Fig. 2. Plantlet regeneration from hypocotyl callus of 'Moriguchi' cultured for 30 days on initiation medium (MS + 0.1 2,4-D + 1.0 mg BA/liter), followed by two subcultures on MS + 1.0 mg BA/liter for another 60 days, and then transplanted onto MS medium.

Fig. 3. Regenerated plant of 'Moriguchi' radish transplanted to vermiculite.

plants, callus usually must be transplanted onto media without 2,4-D, with only cytokinin, or with other auxins, e.g., NAA or IAA, plus cytokinin. In radishes, shoots regenerated on subculture media without 2,4-D in 'Moriguchi' and 'Nerima Shirinaga', as reported for cauliflower (Walkey et al., 1974) and red cabbage (Bajaj and Nietsch, 1975). In the case of 'Comet' on medium containing 2,4-D and kinetin, shoots regenerated directly from hypocotyl cambium. Although many kinds of explants, such as flower organs, leaf and leaf-vine, stem and cotyledon, shoot tip and hypocotyl, have been used for tissue culture of cole crops, the hypocotyl has been optimal for radish callus. Hypocotyls have also been good for callus initiation of broccoli (Hui, 1980), red cabbage, and leaf-mustard (Bajaj and Nietsch, 1975). The number of regenerated plants has varied depending on media and cultivars tested. One explant cut into four sections at every subculture, on average, results in 70.4 'Nerima Shirinaga' and 16.6 'Moriguchi' plants after 4 months of culture.

Literature Cited

- Bajaj, Y.P.S. and P. Nietsch. 1975. In vitro propagation of red cabbage (*Brassica oleracea* L. var. capitata). J. Expt. Bot. 26:883-890.
- Hui, L.H. 1980. The effect of ginseng on the plantlet regeneration percentage of cotyledon and hypocotyl explants of broccoli. Z. Pflanzenphysiol. 96:297-301.
- Murashige, T. and F. Skoog. 1962. A revised medium for the rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 9:1473-497.
- Nakanishi, T. and K. Hinata. 1973. An effective time for CO₂ gas treatment in overcoming self-incompatibility in *Brassica*. Plant Cell Physiol. 14:873-879.

- Walkey, D.G.A., V.C. Cooper, and P. Crisp. 1974. The production of virus-free cauliflowers by tissue culture. J. Hort. Sci. 49:273-275.
- Zee, S.Y. and B.B. Johnson. 1985. Vegetable;

- Cole crops, p. 227-246. In: P.V. Ammirato, D.A. Evans, W.R. Sharp, and Y. Yamada (eds.). Handbook of plant cell culture. vol 3. Macmillan, New York.

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Seed and Embryo Growth in Pod Cultures of *Phaseolus vulgaris* and *P. vulgaris* × *P. acutifolius*

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Abstract. Interspecific hybrid embryos resulting from crosses between *Phaseolus* species generally fail to reach maturity, and embryo rescue techniques are required to recover plants. To determine if ovary (pod) culture could permit interspecific hybrid embryo development, pods of *P. vulgaris* L. × *P. acutifolius* A. Gray and *P. vulgaris* L. (control) were cultured upright, supported by glasswool, in modified Murashige-Skoog liquid medium. The weight of seeds and length of embryos in *P. vulgaris* pods increased significantly during culture. However, usually only one or occasionally two seeds located at the middle positions of the pods developed to maturity. The same pod culture procedure promoted precocious germination of early cotyledonary-stage *P. vulgaris* × *P. acutifolius* embryos without further maturation of the embryos. These findings confirm that developmental arrest of the interspecific hybrid embryos in vivo is due to intrinsic deficiencies.

Phaseolus vulgaris (common bean) is susceptible to several viral, bacterial, and fungal diseases. Resistance has been identified in related species, *P. coccineus* Lam. (runner bean), *P. acutifolius* (teparty bean), and *P. lunatus* L. (lima bean). For instance, *P. acutifolius* contains resistance to common blight (Coyne et al., 1963; Yoshii et al., 1978) and golden mosaic virus (CIAT, 1973). In addition, this species is drought tolerant and adapted to dry-land culture. Transfer of desirable traits from related *Phaseolus* species to *P. vulgaris* could greatly improve agronomic characteristics of the common bean. Therefore, interspecific hybridization has been used to facilitate genetic exchange between *Phaseolus* species.

