Theor Appl Genet (1989) 78: 641–647

Interspecific hybridization between *Vigna radiata* (L.) Wilczek and *V. glabrescens*

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Received May 25, 1988; Accepted June 1, 1988
Communicated by H. F. Linskens

Summary. Interspecific hybrids of the mungbean, *Vigna radiata* (L.) Wilczek (2n = 22) and *V. glabrescens* (2n = 44) were generated with the aid of embryo culture. *V. glabrescens* × *V. radiata* hybrids were recovered via germination of the immature embryos. Reciprocal hybrids were obtained via shoot formation from embryonic callus. The authenticity of the hybrids was determined by morphological characteristics, chromosome number, and isozyme patterns. The hybrids were highly sterile upon selfing, but backcrossing to the diploid parent yielded viable seeds. Some of the plants resembled the diploid parent morphologically while others resembled neither parent. The backcross plants were sufficiently fertile to give a large number of mature, selfed seeds. Plants obtained differed morphologically and in their isozyme patterns from either parent, indicating introgression. These progeny populations will be used as bridging materials to transfer pest resistance from the wild tetraploid to the cultivated mungbean.

Key words: Interspecific gene transfer – Embryo culture – *Vigna radiata* – *Vigna glabrescens*– Mungbean

Introduction

Grain legumes are important sources of dietary protein in Asia, Africa, and Latin America. *Vigna* species, particularly the mungbean (*Vigna radiata* [L.] Wilczek, 2n = 22), are widely cultivated in Asia. Improvement of mungbean has been one of the major objectives of the Asian Vegetable Research and Development Center (AVRDC) since 1972. Advanced selections from AVRDC, such as VC1973A and VC2768A, have provided significant increases in yield (from 0.5 ton/ha to 1.5 ton/ha). However, in *V. radiata,* susceptibility to pests such as bean flies (*Ophiomyia sp.*), cowpea weevil (*Callosobruchus chinensis*) and root and stem rot (*Rhizoctonia* spp.) has often led to substantial yield loss. Recently, a tetraploid accession of *V. glabrescens* (2n = 4 ×), V1160, was identidied as highly resistant to pests of mungbeans (AVRDC, Annual Reports for 1985, 1986). In order to facilitate the transfer of genetic resistance from this tetraploid species to the mungbean, interspecific hybridization was attempted. Although interspecific hybridization between diploid *Vigna* species has been reported (Ahn and Hartmann 1978; Chen et al. 1983; Fatokun and Singh 1987; Gosal and Bajaj 1983; Machado et al. 1982), interploidy crosses within this genus have been rare (Dana 1968; Egawa et al. 1988). In this paper we describe the procedures of hybrid recovery via embryo culture and the successful generation of backcross progeny which were sufficiently fertile to set selfed seed.

Materials and methods

Plant materials

Seeds of *V. radiata* cultivars VC1973A and VC2768A and *V. glabrescens* accession V1160 were obtained from AVRDC. Genotypes of the two species can be distinguished by a number of morphological characteristics and growth habits (Table 1). Plants of the two species were grown in growth chambers under different conditions. For *V. radiata* genotypes, a photoperiod of 12 h and temperature regime of 30°/27 °C (day/night) was used, whereas for *V. glabrescens* the photoperiod was 10 h and the temperature 27°/25 °C (day/night).

Pollination and embryo culture

Flowers were emasculated 1 day before opening and pollinated the next day. Immature embryos resulting from the crosses were cultured following procedures described earlier to recover *Phaseolus* hybrids (Mok et al. 1978). Hybrid embryos were iden-

Table 1. Characteristics of *V. radiata*, *V. glabrescens* and the interspecific hybrids

	V. radiata	*V. glabrescens*	Hybrid
Chromosome no.	2n = 22	2n = 44	2n = 33
Seed color	green	black	maternal
Germination	epigeal	hypogeal	NA
Primary leaves	lanceolate without petiole	deltoid with petiole	deltoid with petiole
Hypocotyl color	green	purple	purple
Growth pattern	determinate	indeterminate	indeterminate
Photoperiod response	insensitive	short day	short day
Start of flowering	30–35 days	75 days	45 days
Flowering pattern	synchronous within 15 days	asynchronous >30 days	asynchronous > 30 days
Corolla size (width/length)	1.6/1.35 cm	2.5/1.3 cm	2.4/1.5 cm
Corolla color	ligth yellow	deep yellow	deep yellow

