration of the embryo had begun, as was reported for embryos of wheat x rye crosses (Taira and Larter, 1978). No other differential effects of initial incubation conditions were detected for other hybrid combinations.

Cotyledon removal treatments (Table 2) did not appear to be different in their effect on the final scores attained by the embryos.

V. radiata x V. umbellata

From the cross of RR1 x UU1, 12 abscised pods at 7-25 days after pollination provided 14 embryos for culture. Embryos were normal in appearance except that one had no cotyledons; it could not be determined whether they had never developed or were lost at excision. Ten embryos grew normally, two failed to develop epicotyls, one produced callus, and one did not grow at all. In four weeks, eight of the ten normal plantlets were transferred to larger vessels containing fresh D5 medium. The other two had poor leaf expansion and ceased growth in stage one. Of the eight plantlets that were transferred, one was lost to contamination, two failed to develop adequately, and five were eventually potted. These embryos were not potted aseptically, and only one of them survived. It took about ten weeks from excision to greenhouse readiness.

Sawa (1973) also used embryo culture to obtain four mature plants from 23 embryos of this hybrid combination. Other workers obtained mature plants from viable seeds without the use of embryo culture (Ahn, 1976; Ahn and Hartmann, 1978c; Asian Vegetable Research and Development Center, 1974, 1975, 1976; Chen, 1980; Chen et al., 1978; Dana, 1966c). The hybrid obtained by embryo culture in the present work need not be described again as it is the same as that obtained from viable seed by Chen (1980). Fertility of the F_1 of this combination is low and no diploid F_2 population has been reported. However, amphidiploids have been derived by treatment with colchicine (Ahn, 1976; Ahn and Hartmann, 1978c; Chen, 1980; Dana, 1966c; Sawa, 1974) and spontaneously (Asian Vegetable Research and Development Center, 1976a, 1976b) which produced viable progeny.

V. umbellata x V. angularis

Two different V. umbellata accessions, UU1 and UU3 were used as the maternal parent in crosses with AA1. From the first of these crosses, UU1 x AA1, nine pods ranging form 12-16 days after pollination provided 19 ovules that contained normal embryos and 2 ovules with small or collapsed embryos. The 19 normal embryos were plated but only six embryos grew. Four of these ceased growth when the shoot reached 5 mm or less in length. Two embryos proceeded to the second stage and were ultimately transplanted to the greenhouse after an average 187 days from excision.

In the second cross involving these two species, that is, UU3 x AA1, eight embryos from one shrivelled, chlorotic, 20-day-old pod were cultured. The embryos were normal in appearance except that two of them had slightly distorted cotyledons. All eight of these embryos grew and developed normally; however, two of them quiesced before attaining 1 mm of shoot growth. The other six plantlets reached the potting stage, but two of these succumbed. Four plantlets were transplanted to the greenhouse in an average of 120 days after excision.

Chen (1980) has already described the mature plants of UU1 x AA1. The mature plants of UU3 x AA1 did not differ greatly from those of UU1 x AA1 except that the former tended to have greater leaf pubescence and a little different growth habit than the latter. Whereas hybrid plants of UU1 x AA1 were of medium height, somewhat trailing, and produced juvenile shoots from the base of the plants, those of UU3 x AA1 were taller, more vining, and produced axillary branches at the higher nodes. These differences may have been partly due to different environmental conditions. Pollen stainability was 82% (mean based on 100 grains from each of eight flowers) and numerous fruits and seeds were produced. However, pod length and number of seeds per pod were not determined.

The cross of _V_. _umbellata_ x _V_. _angularis_ has been reported (Ahn, 1976; Ahn and Hartmann, 1978b, 1978c; Chen, 1980; Sawa, 1973) but only with the aid of embryo culture. Although Evans (1975) reported obtaining seeds, no mention was made of F_1 plants. Ahn and Hartmann (1978b) reported difficulty in obtaining the hybrid plants as many embryos failed to grow in culture or died as seedlings. Poor germination of the UU1 x AA1 embryos was also observed in the present work, although UU3 x AA1 embryos presented no serious difficulty. Sawa (1973) reported the F_1

plants he obtained to be sterile, but the hybrid plants reported by Ahn and Hartmann (1978b) and those in the present investigation were fertile. UA11 had 77% stainable pollen (Chen, 1980) and UA31 had 82%; both produced plump seeds. F_2 plants were grown out by Ahn and Hartmann (1978b) and in this study with no unusual difficulties. Ahn and Hartmann (1978b) also backcrossed the F_1 to both parents, obtaining fertile progeny. Thus, gene transfer between these two species is probable.

An F_2 population of the cross UU1 x AA1 was observed for epicotyl color and the shape of the first foliar leaves. Ahn and Hartmann (1978b) and Chen (1980) reported continuous variation for both of these traits. Numerous leaf forms intermediate between and including cordate and lanceolate were observed in the F_2 population of the present study, indicating complex inheritance. However, the observations for epicotyl color (Table 10) fit a 9:3:4 ratio where one dominant gene determines purple pigmentation and a second dominant gene intensifies that pigmentation to dark purple. This agreed with Kakizaki's (1923) genetic model for stem color in V. angularis, P for purple and I which intensifies P.

V. angularis x V. umbellata

The parent AA2 was crossed with UU2. One bloated, 10-day-old pod and one normal, 11-day-old pod were harvested. Seventeen ovules were obtained with one or two from each pod beginning to discolor with a red or brown hue. Two embryos were damaged during embryo excision so that only 15 were plated. One of the embryos had three cotyledons. Eight of the embryos, including those from discolored ovules, grew, but in either a deformed or a callused manner. Leaves were generally under-developed and several embryos failed to form roots. One of these embryos, however,

Table 10. Genetics of epicotyl coloration in the interspecific cross of *V. umbellata* x *V. angularis*.

Species	Generation	Phenotypic classes			Total
		Purple	Intermediate	Green	
V. umbellata (UU1)	P_1	5	0	0	5
V. angularis (AA1)	P_2	0	0	5	5
Interspecific hybrid (UA11)	F_2	33	9	14	56
Expected (9:3:4) ratio^z	expected F_2	31.5	10.5	14.0	56

$^z\chi^2 = .296$; $p = .80-.90$.

produced a relatively strong shoot and was transferred to a larger vessel. The main shoot soon weakened, but the plantlet began to produce many small shoots, usually with only rudimentary leaves or stipules. Eight of these shoots were excised and placed on D4 medium, but there was virtually no subsequent growth. After nine months in culture, the plantlet developed another strong shoot. This was excised (with some rootlets) and aseptically potted. The hybrid plant was acclimated and transplanted to the greenhouse nearly one year after initiating the culture. It eventually flowered (N. C. Chen, personal communication) but is not described here. The same plant is mentioned by Chen (1980).

Ahn and Hartmann (Ahn, 1976; Ahn and Hartmann, 1978b, 1978c) also reported poor germination and callus growth in their unsuccessful attempts to obtain this hybrid by embryo culture. The parental genotypes may have been responsible for the success of the present attempt. The callus formation and deformity suggest that endogenous auxin might have been in oversupply (Rappaport et al., 1950; Solacolu and Constantinesco, 1936; Yeung and Sussex, 1979) so rescue of this hybrid might be facilitated by use of an auxin antagonist (Newcomb and Wetherell, 1970) or cytokinin (see discussion of RR x VVGG) in the culture medium. Excess gibberellins also tend to induce callus (Cionini et al., 1976; Stewart and Hsu, 1978) but the lack of leaf expansion in the hybrid plants indicated a possible shortage of gibberellins. Other explanations might include above optimal incubation temperature which favors callus growth over differentiation (Taira and Larter, 1978), that additional nutrients such as amino acids were necessary for proper embryo differentiation (Singh and Ahuja, 1974; Taira and Larter, 1978), or that toxins acquired _in ovulo_ needed to be diffused or otherwise dissipated before normal development could proceed (Emsweller et al., 1962). The fact that one embryo did eventually produce normal shoots without any additions to the medium lends credence to the last possibility, but this possibility cannot be substantiated without additional research.