Although fertilization occurs and embryos are formed, interspecific hybrid embryos are generally limited in their developmental potential (Mok et al., 1986). The particular stage at which the developmental arrest occurs depends on the interspecific combination as well as the direction of the cross. The immature embryos, with the exception of those of *P. lunatus* × *P. vulgaris* that develop only to

the four-celled stage, can be cultured in vitro, germinated, and grown to plants (Huel and Scoles, 1985).

The development of a suitable pod culture system for *Phaseolus* may facilitate elucidation of factors influencing the ontogeny of interspecific embryos. Development of alternative or supplemental methods to embryo culture to optimize the recovery of interspecific hybrids would also be desirable. We report here the procedures and results of culturing immature pods of *Phaseolus*, beginning with selfed pods of *P. vulgaris* to establish the protocols and followed by pods

Fig. 1. Embryos of *P. vulgaris* GN (large) and *P. vulgaris* GN × *P. acutifolius* Ac2 (small) 14 days after pollination. Bar = 1 mm.

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Fig. 2. Seed weight (A) and embryo length (B) of *P. vulgaris* GN before and after 21 days of pod culture. Significant differences between 0 culture time and 21 days are denoted by * ($P = 0.05$). Different letters for positions indicate significant differences in LSD test ($P = 0.05$).

Fig. 3. A large and small embryo of *P. vulgaris* GN recovered after 21 days of pod culture. Bar = 2 mm.

of *P. vulgaris* × *P. acutifolius*, to test the feasibility of enhancing interspecific hybrid embryo development.

Pods were obtained from plants grown in growth chambers at a 12-hr photoperiod. Seed and embryo development of *P. vulgaris* cv. Great Northern (GN) and *P. vulgaris* cv. Great Northern × *P. acutifolius* A. Gray P.I. 321637 (GN × Ac2) were examined 3 weeks after pollination. Pods of GN 9.1 to 9.3 cm long (10 days after pollination) were selected for culture. Pods of 'GN × Ac2' were collected

14 days after pollination. The seeds and embryos contained in the selfed and hybrid pods were similar in size. Also, younger pods of GN (6 and 8 days) and GN × Ac2 (10 days) and older pods of GN × Ac2 (21 days) were cultured.

Pods were surface-sterilized in 70% ethanol for 30 sec, then in 35% Clorox (1.8% sodium hypochlorite) for 10 min, followed by rinsing in sterile distilled water and drying with sterile filter paper. Several methods for in vitro culturing of immature GN pods were explored. The pods either were placed flat in a liquid medium or supported upright by a hollow glass rod or glasswool. Glasswool was selected as support, since it proved to be the best in preliminary experiments, resulting in growth of seeds and embryos without browning of the pods. Pods were placed in 150 × 25-mm tubes with the pedicels immersed in 10 ml of liquid medium devised for pea pod culture (Barratt, 1986). The medium contained MS mineral nutrients (Murashige and Skoog, 1962), but with KNO_3 and NH_4NO_3 deleted and 10 mM KCl and 1.5 mM K_2SO_4 added, and only one-half the Fe concentration. Other supplements were 20 g sucrose, 100 mg myo-inositol, 10 mg thiamine-HCl, 1 mg nicotinic acid, 1 mg pyridoxine-HCl, 3 mg glycine, 40 mM glutamine, 20 mM asparagine, and 1 g casein hydrolysate per liter. The cultures were incubated at 27°C with a photoperiod of 16 hr (at photosynthetic photon flux density of 25

$\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$) for 21 days for the 'GN' and 16 days for 'GN × Ac2' pods. The differences in culture periods reflected the differential onset of senescence for the two types of pods.

Seed weight and embryo length were measured at the beginning and end of the culture period for each experimental group of 20 pods. Significant differences were determined by analyses of variance and *t* tests. Least significant differences were calculated for means. Logarithmic transformation of the data for GN seeds and embryos was necessary since variance was nonuniform.

Embryos recovered from cultured pods were placed in sterilized Jiffi Mix Plus (Ball Seed Co., Chicago, Ill.) and incubated under the same light and temperature conditions as the pod cultures. Some hybrid embryos were precultured on embryo culture medium (Rabakoarihanta et al., 1979) for 28 days and then transferred to Jiffi Mix Plus.