Table 2. Summary of crossing data between *V. glabrescens* and *V. radiata*

Cross	No. of flowers pollinated	No. of pods set	No. of embryo/pod	Embryo development	No. of embryos cultured	No. of embryos germinated	No. of plantlets	No. of mature hybrids
V. glabrescens × *V. radiata*	104	57	4.7	cotyledonary	116	116	64	48
V. radiata × *V. glabrescens*	108	72	2.8	heart stage	114	5[a]	13	12

[a] Callus tissues formed from these embryos

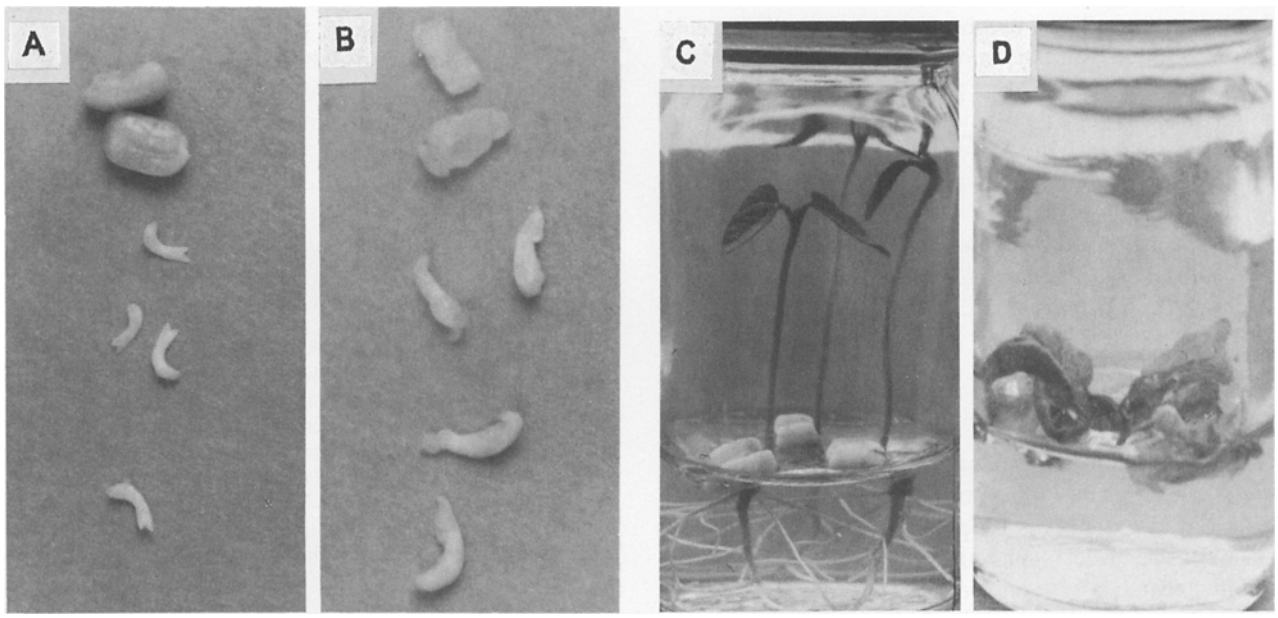

Fig. 1 A–D. Embryo development and germination of *V. glabrescens* versus *V. glabrescens* × *V. radiata*. **A** Normal cotyledons (upper two) and embryo axes (lower four) of *V. glabrescens* embryos. **B** Abnormal cotyledons (upper two) and elongated embryo axes (lower four) of *V. glabrescens* × *V. radiata* embryos. **C** Seedlings of *V. glabrescens*. **D** Plantlet with wrinkled leaves from precocious germination of *V. glabrescens* × *V. radiata* embryos