V. angularis x V. radiata

Two different *V. radiata* genotypes were used to pollinate *V. angularis*. From the cross of AA1 x RR1, three green, bloated pods were obtained 18-22 days after pollination. Embryos were rescued from eight ovules and three of the eight ovules were beginning to turn brown around the hilum. One embryo was somewhat "spongy" or "cottony" in appearance. Two normal pods were harvested at 13 days from pollination and provided six more embryos from ovules slightly discolored around the hilum. One of these embryos had deformed and/or slightly deteriorated cotyledons. All 14 of the embryos were plated. Eight of the embryos (including the one that was "spongy" and the one with deformed cotyledons) grew. Five of these eight succumbed to callus in the first stage. Of the three embryos that grew without callus (all from the pods that were taken only 13 days after pollination) two were transplanted to pots but soon died. Although the two most vigorous plantlets originated from the normal, 13-day-old pods, the original pod condition was not found to have a significant effect on the final growth score.

From the cross of AA1 x RR5, only four embryos from one normal, 14-day-old pod were cultured. Only two embryos grew; one to about 1 mm, and the other as callus.

Other unsuccessful attempts to obtain mature plants from this cross have been reported (Ahn, 1976; Ahn and Hartmann, 1978a, 1978c; Chen, 1980). Ahn (1976) cultured 11 embryos to obtain one etiolated seedling which died. Although the two seedlings in the present study were not etiolated, they died soon after being transplanted to pots. Since the difficulty encountered in the present work is different from that reported by Ahn (1976) and Ahn and Hartmann (1978a, 1978c), it is conceivable that other attempts using different parental lines might be successful. Indeed, two different *Phaseolus vulgaris* cultivars when crossed with *P. coccineus* produced two different developmental abnormalities in the F_1 which were able to be circumvented by using F_1 *P. vulgaris* hybrids or a third *P. vulgaris* cultivar as the maternal parent (Kedar and Bemis, 1960). Chen (1980) also found a significant difference for pod-set between cultivars of the maternal parent in the cross of *V. angularis* x *V. radiata*, although he did not find a significant difference in the time that pods remained on the plant. Nonetheless, it may be possible to obtain a combination of parental types that is successful.

V. mungo x V. angularis

By using various parental lines of these species, two different hybrid combinations were attempted. Two embryos were cultured from a 20-day-old pod of the cross MM3 x AA1, but neither embryo grew.

Four ovules were rescued from 14- and 17-day-old pods of the cross MM1 x AA2. All four embryos initiated growth and three were transferred to larger vessels within four weeks. Unfortunately, one was lost to contamination, one to excessive callus growth, and the other plantlet failed to expand new leaves.

Previous attempts to obtain mature plants of this cross by embryo culture (Ahn, 1976; Ahn and Hartmann, 1978c; Chen, 1980) were also unsuccessful. Those that grew died as seedlings. Poor root development was a common problem in the previous reports, but root development was normal in the present attempt. The chief problem encountered was poor leaf expansion. Chen (1980) was able to obtain one flowering plant from seed of this cross by using intraspecific hybrids as the parents. However, because of the low frequency of viable seeds, embryo culture techniques seem advantageous for producing this hybrid. One remedy for poor leaf expansion might be the incorporation of growth regulators into the medium. For example, Parthadev (1977) was unable to obtain leaf expansion in decotyledonized excised embryos of *V. radiata* without adenine, and GA_3 was found to enhance leaf expansion in *Phaseolus vulgaris* embryos excised 15-18 days after pollination (Skene, 1969). Another remedy might be to graft these hybrid shoots onto parental rootstocks, as Smith (1943) did to successfully overcome chlorophyll deficiencies in the shoots of a *Melilotus* hybrid. A suitable grafting technique for small embryos was developed by Blakeslee (1944) and used by McLean (1946) for overcoming root deficiencies in *Datura* hybrids.

V. mungo x V. radiata

From the cross of MM1 x RR1 one 12-day-old pod was harvested with four ovules. Two embryos were successfully excised. Only the larger embryo developed, attaining a shoot length of about 8 mm; but excessive callus prevented further normal development.

Dana (1966a) attempted to culture embryos from mature seeds of this interspecific cross, but to no avail. The results obtained from culture of immature embryos by Ahn and Hartman (Ahn, 1976; Ahn and Hartmann, 1978c) and Chen (1980) were similar to those reported here; that is, normal embryo development gave way to callus growth. Singh et al. (1964) reported that seed set for this cross was not as good as in selfed parents, but they did not mention whether or not any F_1 seeds reached maturity or were viable. Luyeye (1975) obtained sufficient seed from this cross to analyze dipeptides, but did not indicate that F_1 plants were grown. Gupta and Wagle (1978) have studied the biochemical composition of seeds of *Phaseolus mungoreous*, an amphidiploid of a cross between *V. mungo* and *V. radiata* obtained by pulse breeders at Haryana Agricultural University. They did not make clear which species served as the maternal parent. Whatever the actual status of the cross *V. mungo* x *V. radiata*, mature viable seeds and F_1 plants from the reciprocal cross have been obtained with apparently less difficulty (Ahn, 1976; Ahn and Hartmann, 1978c; Asian Vegetable Research and Development Center, 1974, 1975, 1979; Chen, 1980; Dana, 1966a; De and Krishnan, 1966; Sen and Gosh, 1960).

V. mungo x V. umbellata

By using various parental lines of these species, four different hybrid combinations were attempted. Four embryos were successfully excised from one normal, ten-day-old pod of the cross MM3 x UU1. One embryo (the smallest in all measurements) failed to grow; the other three grew but were lost to excessive callus growth.

The second cross was between MM1 and UU1. One ten-day-old pod provided one ovule. The shoot of the embryo grew to about 4 mm, but was overcome by excessive callus growth.

Five ovules were excised from ten- and 14-day-old pods of the cross

MM1 x UU2. Shoot growth of each embryo was limited to less than 10 mm and all five embryos succumbed to callus.

The fourth cross was between two intraspecific F_1 hybrids, MM31 and UU21. Two normal, green pods were harvested 19 days after pollination which provided nine successfully-excised embryos. All embryos grew, but with callus and/or other deformity. Three developed sufficiently for transfer, but were deformed by a "coiling" of the shoot. There was no expansion of trifoliolate leaves. These embryos from the interspecific cross of intraspecific F_1 parents were older and larger at plating than embryos from pure-line parents. Nonetheless, they did not develop adequately for potting.

Numerous attempts to obtain this hybrid have been reported (Ahn, 1976; Ahn and Hartmann, 1978c; Biswas and Dana, 1975a; Chen, 1980; Chowdhury and Chowdhury, 1977). These workers obtained only shrivelled seeds, so usually used embryo culture. Ahn and Hartmann and Chen cultured immature embryos (10-19 days after pollination) with results similar to those reported here; that is, the embryos produced callus or weak seedlings that died before flowering. Coiling of the shoot was also reported by Ahn and Hartmann. Biswas and Dana (1975a), on the contrary, obtained 12 mature F_1 plants by soaking mature but shrivelled seeds and then culturing the dissected embryonic axes with a sucrose-enriched nutrient solution. Biswas and Dana (1975a) and Chen (1980) found differences among varieties of _V. mungo_ in pod set and/or number of mature, albeit shrivelled, seeds obtained. Ahn and Hartmann (1978c), found no difference in pod set in two lines of _V. mungo_, but did observe a differential effect of _V. umbellata_ pollinators. In the present work, two embryos from the cross using intraspecific hybrids as parents grew to a more advanced stage than those from pure lines, but still not to maturity. Thus, the use of particular parental genotypes for this cross may be as important as in certain Phaseolus crosses (Mok et al., 1978).

The abnormal behavior of the embryos of this cross in vitro suggested that the embryos had an excess supply of auxin, as callus and irregular bending can be induced by auxin application (Rappaport et al., 1950; Solacolu and Constantinesco, 1936; Yeung and Sussex, 1979). By waiting until the ovules matured, Biswas and Dana (1975a) may have allowed the excess auxin, if indeed present, to be metabolized.

V. glabrescens x V. umbellata

From the cross of V. glabrescens x UU5, one 14-day-old pod was harvested. It was still green but beginning to shrivel. Seven plump ovules were excised. Supernumerary cotyledons were the rule; of the seven embryos, one had two extra cotyledons and four had one extra cotyledon. All cotyledons were left intact at plating and the number had no apparent effect on recovery of hybrid plants. All embryos progressed to the second stage within six weeks, and five of them even reached potting within that period. One more was eventually potted, but only five survived to maturity. The average time from plating to the greenhouse was 86 days.