The size of hybrid seeds was smaller than that of selfed seeds of the same age. At 14 days after pollination, hybrid embryos, characterized by their open and often uneven cotyledons, were much smaller than the selfed embryos (2 vs. 4 mm; Fig. 1). The size difference was even more pronounced for embryos collected 21 days after pollination (3 vs. 10 mm). The hybrid embryos failed to grow further, and pods abscised soon thereafter.

The fresh weight of seeds (Fig. 2A) and length of embryos (Fig. 2B) in GN pods increased substantially during the 21-day pod culture. The increases were due to growth of only one seed and, occasionally, two seeds per pod. The location of the embryo in the pod was an important factor affecting continued development. The growth of seeds and embryos located at the middle positions was significantly greater than those at the terminal positions. Differences in seed weight and embryo length before and after culture were significant for almost all positions; data for the sixth position were omitted since many of the pods contained only five seeds. The final weight of many of the developing seeds was similar to that of mature seed (≈ 400 mg) obtained from pods collected from the plants. The difference between well-developed and poorly developed embryos recovered from the same cultured pod is illustrated in Fig. 3.

The culture period for the hybrid pods was shorter than that for selfed pods since the hybrid pods senesced earlier than the selfed pods. The hybrid embryos had reached their maximum size after 16 days in culture and started to deteriorate with pod senescence. Seeds and embryos of GN × Ac2 increased significantly in weight (Fig. 4A) and length (Fig. 4B) during the culture period. However, there were no significant position effects, and hybrid embryos did not advance to the same extent as the parental embryos. The seed weight and embryo length increases were the result of root formation rather than advancement in embryo development (Fig. 5).

Younger pods of GN with 1- to 5-mg seeds

Fig. 4. Seed weight (A) and embryo length (B) of *P. vulgaris* GN $\times$ *P. acutifolius* Ac2 before and after 16 days of pod culture. Difference between 0 culture time and 16 days is denoted by * ($P = 0.05$).

Fig. 5. Embryos of *P. vulgaris* GN $\times$ *P. acutifolius* Ac2 recovered after 16 days of pod culture. Bar = 2 mm.

and embryos <1 mm long were cultured successfully, although the percentage of developing seeds was lower than that for the older pods. However, pods of GN $\times$ Ac2 younger than 14 days gave poor results in culture; the embryos shriveled after 7 or 10 days in culture. Older (21-day post-pollination) pods of GN $\times$ Ac2 responded similarly to 14-day-old pods; the embryos germinated in cultured pods but their cotyledons did not enlarge.

Embryos recovered from cultured pods were placed in sterile soil. The larger embryos of GN germinated readily and gave rise to viable plants. The hybrid embryos also germinated but were weak and only few of those placed in soil survived. However, an intervening conditioning phase in embryo culture medium before planting in soil ensured the survival of $\approx 90\%$ of the hybrids.

The results demonstrate that seed and embryo growth can occur using this pod culture protocol. Selfed seeds weighing ≈ 5 mg and containing embryos <1 mm long initially could reach the size attained in vivo, indicating that pod culture provides the conditions necessary for growth and development. The same methodology was successfully applied to obtain mature selfed seeds of *P. acutifolius* (Ac2) weighing >400 mg, starting with pods containing embryos of similar size as those used for GN (unpublished results).

Usually only one selfed seed per cultured pod showed significant development, which may indicate competition for nutrients between developing embryos. In our experiments, these seeds were more often located in the middle of the pod than at the ends, which is in agreement with the observation for *Glycine max* (soybean) pod cultures (Obendorf et al., 1983). However, in their experiments, the position effect depended on the culture system. When the whole pod was immersed in the medium, the seed at the tip showed better development than the ones in

the middle or at the peduncle end. Optimum seed growth occurred when the pods were placed flat in the culture medium. Submersion of pods or laying pods flat in a shallow layer of medium did not stimulate embryo growth in our system. However, the initial weight of the soybean seeds was ≈ 50 mg, whereas *Phaseolus* seed weights ranged from 1 to 15 mg.