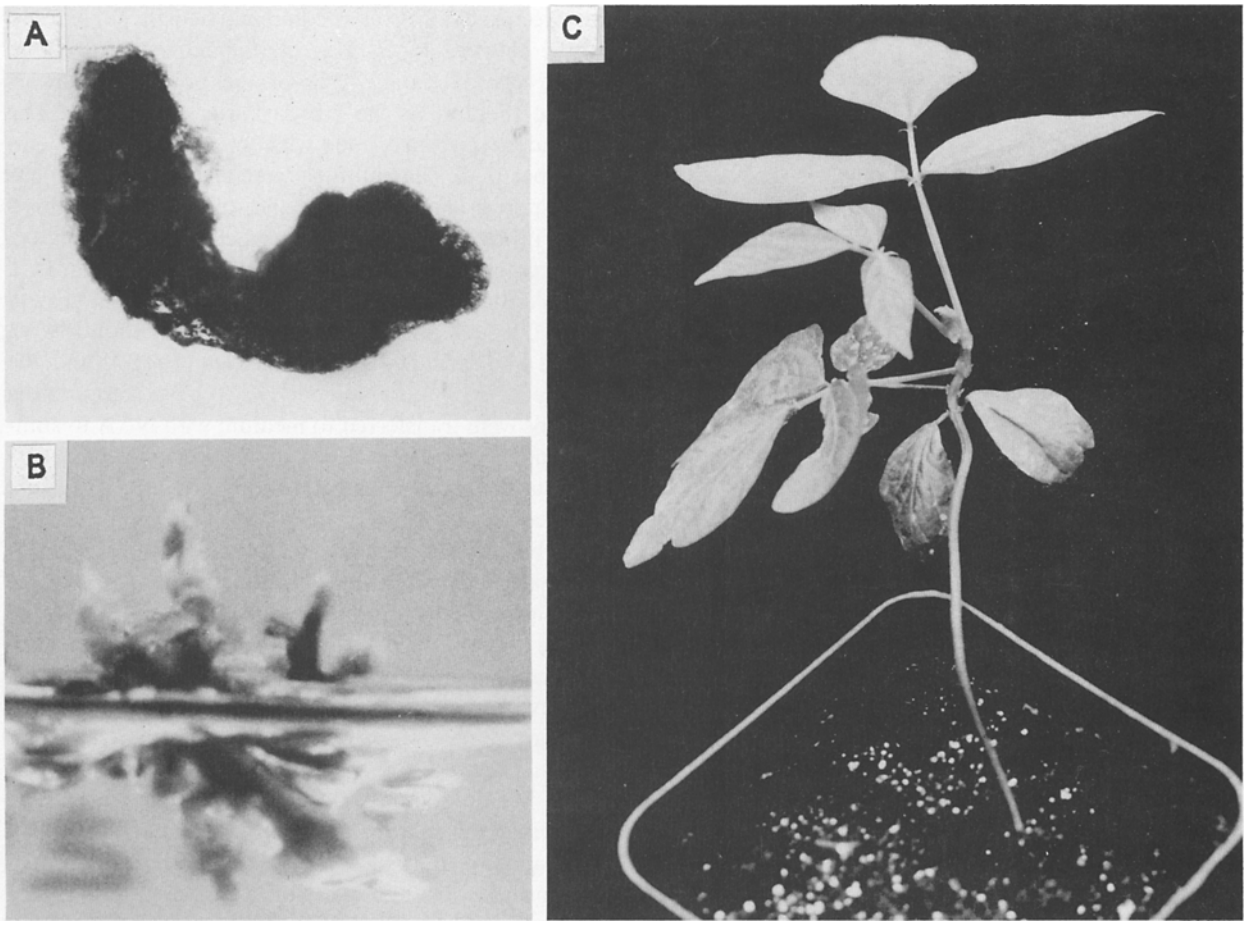

Fig. 2A–C. Embryos and plantlet of *V. radiata* × *V. glabrescens*. **A** Abnormal embryo of *V. radiata* × *V. glabrescens*. **B** Multiple shoots from embryonic callus of *V. radiata* × *V. glabrescens*. **C** Plantlet of *V. radiata* × *V. glabrescens*

tified by their distinctive morphology (Table 1). The embryo culture medium consisted of mineral nutrients as described by Murashige and Skoog 1962) with the addition of sucrose (30 g/l), *myo*-inositol (100 mg/l), glutamine (100 mg/l), thiamine (1 mg/l), nicotinic acid (5 mg/l) and pyridoxin (0.5 mg/l). The medium was adjusted to pH 5.7 and solidified by 0.8% Difco Bacto agar.