Although this hybrid combination has been described previously (Dana, 1964, 1965a) its description will again be given since Dana's two different descriptions indicate that different parental genotypes have a considerable effect on the characteristics of the hybrid. The five hybrid plants obtained by embryo culture in the present study favored the maternal parent in broadly ovate leaf shape and entire leaf margins. The hybrid plants were intermediate between the parents for pubescence and growth habit. The greater degree of pubescence and the presence of trailing branches in the hybrid distinguished it from the maternal parent which was more glabrous in texture and upright in stature (Table 11, Figure 2). The hybrid plant was triploid (33 chromosomes) as determined by root tip squashes performed by G. R. Bauchan. Flowering was profuse, but the few pods that set abscised within a day or two.

Dana (1964, 1965a) successfully made this cross with two different accessions of V. umbellata. He, too, observed that pods began to shrivel two to three weeks after pollination, but he was able to obtain some shrunken, viable seeds from one of these crosses. Nonetheless, he obtained mature plants only by employing in vitro culture of immature embryos. He noted a lower percentage of recovery than that of the present study, and a considerable loss of plants after potting. This may have been due in part to the fact that he excised the embryos at a slightly greater age. Parental differences may also have been acting, as evidenced by the abnormalities he reports for one of his crosses (1965a). Dana reported low fertility for these hybrids (8 and 11% stainable pollen), but pollen stainability was increased to 76% and 70% respectively, by treating apices with colchicine (Dana, 1964; Biswas and Dana, 1976). This allowed Biswas and Dana to obtain a C_2 generation, but further progress is unknown.

V. glabrescens x V. radiata

The natural amphidiploid, V. glabrescens, was crossed with a cultivar (RR1) and an intraspecific hybrid (RR2 x RR1) of V. radiata. In the first of these crosses, 20 ovules were obtained from three pods

Table 11. Characteristics of *V. glabrescens* (VVGG), *V. umbellata* (UU5), and their interspecific hybrid (VGU5).

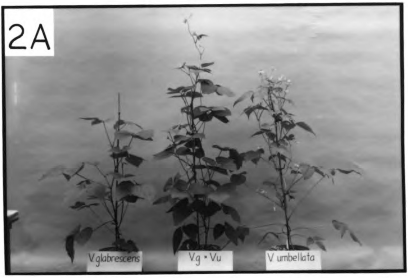
Characteristic	Maternal parent *V. glabrescens*	Hybrid	Paternal parent *V. umbellata*
Vegetative growth	indeterminate	indeterminate	indeterminate
Habit	erect with sub-erect branches	sub-erect with trailing branches	twining to trailing
Terminal bladelet shape	usually broadly ovate occasionally deltoid	broadly ovate	ovate to lanceolate
Terminal bladelet margin	entire	entire	usually entire occasionally 3-lobed
Leaf pubescence	slightly hairy	moderately hairy	very downy

Figure 2. *V. glabrescens* (VVGG), *V. umbellata* (UU5), and their F_1 interspecific hybrid (VGU5).

A. Left to right: Plants of *V. glabrescens* (VVGG), F_1 interspecific hybrid (VGU5), and *V. umbellata* (UU5).

B. Close-up of an older specimen of the F_1 hybrid (VGU5) showing the trailing branches.

2A



2B

harvested 11-15 days after pollination. The cotyledons of the five embryos from the oldest pod were deteriorated, so were detached without being measured. The cotyledons of the remaining 15 embryos appeared normal. One or both cotyledons were removed from 13 of these embryos (Table 2). All of the embryos grew vigorously to the transfer stage within six weeks, but growth was not entirely normal. Sixteen of the plantlets had some degree of callus, and 11 had some degree of deformity such as coiling of the shoot and growths on the stem and cotyledons. Certain growths on the cotyledons appeared bud-like, but attempts to regenerate them on shoot-inducing media S1, S2, S3, and S4 (Table 5), failed. One plantlet had three first-foliar leaves. In addition, epicotyls of most of the embryos grew into abnormally long, spindly shoots primarily in the internode above the first foliar leaves. The first foliar leaves were well expanded and green, but the first trifoliolate leaves often remained rudimentary or even concealed by the stipules for an extended period. The cool white light regime (environment H, Table 4) was changed to include one half warm white fluorescent (envrionment J) because more red light might alleviate the problem (Borthwick and Hendricks, 1960), but elongation continued and the spindly and fragile internodes began to break. Axillary shoots also began to grow in a similar manner. The shoot tips were excised (about 10 mm in length) and the cut end of each was pushed about 2-4 mm into new D4 medium. Eighteen primary and axillary cuttings were taken. These cuttings often continued to elongate such that excision of the tip was required as many as three more times. Eventually ten of the cuttings (from eight embryos) assumed a more normal growth pattern, rooted, and were transplanted to pots. Nine of these were transplanted to the greenhouse over a period of 90-285 days from the original plating of the embryos.

It has already been stated that no effect of cotyledon removal was detected for any of the hybrids. Nonetheless, a word about cotyledon removal seems appropriate here. Cotyledon removal has been reported to be helpful for hybrid embryos that have begun to deteriorate (Skirm, 1942). It has also been suggested (P. D. Ascher, personal communication) that cotyledons of some species contain inhibitors and that removal is advantageous for immature embryos _in_ _vitro_. In the present work, however, cotyledons were removed from all the embryos that were obviously deteriorated so that no control group existed. Within the group of embryos that had not begun to show deterioration and were otherwise treated alike, no significant difference in the ultimate rescue of plantlets was detected among cotyledon treatments.

In the second cross of these species, using an intraspecific F_1 _V. radiata_ pollen parent (RR2 x RR1), seven ovules were obtained from a 14-day-old pod. Cotyledons were normal in appearance. Two of the embryos grew in a very deformed manner and the rest had minor deformities and/or callus growth. Nonetheless, six embryos (the largest six for all measurements) were transferred to larger vessels. All of these displayed abnormal elongation. The cutting procedure was applied to six primary and one axillary shoot tips. One of these was lost to contamination and one failed to root, but five (originally from five different embryos) were transplanted and reached the greenhouse in an average of 114 days from initial embryo excision.

In the greenhouse, hybrid plants from these two crosses were phenotypically very similar. They were strongly matroclinous for indeterminate growth, but slightly less so for the bright yellow color of the standard. Leaves of the hybrid plants appeared slightly narrower than those of either parent. For other traits, the hybrid plants were intermediate between the parents. The intermediate characters showing the greatest influence of the paternal parent were the leaf and stem pubescence, the greyish-green cast of the keel, and the extension of the keel beyond the wing petal (Table 12, Figure 3). Root tip squashes performed by G. R. Bauchan revealed a somatic chromosomal number of 33. Although flowering was profuse, these hybrids failed to retain pods for more than a day or two.

Some particularly interesting features were observed during the development of this hybrid. Pods of this cross frequently reached maturity with no external signs of degeneration. Dana (1965b) also obtained mature pods with well-developed seeds, but these contained only shrivelled, non-viable embryos. During the course of the present investigation, it was found that the axes of embryos excised 15 or more days after pollination appeared to have elongated and pushed the plumule of the embryo into the seed coat. The lack of mature viable seeds in this cross appeared to be more a case of abnormal growth than arrested development. Even _in vitro_, abnormal excessive development was the problem. Embryos _in vitro_ demonstrated high (100%) viability, but the internode just above the first foliar leaves continued to elongate. Other difficulties included callus and growths on the stem and cotyledons, coiling of the shoot, and slow development or suppressed initiation of the first trifoliolate leaves. These observations suggested a hormonal imbalance in the embryo and young plantlet, possibly an over-supply of gibberellin and/or auxin (Cionini et al., 1976; Mok et

Table 12. Characteristics of *V. glabrescens* (VVGG), *V. radiata* 'Tainan # 1' (RR1), and their interspecific hybrid (VGR1).

Characteristic	Maternal parent *V. glabrescens*	Hybrid	Paternal parent *V. radiata*
Vegetative growth	indeterminate	indeterminate	determinate
Terminal bladelet shape	usually broadly ovate occasionally deltoid	ovate to broadly ovate	deltoid to broadly ovate
Leaf pubescence	slightly hairy	moderately hairy	very hairy
Main color of standard	bright yellow	bright yellow	pale yellow
Keel color	yellow with slight green	yellow with green-grey	yellow with green-gray
Keel extension (in mm)^z	1.7 ± 0.33	2.2 ± 0.17	4.2 ± 0.17

^{z}Each mean and its $sd_{\bar{x}}$ based on 3 flowers (from 4 P_1 plants, 4 P_2 plants, and 4 cuttings from the F_1 generation).