The position effects may in part be related to the differential development of seeds in the same pod in vivo. Bean seeds were aborted more frequently at the pedicel end than at the styler end of the pod (Harris, 1915; Nakamura, 1988), and developing bean embryos were larger at the styler end (Gabelman and Williams, 1962). Although in our system the growth of embryos at the pedicel end was limited, the seeds near the styler end did not develop as much as the middle seeds, possibly due to the occurrence of a nutrient gradient.

In contrast to the selfed seeds, the cotyledons of interspecific hybrid seeds in cultured pods did not grow; instead, the embryos germinated precociously. Delayed endosperm division and slower embryo growth have been shown for interspecific crosses of *Phaseolus*, including *P. vulgaris* $\times$ *P. acutifolius* (Mok et al., 1978; Rabakoarimanta et al., 1979; Thomas, 1964). Thus, the early growth rates as well as the later development of the interspecific hybrid embryos appear to be intrinsically limited.

The physiological basis of hybrid embryo arrest is not known. *P. vulgaris* $\times$ *P. acutifolius* embryos contain lower levels of extractable cytokinins than those of the parental species (Nesling and Morris, 1979), which indicates the possibility of an imbalance in hormonal biosynthesis and metabolism. Possibly related to this are the effects of N^6 -benzyladenine in advancing *P. lunatus* $\times$ *P. vulgaris* embryos from the four-celled stage to the globular stage (Mok et al., 1986). The pod culture system described here may be employed to examine the effects of growth regulators on the development of interspecific hybrid embryos.

The ability to substitute embryo rescue with pod culture to recover interspecific hybrids is of practical value. Although mature hybrid seeds of *P. vulgaris* $\times$ *P. acutifolius* have not been obtained in pod culture, hybrid plantlets can be obtained after a brief phase of culturing the embryos in embryo culture medium. However, the plantlets are still not as strong as those obtained from mature selfed seed and special care must be taken to prevent wilting. Further studies of the factors involved in developmental arrest and precocious germination may aid the design of an optimum pod culture medium leading to formation of better-developed interspecific hybrid seeds.

Literature Cited

- Barratt, D.H.P. 1986. In vitro pod culture of *Pisum sativum*. Plant Sci. 43:223-228.
- CIAT, 1973. Bean program report. Centro Internacional de Agricultura Tropical, Cali, Colombia.

- Coyne, D.P., M.L. Schuster, and S. Al-Yasiri. 1963. Reaction studies of bean species and varieties to common blight and bacterial wilt. *Plant Dis. Rptr.* 57:111-114.
- Gabelman, W.H. and D.F. Williams. 1962. Water relations affecting pod set of green beans. *Proc. Plant Sci. Symp. Campbell Soup, Camden, N.J.* p. 25-35.
- Harris, J.A. 1915. The influence of position in the pod upon the weight of the bean seed. *Amer. Naturalist* 49:44-47.
- Hucl, P. and G.J. Scoles. 1985. Interspecific hybridization in the common bean: A review. *HortScience* 20:352-357.
- Mok, D.W.S., M.C. Mok, and A. Rabakoarihanta. 1978. Interspecific hybridization of *Phaseolus vulgaris* with *P. lunatus* and *P. acutifolius*. *Theor. Applied Genet.* 52:209-215.
- Mok, D.W.S., M.C. Mok, A. Rabakoarihanta, and C.T. Shii. 1986. *Phaseolus*—Wide hybridization through embryo culture, p. 309-318. In: Y.P.S. Bajaj (ed.). *Biotechnology in agriculture and forestry*. vol. 2. Springer-Verlag, Berlin.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473-497.
- Nakamura, R.R. 1988. Seed abortion and seed size variation within fruits of *Phaseolus vulgaris*: Pollen donor and resource limitation effects. *Amer. J. Bot.* 75:1003-1010.
- Nesling, F.A.V. and D.A. Morris. 1979. Cytokinin levels and embryo abortion in interspecific *Phaseolus* crosses. *Z. Pflanzenphysiol.* 91:345-358.
- Obendorf, R.L., G.T. Rytco, and M.C. Byrne. 1983. Soya bean seed growth and maturation by *in vitro* pod culture. *Ann. Bot.* 51:217-227.
- Rabakoarihanta, A., D.W.S. Mok, and M.C. Mok. 1979. Fertilization and early embryo development in reciprocal interspecific crosses of *Phaseolus*. *Theor. Applied Genet.* 54:55-59.
- Thomas, H. 1964. Investigation into the inter-relationships of *Phaseolus vulgaris* L. and *P. coccineus* Lam. *Genetica* 35:59-72.
- Yoshii, K., G.E. Galvez-E, and G. Alvarez-A. 1978. Screening bean germplasm for tolerance to common blight caused by *Xanthomonas phaseolii* and the importance of pathogenic variation to varietal improvement. *Plant Dis. Rptr.* 62:343-347.