Plantlets derived from germinating embryos of *V. glabrescens* × *V. radiata* were transferred to medium containing 0.01 mg/l α-naphthalene acetic acid (NAA) for rooting. Shoot formation was induced in callus tissues originating from embryos of *V. radiata* × *V. glabrescens* using embryo culture medium supplemented with adenine sulfate (40 mg/l), NaH_2PO_4 (170 mg/l), N^6-benzyladenine (2 mg/l), and NAA (0.05 mg/l). Shoots were transferred to medium containing NAA (0.01 mg/l) for rooting. Rooted plantlets were transferred to pots containing Jiffy Mix Plus.

Electrophoresis of selected enzymes

Three enzyme systems – shikimate dehydrogenase (SDH), 6-phospho-gluconate dehydrogenase (6-PGD) and esterase (ES) – with distinct banding patterns between parents, were chosen as biochemical markers to authenticate the hybrids obtained. Malate dehydrogenase (MD) and ES were used to identify backcross progeny. Embryos or leaves were homogenized in 0.1 *M* phosphate buffer (pH 7.5) with a Tissuemizer (Tekmar) equipped with a microprobe shaft. The samples were centrifuged at 12,000 g for 20 min and the supernatant was concentrated and analyzed by PAGE. The polyacrylamide concentrations of the stacking and separating gels were 2.5% and 8.0%, respectively. The running buffer consisted of TRIS-HCl (0.005 *M*) and glycine (0.038 *M*) at pH 8.5. Isozymes were separated at 150 V for 0.5 h and then at 200 V for 2 h. Sample preparation and electrophoresis were carried out at 4 °C. The staining procedures for the isozymes of SDH and ES were those of Orton and Rick (1980a, b). Staining solution (150 ml) for 6-PGD contained 30 mg phosphogluconate, 30 ml TRIS (0.5 *M*, pH 8.5), 6 ml $MgCl_2$ (0.1 *M*), 15 mg NADP, 30 mg nitro blue tetrazolium (NBT), and 3 mg phenazine methosulfate (PMS). MDH staining solution (150 ml) contained 30 ml malate buffer (0.05 *M*, pH 6.0), 30 ml TRIS-HCl (0.5 *M*, pH 8.5), 30 mg NAD, 30 mg NBT, and 3 mg PMS. Gels were stained for 1.5 h at 4 °C.

Cytology

Root tips and flower buds were fixed in ethanol/glacial acetic acid (3:1, v/v) for 24 h. Mitosis and microsporogenesis were examined using the squash technique. Fertility was estimated based on pollen stainability (with 1% acetocarmine).