Figure 3. *V. glabrescens* (VVGG), *V. radiata* (RR1), and their F_1 interspecific hybrid (VGR1).

A. Left to right: Plants of *V. glabrescens* (VVGG), F_1 interspecific hybrid (VGR1), and *V. radiata* (RR1).

B. Left to right: Flowers in the same order. Note the extension of the keel beyond the wing petal.


3A
V.glabrescens
V.g × V.r
V. radiata


3B
4
5
6
7
8
9
10
11
V. g
V. g x V. r
V. r

al., 1978; Murashige, 1961; Rappaport et al., 1950; Solacolu and Constantinesco, 1936). If excess gibberellin is the cause of these phenomena, application to the maternal parent of an inhibitor of gibberellin biosynthesis such as AMO-1618 that can be translocated into developing seeds (Marth and Mitchell, 1961) may allow recovery of this hybrid without the need of embryo culture. If excess auxin is the problem, it may be possible to facilitate this cross by use of an auxin antagonist _in vitro_ (Newcomb and Wetherell, 1970).

V. radiata x V. glabrescens

An intraspecific F_1 of _V. radiata_ (RR2 x RR1) was also used as the maternal parent in a cross with _V. glabrescens_. Ten ovules were obtained from a pod harvested 14 days after pollination. The embryos appeared "calcified"; that is, more dry and opaque than other embryos. Two embryos failed to grow, and the other eight grew as mostly disorganized callus. At five weeks, one shoot tip was excised from the embryo obtained from the largest ovule. It rooted, developed normally, and was transplanted to the greenhouse in 83 days. The other embryos, all heavily callused, were transferred to S1, S2, or S3 medium and moved to continuous light (environment K). The calli were subdivided occasionally (with any brown callus being scraped off at that time) and transferred to fresh medium. After 80 days on the S2 medium, one of the pieces (from an embryo above the median in all measurements) began to produce shoots. Ten of these were excised over the next six weeks, moved to D4 medium, and back to a diurnal light regime (environment J, then H). Nine of these shoots developed into plantlets for potting, but only eight were potted. Six of these were transplanted to the greenhouse after an average of 226 days from initial culture.

Mature hybrid plants of this cross were similar to the *V. glabrescens* x *V. radiata* plants already described. Average pollen stainability was 11% (mean based on 100 grains from each of six flowers) and the hybrid plants failed to set pods.

Other workers have obtained mature viable seeds from this cross, but at low frequency. Chen (unpublished data) reared one hybrid plant to flowering from a mature seed. It was phenotypically similar to the hybrid plants obtained in the present study. Dana (1965b) obtained 22 shrunken seeds from 250 pollination attempts, but only one mature hybrid plant which died soon after flowering. Using an autotetraploid of *V. radiata*, Krishnan and De (1968) obtained a number of seeds, but only one mature F_1 plant. They successfully backcrossed that hybrid to *V. glabrescens* and to the *V. radiata* autotetraploid, obtaining a few viable seeds and backcross plants in each case.

The observations that most of the hybrid embryos in the present work grew as callus, and that shoots were obtained from one callused embryo after transfer to a medium containing cytokinin, suggested that the endogenous auxin level may have been supra-optimal or that cytokinin was sub-optimal in the hybrid embryos. Nesling and Morris (1979) suggested that reduced levels of cytokinin in ovules of interspecific *Phaseolus* hybrids may be causally related to abortion of the hybrid embryos. The addition of cytokinin to the medium in the present study may have optimized the auxin-cytokinin balance to allow formation of shoots (Skoog and Miller, 1957).

V. glabrescens x V. mungo

From the cross of *V. glabrescens* x MM1, four normal pods, 15 days after pollination, provided 11 ovules. Growth was weak and spindly in one embryo, but proceeded normally in the other ten which reached the transfer stage within three weeks. Two of these plantlets, transferred to a possibly-defective medium (dD4), succumbed. Of the eight embryos transferred in the standard manner, one ceased growth, another produced a spindly shoot, and six were aseptically potted. A total of five plants were transplanted to the greenhouse in an average of 129 days from plating.

Hybridity was obvious because of the wide divergence of the parents. The hybrid plants were less erect than either of the parents, favored the maternal parent in indeterminate growth and leaf pubescence, were like the paternal parent in that some of the leaflets were acuminate, and intermediate for other phenotypic characters (Table 13, Figure 4). In addition to the gross differences in phenotypes, the difference between hybrid and maternal plants was significant for the leaf characters of length, width and l/w ratio (Table 13). The hybrid plants flowered profusely, but pollen stainability was only 0.3%, compared to more than 95% for the parents. The few pods that were set abscised within three or four days of flowering.

Dana (1968) attempted the reciprocal cross of these two species and obtained partially filled but viable seeds. Eight of these plants flowered, but there was no pod set and average pollen stainability was only 0.8%. After treatment of shoots with colchicine, however, Dana obtained a few viable seeds. These progeny reached flowering and had 53% pollen stainability.

Table 13. Characteristics of *V. glabrescens* (VVGG), *V. mungo* 'T-9' (MM1), and their interspecific hybrid (VGM1).

Characteristic	Maternal parent *V. glabrescens*	Hybrid	Paternal parent *V. mungo*
Vegetative growth	indeterminate	indeterminate	determinate
Habit	erect with sub-erect branches	sub-erect to trailing or twining	erect
Terminal bladelet[z]			
length (in mm)	132 ± 2.6	84 ± 2.8	69 ± 4.0
width (in mm)	102 ± 2.7	54 ± 2.5	34 ± 0.5
length/width ratio	1.3 ± 0.00	1.6 ± 0.05	2.0 ± 0.10
Leaf pubescence	slightly hairy	slightly hairy	slightly to moderately hairy
Pollen stainability (%)[y]	97.3	0.3	95.3

[z] Each mean and its $sd_{\bar{x}}$ based on leaves (4 from P_1, 2 from P_2, 4 from F_1) taken from the central portion of each plant after flowering had commenced. Two individuals of each genotype were measured, and no 2 individuals of the same genotype were significantly ($p = .05$) different from each other for any leaf characters. Both hybrid plants checked were significantly different from maternal plants for these characters. The F_1 values reported are those of the plant that was least different from the maternal species for all 3 characters based on $\Sigma t.val_{cal} - t.val_{tab}$.

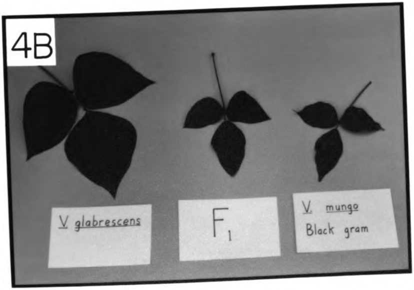
[y] Each mean is based on a count of 100 grains from each of 3 flowers.

Figure 4. *V. glabrescens* (VVGG), *V. mungo* (MM1), and their F_1 interspecific hybrid (VGM1).

A. Left to right: Plants of *V. glabrescens* (VVGG), F_1 interspecific hybrid (VGM1), and *V. mungo* (MM1).

B. Left to right: Leaves in the same order.


4A
V. glabrescens
Vg × Vm
V. mungo


4B
V. glabrescens
F_1
V. mungo
Black gram

(V. radiata x V. umbellata) amphidiploid x V. umbellata

The amphidiploid, RRUU62, and the diploid, UU1, were hybridized. Thirty-three embryos were obtained from seven abscised pods 11-17 days after pollination. Cotyledons of these embryos were poorly developed. Twenty-one embryos initiated growth, but seven succumbed to callus and/or deformed growth, and the remainder ceased growth before the shoot exceeded 5 mm in length.

This backcross would lead to introgression of V. radiata genes into V. umbellata, the less cultivated of the two species. Nonetheless, since V. umbellata is a crop plant in China and India and because it produces fertile hybrids with V. angularis (and thus is a potential bridge from V. radiata to V. angularis), introgression of V. radiata genes into V. umbellata could be valuable.