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In Vitro Propagation of *Miscanthus sinensis*

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Abstract. Immature inflorescences of *Miscanthus sinensis* Andress. 'Gracillimus', 'Variegatus', and 'Zebrinus' were cultured on modified MS medium with 9.0 μM 2,4-D, 20 g sucrose/liter, 2.0 g Gelrite/liter, and 0.75 g MgCl_2 /liter. Organogenesis was observed 8 to 12 weeks after callus initiation. Shoots were rooted on half-strength MS medium without growth regulators. After rooting, tillers were initiated. When transferred to soil, plants matured to flowering quickly and retained their variegation patterns. Propagation through in vitro tillering is suggested. Chemical name used: 2,4-dichlorophenoxyacetic acid (2,4-D).

Interest in new, low-maintenance landscape plants has increased in recent years (Weatherly, 1988). *Miscanthus sinensis* 'Gracillimus', 'Variegatus', and 'Zebrinus' are ornamental grasses that are low maintenance, drought tolerant, adaptable to poor soils, and hardy through zone 4 (Corley, 1983; Gibson, 1986). Each of these cultivars exhibits a unique variegation pattern: 'Gracillimus'—green blade with white midrib, 'Variegatus'—vertical variegation, and 'Zebrinus'—horizontal variegation. These cultivars are sterile and must be propagated through division (Gibson, 1986) and, therefore, have restricted commercial availability. More efficient propagation could enhance the availability and use of *M. sinensis*. This report describes an efficient procedure for in vitro regeneration of these cultivars.

In preliminary regeneration experiments,

immature inflorescences, mature leaves, immature leaves, nodal segments, internodal segments, meristematic regions, and ovules were tested for regeneration (Gawel et al., 1987). Explants were cultured on Murashige and Skoog (1962) (MS) medium containing a wide range of auxin types and concentrations. The only explant types to produce callus were immature inflorescences and, therefore, were used as the sole explants in this study. Only media containing 4-amino-3,5,6 trichloropicolinic acid (Picloram) or 2,4-D with no cytokinin produced the regeneration response.

All explants were harvested from 7-year-old, field-grown plants. Surface sterilization consisted of a 20-sec dip in 95% (v/v) ethanol and 20 min in 0.525% (v/v) sodium hypochlorite followed by three rinses in sterile, distilled water. Explants were surface-sterilized as intact stems containing unelongated inflorescences, then dissected to remove the immature inflorescence. Unless otherwise noted, the basal medium consisted of MS salts and vitamins supplemented with 20 g sucrose, 100 mg myo-inositol, 2.0 g Gelrite (Scott Laboratories, Atlanta), and 0.75 g MgCl_2 (all per liter). The MgCl_2 was added to replace divalent cations sequestered by the

Gelrite during gelling. Growth regulators were added, and pH was adjusted to 5.5 with 1 N NaOH before sterilization. The media were dispensed as 8-ml aliquots into 14-ml glass vials and autoclaved for 20 min at 121°C. Cultures were maintained at 27 to 30°C under 16 hr of light provided by 110-W wide-spectrum fluorescent bulbs (70 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$).

Auxin type and concentration for optimum callus initiation were determined by placing 2-cm-long sections of immature inflorescences on basal medium supplemented with either 4.5, 9.0, 18.1, or 36.2 μM 2,4-D or 4.1, 8.3, 16.6, or 33.2 μM picloram. Each auxin type/concentration/cultivar combination was replicated 10 times. Cream-colored callus began to form within 10 to 14 days. Cultures were scored for callogenesis after 6 weeks. The most efficient treatment was 9.0 μM 2,4-D (Table 1). Overall, 93% of the explants produced callus with this treatment.

Callus initiation was influenced by the developmental stage of the inflorescences. Im-

Fig. 1. Highly meristematic shoot primordia of *M. sinensis* 'Variegatus' callus ($\times 17$).

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