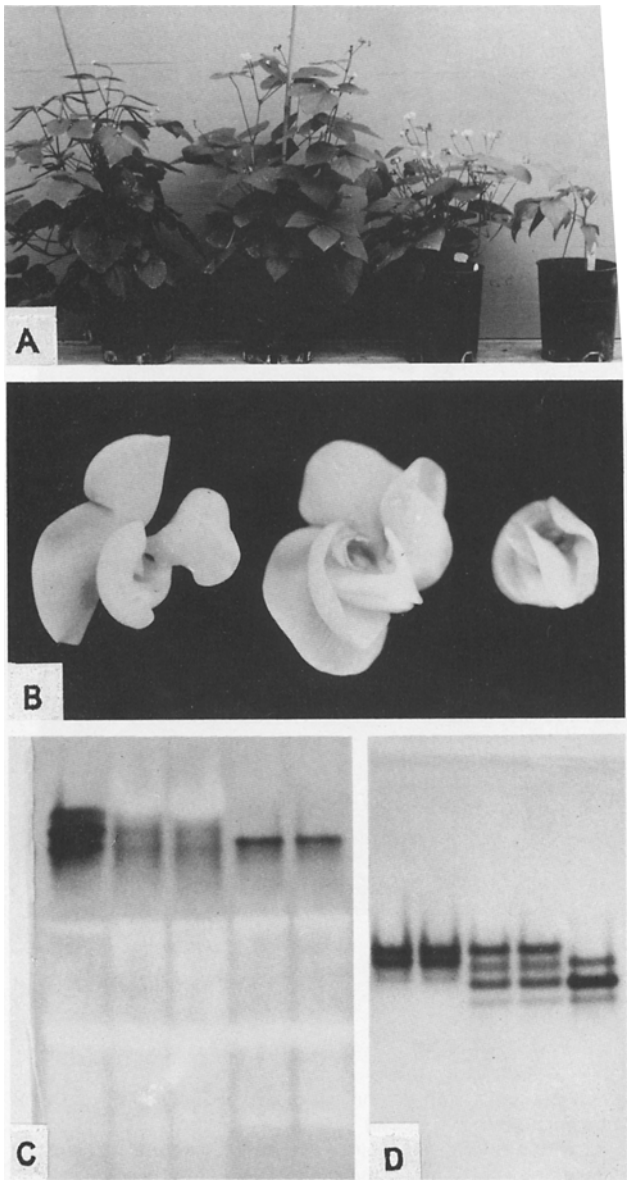

Fig. 3A–D. Morphology and isozymes of interspecific hybrids. **A** Plants of *V. glabrescens*, *V. glabrescens* × *V. radiata*, *V. radiata* × *V. glabrescens*, and *V. radiata* (left to right). **B** Corolla of *V. glabrescens* (left), *V. glabrescens* × *V. radiata* (center), and *V. radiata* (right). **C** 6-PGD isozymes (from left to right) of *V. glabrescens*, *V. glabrescens* × *V. radiata* cv VC1973A, *V. glabrescens* × *V. radiata* cv VC2708A, *V. radiata* cv VC1973A, and *V. radiata* cv VC2708A. **D** SDH isozymes (from left to right) of *V. radiata* cv VC1973A, *V. radiata* cv VC2708A, *V. glabrescens* × *V. radiata* cv VC1973A, *V, glabrescens* × *V. radiata* cv VC2708A, and *V. glabrescens*

Results

Pod set and development of hybrid embryos

The frequency of pod and seed set, the development of hybrid embryos and the number of plantlets obtained from reciprocal crosses are summarized in Table 2. In crosses between *V. glabrescens* (female) and *V. radiata* (male), approximately 45% of the pollinated flowers aborted the 2nd or the 7th day after pollination. The remainder developed pods with an average of 4.7 embryos per pod. The embryos were characterized by uneven, sponge-like cotyledons and an elongated embryo axis (Fig. 1 A and B). All cultured embryos germinated. However, compared with normal germination (Fig. 1 C), most plantlets exhibited some form of abnormal growth (Fig. 1 D), such as lack of hypocotyl elongation (89%), uneven and wrinkled primary leaves (100%), and the absence of root development (92%). The germinating embryos were transferred to medium with NAA to stimulate root development. Rooted plantlets (64) were transferred to the growth chamber and 48 hybrid plants were obtained.

Hybridization between *V. radiata* (female) and *V. glabrescens* (male) resulted in slightly higher pod set than the reciprocal cross (Table 2). Approximately 33% of the flowers aborted the day following pollination. However, the average number of embryos per pod was lower (2.8). The development of the hybrid embryos was arrested at the heart-shaped stage (Fig. 2 A). Of the 114 embryos cultured, none germinated but callus formed on 5 embryos. Multiple shoots (Fig. 2 B) occurred when these callus tissues were transferred to medium containing N^6-benzyladenine and NAA. These shoots could be rooted by transfer to medium containing NAA alone. Thirteen hybrid plants were obtained after transplanting to soil. Interestingly, one mature seed was obtained and the seedling resembled the male parent morphologically (Fig. 2 C).