The single most important barrier observed in embryo culture of this hybrid was failure of the embryos to develop beyond a few millimeters in length. These embryos were very small at excision, and van Overbeek et al. (1942) suggested that very young embryos may be unable to synthesize an adequate amount of some endogenous growth regulator(s) or other substance(s) to maintain growth. They found that progressively younger Datura embryos required progressively more complex media to grow to transplantable seedlings. Went (1954) suggested that factors needed for growth and differentiation might diffuse into the medium and away from the embryo so that an inadequacy develops even though the embryo might be able to produce sufficient quantities. Skene (1969) found that development of immature embryos (15-18 days after pollination) of Phaseolus vulgaris 'Hawkesbury Wonder' was restricted unless GA_3 was supplied in the medium. Although many hybrids were obtained in the present investigation without the use of GA_3, this particular hybrid may benefit from its presence in the medium.

(V. radiata x V. umbellata) amphidiploid x V. radiata

Three different amphidiploid x V. radiata combinations were attempted. From the cross of RRUU62 x RR1, sixty-one ovules from 11 pods that had abscised 11-16 days after pollination were prepared for embryo culture. Minor abnormalities were noted, such as two embryos that had three cotyledons and 16 embryos that had only one cotyledon. Growth commenced in 44 embryos, but soon ceased. Four embryos were prematurely transferred to fresh media (dF1, dF2, dF3, or dF4) in larger vessels in an effort to overcome the barrier, but to no avail. Eventually some of the 40 remaining embryos resumed growth and 12 reached the transfer stage. Nine of these were transferred to fresh media (D5, dF1, dF2, dF3, or dF4) in larger vessels, and the other three were transferred to larger vessels without renewing the media. Four were eventually transplanted to pots, one was lost to mechanical error, and seven ceased growth a second time and eventually died, possibly due in part to the defective media. The first of the plantlets to be transplanted to a pot died, so the other three were potted aseptically, and survived to flowering. Average time from initial plating to establishment in the greenhouse was 153 days.

RR2 was also used to pollinate RRUU62. Four abscised pods provided 18 ovules. Cotyledon abnormalities were again noted, both in number and in size. (Two embryos had only one cotyledon and a third had three; the cotyledons of embryos that had two cotyledons were often very unequal in size.) All 18 embryos began to grow, but most failed to develop roots. About one third of the embryos exhibited a slight degree of callus and/or deformed growth. Four embryos were transferred prematurely to new medium (dF1, dF2, dF3, and dF4) in larger vessels and succumbed. Only two of the 14 remaining embryos reached the necessary size for first transfer. These were eventually transplanted to the greenhouse in an average of 128 days from initial plating.

A second amphidiploid, RRUU87, was crossed with RR1. Abscised pods 8-14 days after pollination provided 22 small, but normal-appearing embryos for culture. Of the 19 embryos that grew, two became slightly deformed and none attained more than 3 mm of shoot length.

The five hybrid progeny of RRUU62 that reached the greenhouse had either RR1 or RR2 as the paternal parent. The two groups of hybrid plants were phenotypically similar to each other and were intermediate for nearly every character in which the parents were different (Table 14, Figure 5). The growth habit of the hybrid was more similar to that of the maternal parent, but the leaf and pod pubescence and the floral color and structure of the hybrid showed the influence of the paternal parent. The leaves and pods were more coarsely pubescent in the hybrid than in the maternal parent. The green and grey coloration of the keel, as well as the keel's extension beyond the wing petal, was greater in the hybrid than in the maternal parent. The hybrid was triploid (33 chromosomes) as determined by root tip squashes performed by M. Machado.

After about a month of flowering, these backcross plants began to set pods, both by natural self-pollination and by backcrossing again to _V. radiata_. Many of these pods, especially the first ones to set, abscised within two weeks but did provide some embryos for culture. Some of the pods that set later reached maturity and produced viable seeds.

The successful back-crossing of the amphidiploid only to its

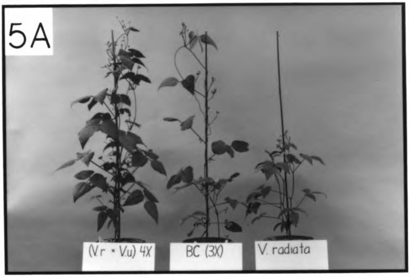
Table 14. Characteristics of [(*V. radiata* x *V. umbellata*) amphidiploid] (RRUU62), *V. radiata* 'Tainan #1' (RR1), and their backcross hybrid (RUR621).

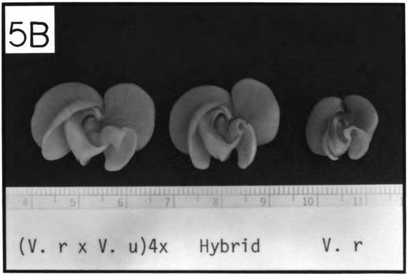
Characteristic	Maternal parent amphidiploid RRUU62	Hybrid	Paternal parent *V. radiata*
Vegetative growth	somewhat indeterminate	somewhat indeterminate	determinate
Habit	moderately twining	moderately twining	erect
Leaf pubescence	downy to very hairy	very hairy	very hairy
Main color of standard	bright yellow	dull to nearly bright yellow	pale yellow
Keel color	yellow with moderate green	yellow with green-grey	yellow with green-grey
Keel extension (in mm)[z]	2.7 ± 0.17	3.7 ± 0.17	4.7 ± 0.17
Pollen stainability (%)[y]	41.3	20.6	97.4
Pod pubescence	slightly hairy	moderately to very hairy	very hairy

[z] Each mean and its $sd_{\bar{x}}$ based on 3 flowers (from 4 P_1 plants, 4 P_2 plants, and 4 cuttings from the backcross generation).

[y] Each mean is based on a count of 100 pollen grains from each of 7 flowers.

Figure 5. [(*V. radiata* x *V. umbellata*) amphidiploid] (RRUU62), *V. radiata* (RR1), and their backcross hybrid (RUR621).
A. Left to right: Plants of [(*V. radiata* x *V. umbellata*) amphidiploid] (RRUU62), the backcross hybrid (RUR621), and *V. radiata* (RR1).
B. Left to right: Flowers in the same order. Note the extension of the keel beyond the wing petal.


5A
(V.r × V.u) 4X
BC (3X)
V. radiata


5B
4
5
6
7
8
9
10
11
(V. r x V. u)4x
Hybrid
V. r

maternal parent species suggested a possible cytoplasmic-genic interaction. The cytoplasm of the amphidiploid was that of *V. radiata*. The triploid obtained by backcrossing this amphidiploid to *V. radiata*, in which two haploid genomes of *V. radiata* and one haploid genome of *V. umbellata* are present in *V. radiata* cytoplasm, was successful. The backcross to *V. umbellata*, in which two haploid genomes of *V. umbellata* and only one haploid genome of *V. radiata* would be present in *V. radiata* cytoplasm, was not successful. Braak and Kooistra (1975) obtained similar results from backcrosses of their *Phaseolus vulgaris* x *P. ritensis* amphidiploid to the two parental species. Nonetheless, the backcross to *V. umbellata* might be attainable by employing supplemental techniques, such as addition of GA to the culture medium as discussed in the previous section.

V. radiata x (V. radiata x V. umbellata) amphidiploid

One pod was harvested 14 days after pollination from the cross RR1 x RRUU87 providing nine ovules for excision. The three smallest ovules appeared deteriorated as evidence by an overall brownish cast and a reddish hilum. The cotyledons were partially fused together in each embryo. No embryo growth was observed.

Relatively few embryos of this cross were plated and all were from the same pod, so the cause of germination failure cannot be ascertained. Since some of the ovules appeared deteriorated, it is possible that the embryos had already atrophied *in vivo* prior to excision. Taira and Larter (1978) reported that wheat x rye embryos older than 14 days after pollination rapidly atrophied *in vivo* and responded poorly *in vitro*.

(V. radiata x V. umbellata) amphidiploid x V. radiata, BC2

It was mentioned in a preceding section that the allotriploid individuals produced by backcrossing RRUU62 to RR1 were backcrossed again to *V. radiata* and that the first of these pods to set abscised before maturity. Therefore, embryo culture was employed until these allotriploids began to produce viable seeds. From a cross using RR21 as the paternal parent, two embryos were excised from one abscised 19-day-old pod. Neither embryo developed *in vitro*.