Characterization of hybrids

Plants obtained from reciprocal crosses exhibited dominant traits of the tetraploid parent (Table 1). For example, the purple hypocotyl, indeterminate growth habit (Fig. 3 A), long flowering period, and larger corolla (Fig. 3 B) were all characteristics of *V. glabrescens*. The authenticity of the hybrids was determined by isozyme banding patterns. *V. radiata* genotypes (VC1973A and VC2768A) had identical isozymes for 6-PGD and SDH extracted from immature embryos, which were distinct from those of *V. glabrescens* (Fig. 3 C and D). Immature embryos resulting from artificial pollination exhibited all parental SDH isozymes and a new 6-PGD pattern resembling the tetraploid parent more than the diploid parent. With regard to ES isozymes, plants obtained from reciprocal crosses showed banding patterns representing a combination of both parents. Thus, the plants obtained via embryo culture and/or adventitious shoot formation were clearly interspecific hybrids.

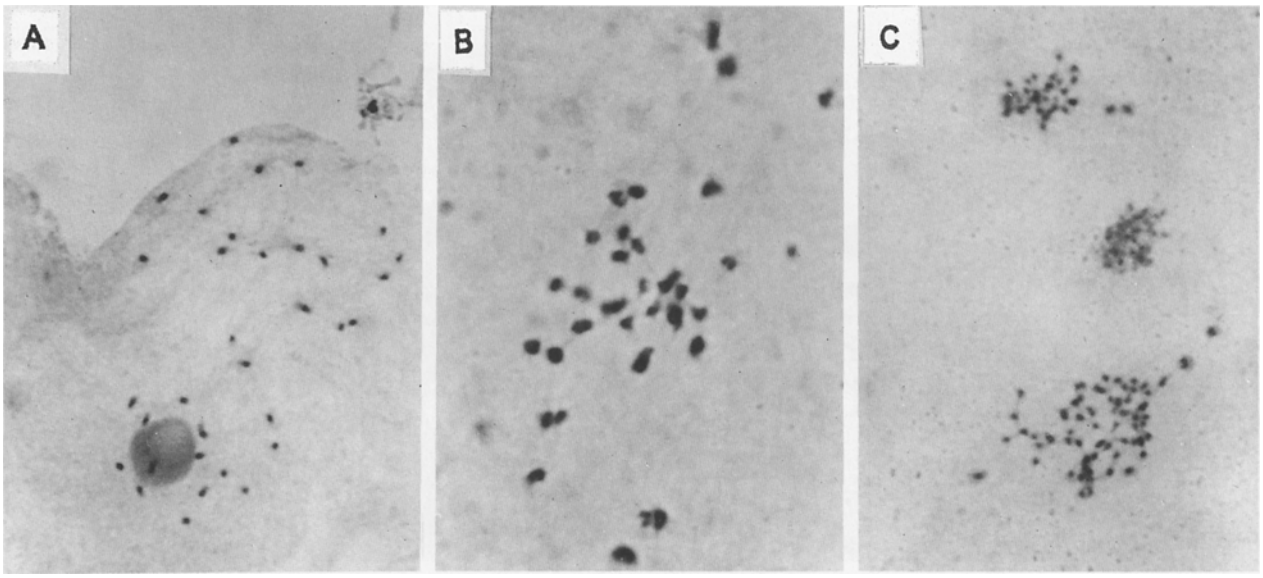

Fig. 4A–C. Meiosis of *V. glabrescens* × *V. radiata* hybrid. **A** Diakinesis with 33 univalents. **B** Met I with 29 univalents and 2 bivalents. **C** Ana I with unequal distribution of chromosomes and laggards

Cytology and crossing behavior of hybrids

The chromosome number of *V. glabrescens* was confirmed as 2n = 44 at mitosis. Hybrids obtained from reciprocal crosses had the expected triploid chromosome number of 2n = 33. Examination at meiosis indicated that parental species had regular chromosome pairing at Metaphase I with 22 and 11 bivalents, respectively, for the tetraploid and diploid species. Hybrids of *V. glabrescens* × *V. radiata* showed very low frequency of chromosome association (Fig. 4A and B). The number of bivalents at Metaphase I ranged from three to eight. On the average, 20 univalents, 5.7 bivalents, 0.43 trivalents and 0.06 quadrivalents were observed per pollen mother cell. Polyploidy was observed occasionally. Among 84 PMCs examined, cells with chromosome numbers of 44 (one), 66 (two), and 99 (one) were found. Other meiotic abnormalities included laggards at Anaphase I (Fig. 4C), uneven chromosome distribution at Telophase I, and unbalanced tetrads with micronuclei.