When RR1 was used as the paternal parent, many pods that set began to abscise nine to 14 days after pollination. Fifty-four embryos were obtained from 19 of these pods. The cotyledons of one embryo appeared fused together, and another embryo had only one cotyledon. About one third of the 26 embryos that grew had some degree of callus or deformity, two were slightly spindly, and the rest were normal. Seven developed sufficiently (in an averge of 22 days) to permit transfer to larger vessels. Two of the seven plantlets were placed on dD1 medium and succumbed. Two of the other five that were transferred reached the potting stage, but only one survived to flowering. Ninety-four days elapsed between initial plating for embryo culture and transplanting to the greenhouse.

In the greenhouse, phenotypic characters of *V. umbellata* (brighter yellow flowers and more indeterminate growth) were still evident in this second-backcross-generation plant. Flowers were abundant, but pods, if set, abscised within a couple of days.

A range in morphological characters and fertility levels were observed in plants that were derived via viable seeds from backcrosses and self-pollination of the same allotriploid (Chen, 1980). M. Machado performed root tip squashes on a number of these plants and found them to be 2n=2x=22 and 2n=2x+1=23 for chromosomal numbers.

The in vitro recovery rate of these embryos from the triploid was very low. It is likely that many of the embryos in the abscising pods were aneuploids of various sorts, and less vigorous due to chromosomal imbalances.

(V. radiata x V. umbellata) amphidiploid x V. glabrescens

An F_1 hybrid plant between the two different V. radiata x V. umbellata amphidiploids, RRUU62 x RRUU87, was hybridized with V. glabrescens. Pods that set began to shrivel about 15 days after pollination, so one pod was harvested at 13 days which yielded nine ovules. The embryos appeared normal except that the two cotyledons of each embryo seemed to be partially fused together. All nine embryos grew normally at first, but two turned chlorotic so that only seven were transferred to larger jars. Five were transplanted to pots and then to the greenhouse. The average time of this culture period was 127 days.

The five hybrid plants grew vigorously in the greenhouse and were intermediate between their parents in general appearance (Figure 6). However, the five plants were phenotypically different from each other. Greater detail is given for one of them in Table 15. This slight variation among individual hybrid progeny can be explained by the fact that the maternal parent was an F_1 between two different RRUU amphidiploids and was heterozygous. One of the hybrid plants exhibited an anomaly in the growth of some of its shoots, resulting in a twisted and sometimes a "sheared-looking" stem. It appeared to be caused by a partial obstruction of the growing point by the petiole of the

Table 15. Characteristics of [(*V. radiata* x *V. umbellata*) amphidiploid] (RRUU6827), *V. glabrescens* (VVGG), and their interspecific hybrid (RUVG68-27).

Characteristic	Maternal parent (RRUU62 x RRUU87)[z]	Hybrid[y]	Paternal parent *V. glabrescens*
Leaf pubescence	moderate	intermediate	slight
Keel extension (in mm)[x]	2.3 ± 0.17	1.3 ± 0.33	0.5 ± 0.00
Pollen stainability (%)[w]	58.0	plant #1: 10.5 plant #2: 6.0	90.5

[z] The F_1 between these two (*V. radiata* x *V. umbellata*) amphidiploids served as the maternal parent. Description given for the maternal parent is that of the F_2 generation, except keel extension which is that of the F_1 generation.

[y] Data are for hybrid plant #1 only except for pollen stainability. Data from other hybrid plants were not recorded.

[x] Each mean and its $sd_{\bar{x}}$ based on 3 flowers (from 4 P_1 plants, 4 P_2 plants, and one interspecific hybrid plant).

[w] Each mean is based on a count of 100 pollen grains from each of 2 flowers.

Figure 6. [(*V.* *radiata* x *V.* *umbellata*) amphidiploid] (RRUU6827), *V.* *glabrescens* (VVGG), and their F_1 interspecific hybrid (RUVG*68*-*27*).


6
(V.r × V.u) 4X
[(V.r × V.u) 4X] × V.g
V. glabrescens

newest leaf bending back over the shoot tip. It was not a serious problem, however, as the shoot tip usually out-grew the condition after a few nodes. Two of the hybrids were placed under short day conditions and flowered, but failed to set pods. Pollen stainability was 10% and 6% for these two plants. Pollen stainabilities for the paternal parent and the F_2 progeny of the maternal parent were 90% and 58% respectively. Dana (1964) found that one genome of V. glabrescens was homologous with the genome of V. umbellata. Although a cytogenetic analysis of the present hybrid plants was not performed, the low fertility suggested that the other genome of V. glabrescens is not homologous with that of V. radiata, unless the low fertility is due to genic rather than chromosomal factors. Krishnan and De (1968) found a maximum of eight V. radiata chromosomes homologous with those of V. glabrescens.

V. radiata x (V. umbellata x V. angularis)

The intraspecific F_1 hybrid of RR1 x RR5 was crossed with the F_1 interspecific hybrid of UU3 x AA1. Five ovules were obtained from one 17-day-old abscised pod and one 16-day-old pod near abscission. Four of the embryos grew. One produced a shoot of a few millimeters and then ceased growth. One grew normally, but as the root system deteriorated, the shoot tip was rescued and rooted. The other two grew spindly shoots, so shoot tip cuttings were taken of these also. All 3 shoot tips rooted and were transferred to larger vessels, and one plantlet was transplanted to a pot; however, all plantlets were weak and eventually succumbed.

This three-species cross was attempted to overcome the incompatibility between V. radiata and V. angularis, and to possibly (remotely) produce a fertile diploid incorporating a genome of V. radiata and some chromosomes of *V. umbellata*. Chen (1980) cited previous successful attempts to overcome interspecific incompatibility by use of third-species bridges; Chen himself successfully used *V. radiata* var. *sublobata* as a bridge between *V. radiata* var. *radiata* and *V. umbellata*, obtaining 25 to 45 times more viable seeds per 100 attempts than by crossing *V. radiata* var. *radiata* x *V. umbellata* directly. In the cross of (*V. umbellata* x *V. angularis*) x *V. radiata*, he obtained pod set but no viable seed. The reciprocal of that cross, undertaken here, provided some embryos for culture but none reached maturity. The few embryos cultured *in vitro* indicated a general rather than any common, specific weakness. Perhaps a greater number of embryos from the same cross, or the use of different parental cultivars would allow successful recovery of mature plants.

(V. umbellata x V. angularis) x V. mungo

The F_1 interspecific hybrid UA31 was crossed with MM3. One attached 15-day-old pod and one abscised 14-day-old pod provided seven ovules. Three embryos failed to grow and the other four ceased after attaining a few millimeters of shoot growth. Two had expanded the first foliar leaves, but none developed roots. All embryos that grew had a slight degree of callus.

Crossing either *V. umbellata* or *V. angularis* in any combination with *V. mungo* appears difficult as indicated by the number of unsuccessful reports. Many attempts to produce these combinations (Ahn, 1976; Ahn and Hartmann, 1978c; Biswas and Dana, 1975a; Chen, 1980) have yielded only one mature plant from *V. mungo* x *V. angularis* (Chen, 1980) and 12 individual plants of *V. mungo* x *V. umbellata* (Biswas and Dana, 1975a).

As discussed in the previous section, a three-species cross was considered worthwhile. Although some viable embryos were obtained for culture, none reached maturity. The problems encountered *in vitro* with the present cross are similar to those that Skene (1969) observed and was able to remedy with 10^{-4}M GA_3.

General Considerations and Summary

The first step in the procedure of embryo culture is the selection of material. One is naturally inclined to allow the interspecific embryo to reach its maximum normal development *in vivo*, yet care must be taken to rescue it before the onset of atrophy (Taira and Larter, 1978). Although experiments were not performed during the present study to determine the optimum age for embryo excision, some observations were made that illustrate the relationship between age or embryo condition and ultimate recovery, and these could be helpful to future interspecific hybridization programs.

First of all, it was observed that some hybrid combinations (such as VVGG x RR and RRUU x VVGG) failed at a particular point in time after pollination, and could, therefore, be harvested just before the onset of atrophy according to a calendar schedule. Other crosses (such as RR x UU, RRUU x UU, RRUU x RR, and RUR x RR) had a broader range of time over which the pods began to abscise or show other signs of failure. Frequently, embryos were successfully rescued and grown into mature plants if secured the day the pod abscised or began to show signs of degeneration. However, this criterion of visible pod degeneration was not always a reliable indicator of embryo condition. This was most evident in the cross *V. glabrescens* x *V. radiata* in which pods reached maturity with no external signs of degeneration although the embryos began to decline about 15 days after pollination, as could be seen immediately upon embryo excision.

Although embryos of many ages were grown in culture, due in large part to confounding with other factors, specific age effects in vitro were generally not clear. As has already been mentioned, 14- to 16-day-old embryos of RRUU62 x UU1 outperformed those of 11 and 17 days, but this difference might have been due to media differences. In the backcross of RUR621 x RR1, the 12-day-old embryos did not perform as well as those only nine days old, but no other age effects for nine- through 14-day-old embryos of this cross were apparent.

There seemed to be a difference among pods in that the percentage of germination of the embryos from five pods was higher that that of two other pods in the cross RRUU62 x RR1. One of these pods whose embryos performed poorly was older than the others (16 days after pollination vs 11 to 15) suggesting that age might be responsible for part of this pod effect. However, the other pod whose embryos performed poorly abscised 12 days after pollination, while embryos from both 11- and 13-day-old pods performed quite well. So this pod effect, if real, might have been due to conditions of the maternal plants in the greenhouse.

In the cross of RUR621 x RR1, a number of ovules were beginning to shrivel at the time of excision. Not surprisingly, the performance of embryos from plump ovules appeared better than that of embryos from shrivelled ovules (.84 vs .14 on the 1 to 4 scale).

There appeared to be a positive linear correlation (r=.67) between ovule size and recovery of embryos from the cross of RR x VVGG. It is interesting to note that the ten embryos plated were from the same pod, and represented quite a range in development, as judged by ovule size. (It was not noted whether placement in the pod was correlated with ovule size.) The embryo that grew to maturity only after having been placed on cytokinin-containing medium was from the second largest ovule (actually tied with three others for this rank) and the embryo that grew to maturity without exogenous cytokinin was from the largest ovule. Perhaps the ovules on the next step up in size are those few that are capable of going on to develop into viable seeds.

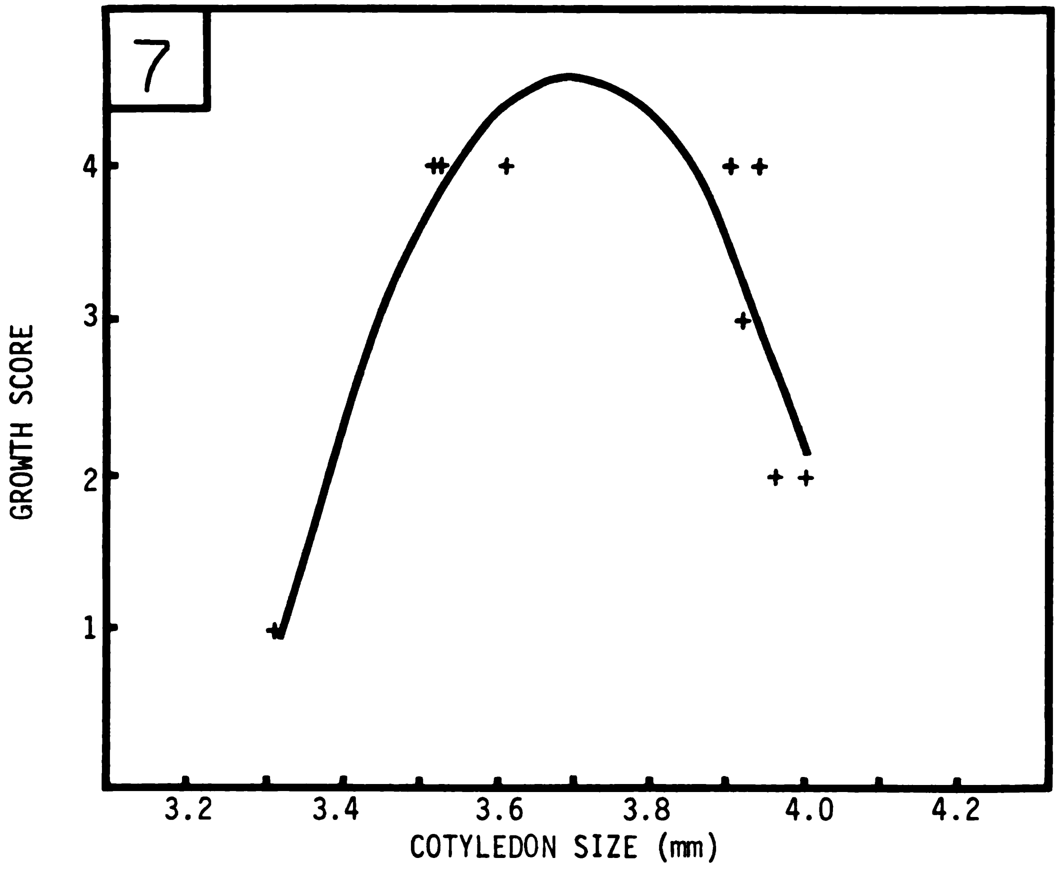
Size factors of the embryos themselves also seemed to be linearly correlated with recovery in the cross of AA1 x RR1. The correlation coefficient between cotyledon size and the growth score obtained by the embryo _in vitro_ was .95 and the correlation between the total embryo size (cotyledon + hypocotyl) and growth score was .89. However, some factor other than age must have been responsible for the degree of development obtained _in vivo_ since no linear correlation between age and these two size factors was apparent.

In two other hybrid combinations, embryo size factors and recovery of the embryos _in vitro_ appeared curvilinearly related. In the cross of VVGG x MM1, the coefficient of determination was .81 for the quadratic regression of final score on cotyledon size (Figure 7). The coefficient of determination was .59 for the quadratic and .92 for the cubic regressions of final embryo score on total embryo size (cotyledon + hypocotyl) for the cross RRUU x VVGG (Figure 8). The existence of a peak or optimum size for recovery of these two hybrid combinations suggests that time of excision is critical -- too soon and the embryos are insufficiently developed to survive -- too late and the embryos, though larger, have begun to atrophy.

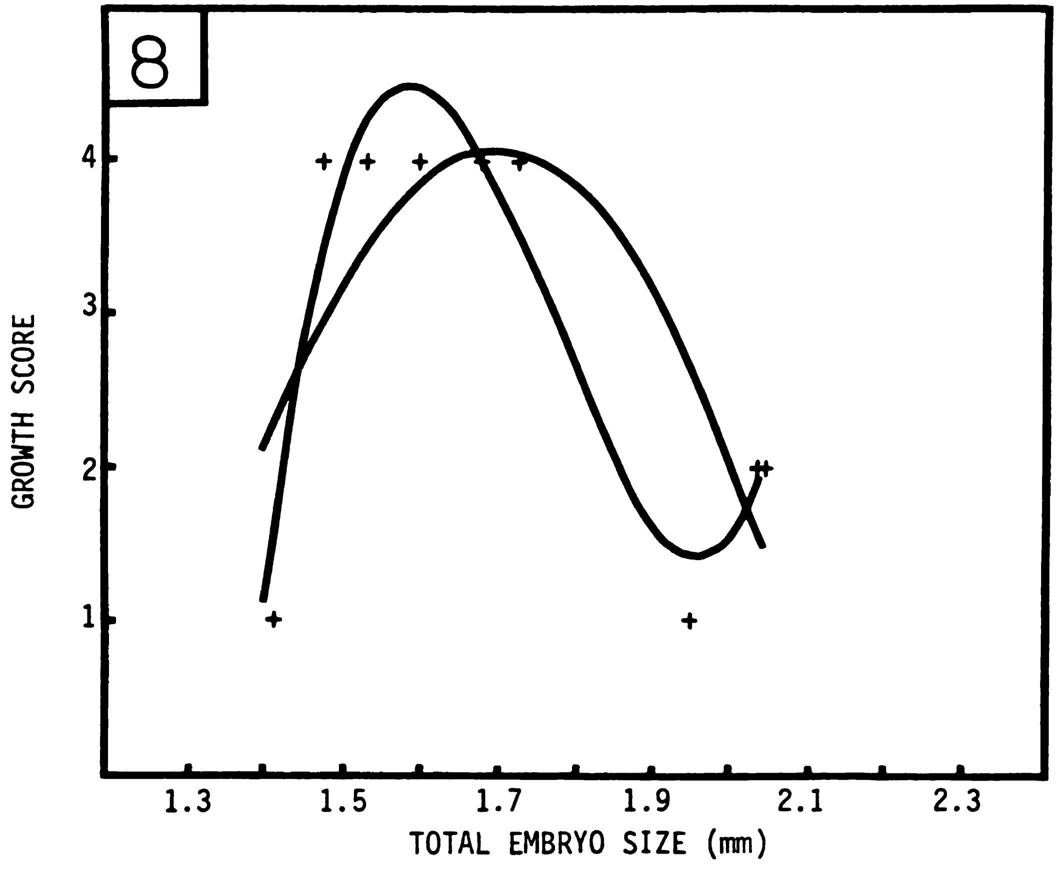
Figure 7. Relationship between cotyledon size and recovery *in vitro* of hybrid embryos of *V. glabrescens* x *V. mungo* ($Y = -351.19 + 192.61X - 26.07X^2$; $r^2=0.81$)

Figure 8. Relationship between total embryo size and recovery *in vitro* of hybrid embryos of (*V. radiata* x *V. umbellata*) amphidiploid x *V. glabrescens* (Quadratic: $Y = -59.53 + 74.97X - 22.10X^2$; $r^2=0.59$. Cubic: $Y = -677.28 + 1175.70X - 669.89X^2 + 125.87X^3$; $r^2=0.92$).