Backcross progeny

Pollen stainability of reciprocal hybrids ranged from 0.73% to 6.43% with an average of 2.6%. Selfing resulted in no pod set. However, backcrossing to *V. radiata* resulted in two pods containing a total of ten seeds. These seeds (BC) germinated when planted in soil (Fig. 5A). Seven plants resembled the diploid parent morphologically. The remaining three plants were characterized by pentafoliate leaflets (Fig. 5B), a trait not found in either parental genotype. All backcross plants flowered early and had a short flowering period similar to that of the *V. radiata* parent. Chromosome numbers as determined at microsporogenesis ranged from 22 to 44. The fertility was much higher than that of the F_1 hybrids, with pollen stainability ranging from 4% to 51%, with an average of 39%.

Twenty-five selfed pods containing 110 seeds (BC S_1) were obtained from the ten backcross progeny. These plants segregated for MDH and ES (Fig. 5C) isozymes and the patterns were not identical to either parent, suggesting recombination between the parental genomes. The pentafoliate leaf character was transmitted as a recessive trait (Fig. 5D).

Discussion

The primary barriers to gene transfer between *V. glabrescens* and *V. radiata* are the arrest of hybrid embryo development in vivo and the low fertility of the reciprocal F_1s. The results described in this paper demonstrate that embryo abortion can be circumvented by culturing the immature embryos. Large numbers of hybrid plantlets are now available. Selfing of the hybrids was not successful due to low fertility. Nevertheless, adequate numbers of viable pollen were formed by the hybrids, resulting in the recovery of backcross (BC) progeny with *V. radiata* as the female parent. More importantly, BC plants were sufficiently self-fertile, giving rise to large number of mature, selfed seeds (BC S_1). The retention of morphological traits and isozyme bands of *V. glabrescens* by BC and BC S_1 plants indicate that introgression has taken place. Therefore, these interspecific progeny populations may

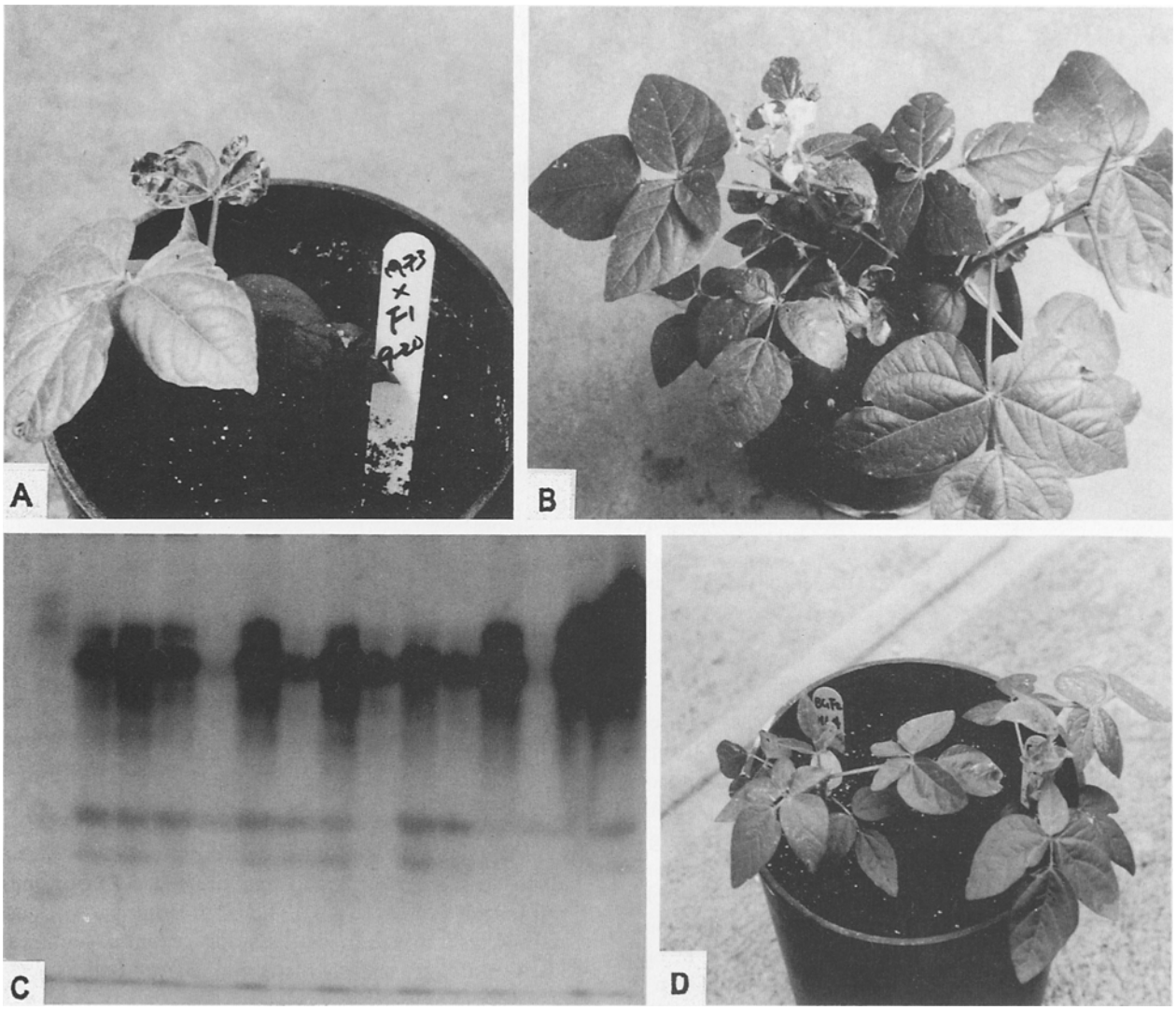

Fig. 5A–D. Backcross plants and their selfed progeny. **A** Backcross plants of *V. radiata* × (*V. glabrescens* × *V. radiata*). **B** Backcross plant at flowering and with pentafoliate leaves. **C** ES isozymes of selfed offsprings of backcross plant showing segregation. **D** Selfed progeny of backcross plant

be valuable bridging materials for the transfer of characters from *V. glabrescens* to the cultivated forms of mungbean. The immediate goal will be the selection of individuals with pest resistance of *V. glabrescens* and the economic characteristics of *V. radiata.*

The origin and genomic constitution of *V. glabrescens* is uncertain. According to Egawa et al. (1988), hybrids of *V. glabrescens* and *V. umbellata* showed 11 bivalents and 11 univalents at meiosis. However, as the morphology of *V. glabrescens* resembles that of *V. angularis* (which, when crossed with *V. umbellata,* gives progeny with 11 bivalents at meiosis), it was suggested that *V. angularis* was the donor of one of the *V. glabrescens* genomes. The hybrids obtained in our study had very low frequency of bivalents, indicating that *V. radiata* is probably not a progenitor of the tetraploid *V. glabrescens.*

Hybrid embryos of *V. glabrescens* × *V. radiata* developed to the cotyledonary stage and were capable of precocious germination on embryo culture medium. Hybrid embryos obtained from the reciprocal cross were unable to germinate but formed callus. The embryo-derived callus, however, was responsive to hormone treatment, resulting in multiple shoot formation. Plantlets obtained from either method could be stimulated to give adventitious shoots. These observations suggest that the distinct developmental programs of reciprocal hybrid embryos may be related to their ability to undergo redifferentiation. This is particularly important since regeneration from cell cultures of large-seeded legumes has been difficult. Preliminary experiments are underway utilizing this interspecific system to study the control of somatic embryogenesis.

Acknowledgements. This research was supported by the Oregon Agricultural Experiment Station and a grant (87-CRSR-2-3185) from IARC Special Constraints Research Grants Program of AID/USDA/CSRS. Technical paper No. 8869 of Oregon Agricultural Experiment Station.

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