7


GROWTH SCORE
1
2
3
4
3.2
3.4
3.6
3.8
4.0
4.2
COTYLEDON SIZE (mm)

8


GROWTH SCORE
1
2
3
4
1.3
1.5
1.7
1.9
2.1
2.3
TOTAL EMBRYO SIZE (mm)

Knowledge of these relationships observed between ovule or embryo condition (plumpness and size) and ultimate recovery *in vitro* are really not very useful in an interspecific breeding program where few embryos are obtainable, unless a non-destructive *in vivo* measure to determine ovule and embryo condition exists. While age is a useful measure in determining excision time for hybrids that have a predictable growth rate or a particular time of breakdown, another monitor would be useful for those hybrids that do not have either of these characteristics. Although Savithri et al. (1978) did not mention whether or not there was a significant correlation between pod diameter and seed fresh weight in their study of *V. radiata* development, their figures indicate that development of these two characters are nearly parallel; thus, measurement of pod diameter might prove to be a good, non-destructive monitoring technique to help determine the optimum time for excision of hybrid embryos.

The presence or absence of the suspensor was not often found to have an effect on recovery. In the cross of RUR621 x RR1, however, embryos with suspensor intact appeared to outperform those without suspensors (1.2 vs 0.5). On the other hand, the suspensor-deprived embryos from the ten-day-old pod of the cross AA2 x UU2 seemed to outperform those with suspensor intact (0.83 vs 0.0); this relationship did not hold for AA2 x UU2 embryos overall (both ten- and 11-day-old embryos). Although these results appear somewhat inconsistent, and the observed inhibitory effect of the suspensor seems to be in conflict with other work (Cionini et al., 1976; Yeung and Sussex, 1979); the fact that some inhibitors are indeed present in young bean suspensors (Alpi et al., 1975) offers a possible explanation. It may be that the suspensors of AA2 x UU2 produced inhibitors (or transported them from maternal tissues) in the early stages of *in vivo* development, which would normally have been deactivated a little later *in vivo*. Suspensors of embryos excised at ten days may have been deprived of the opportunity to deactivate this hypothetical inhibitor while the suspensors of embryos excised at 11 days did have the opportunity to deactivate it. A study of the possible role of these inhibitors in interspecific hybrids might be profitable.

Embryos of a few of the hybrid combinations were cultured *in vitro* and established in the greenhouse without any particular difficulties. More often, however, each hybrid combination exhibited one or more characteristic problems *in vitro* that made the recovery procedure more difficult, decreased the number of plantlets that might otherwise have been obtained, or entirely prevented successful recovery. The most commonly encountered difficulties included low percentage of germination, cessation of growth shortly after its commencement, deformity or callus, difficulty in initiation or expansion of leaves, weak root systems, and problems in acclimation to a potting mixture.

No treatments were applied specifically to the problem of low germination when it was encountered (RR1 x RRUU87, UU1 x AA1, and RUR621 x RR); however, all but the first of these hybrids were recovered by plating a greater number of embryos.

In an attempt to overcome the problem of cessation of growth shortly after germination, a few embryos of the cross RRUU62 x RR1 were transferred to fresh media. This treatment was not found to be effective, but it was later discovered that the fresh media to which they had been transferred were probably defective. Consequently, the usefulness of transferring to fresh medium to remedy the problem of cessation of growth needs further investigation. Some causes and other possible remedies for this problem were posed in the discussions of the crosses RRUU x UU, RUR x RR, and UA x MM.

Deformity or callus were common maladies for several combinations (AA x UU, MM x RR, MM x UU, VVGG x RR, RR x VVGG, RRUU x UU, and UA x MM). One embryo of AA x UU eventually outgrew its callus and deformity. A cutting procedure was successfully applied to the abnormal elongation problem of V. glabrescens x V. radiata. Heavily callused embryos of RR x VVGG were transferred to media containing cytokinin and one of these embryos subsequently began to produce a number of shoots that were then excised, rooted, and transplanted to the greenhouse. The discussion of MM x UU and RRUU x UU also include comments about possible causes and remedies for deformity and callus.

Some of the hybrid combinations, notably AA x UU, MM x AA, MM x UU, VVGG x RR, and UA x MM, had difficulty in expanding or initiating leaves. The embryos of VVGG x RR were incubated with an increased amount of red light (supplied by warm white fluorescent tubes) in an effort to increase leaf expansion. Many of these did eventually expand leaves, but no conclusion can be drawn from this since a control group of embryos for this hybrid (incubation continued in cool white only) did not exist. It should be added that many other hybrid combinations had no difficulty expanding leaves under cool white light, and even some that had initial difficulty eventually expanded leaves without changing the light regime from all cool white light. The use of GA_3, adenine, or grafting have also been suggested to increase leaf expansion in the discussions of AA x UU and MM x AA.

The root system of one embryo from the cross RR x UA developed then deteriorated. By taking a shoot-tip cutting a new root system was established. Three hybrid combinations (AA x UU, RRUU x RR, and UA x MM) exhibited weak root systems _in vitro_. This did not seem to be the main limiting factor for any of these hybrids, but GA_3 and grafting are mentioned as possible remedies for it in the discussions of MM x AA and UA x MM.

The last step in the rescue of hybrid plants by embryo culture is adapting them to the _ex vitro_ environment. A number of individual plants from the cross RR x UU were lost to dessication and one of the plantlets from the cross RRUU x RR succumbed to what was assumed to be damping off. A humid, aseptic potting and acclimation system was developed and proved successful. Nonetheless, plants of AA x RR and RR x UA failed to acclimate under this system. These failures could be attributed to occasional flaws in the system (micro-organisms were isolated from one supposedly-aseptic pot) or possibly to inherent weaknesses of the particular hybrid combination to advance beyond the young seedling stage.

All of the interspecific hybrids that survived acclimatization grew vigorously upon transfer to the greenhouse. All of these plants survived long enough to reach flowering but some individuals were maintained at non-inductive photoperiods to maintain them vegetatively for asexual propagation. Because of the low number of individual plants of most combinations obtained, it was necessary to propagate asexually many of the hybrid plants (six of the ten interspecific hybrid combinations obtained) to maintain them for further investigations and to increase them so as to obtain a greater number of progeny from those hybrids that were fertile. Although colchicine doubling was beyond the scope of this project, it is a logical next step in advancing sterile hybrids. Asexual propagation would also be useful to facilitate the colchicine procedure. Asexual propagation by cuttings was routinely and successfully performed for these interspecific hybrid combinations. However, asexually propagated hybrid plants differed in their vigor. If at least one parent of hybrids involving two species was strongly indeterminate, then problems related to vegetative vigor were not encountered in plants grown from cuttings. However, in the backcrosses where the parental determinate:indeterminate ratio was 2:1, both the number of cuttings that could be obtained and the vigor of these cuttings were restricted. Furthermore, the photoperiod sensitivity of *V. glabrescens* and *V. umbellata* parents allowed the vegetative growth in some of the hybrids (particularly UA31, VGU, VGR, and RVG) to be conveniently controlled by daylength. The utilization of parental germplasm with strong indeterminate growth habit is an important aspect to consider and is a possible key to successful interspecific hybridization programs.

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