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Title: Seed and Embryo Development in Pod Culture of Phaseolus vulgaris and P. vulgaris x P. acutifolius.

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Phaseolus vulgaris, the common bean, is susceptible to many bacterial, fungal and viral diseases. However, resistance to several diseases has been identified in related Phaseolus species, P. coccineus, P. acutifolius and P. lunatus. Thus, interspecific hybridization could result in transfer of beneficial characteristics. One of the problems encountered in interspecific hybridization of Phaseolus resides in the failure of the hybrid embryos to reach maturity. Embryo rescue techniques are required to recover interspecific hybrid plants. In vitro culturing of immature pods represents an alternative to embryo rescue.

In order to develop appropriate pod culture methodology, immature selfed pods of P. vulgaris cv. Great Northern were cultured at three stages of development (containing embryos of 0.5, 0.6-1.0 and 1.5-2.5 mm in length respectively). Seed weights and embryo lengths increased significantly for all three classes of pods when cultured upright, supported by glass wool, in tubes containing a liquid medium. One or two embryos per pod, usually located at the middle positions, could develop further. The same pod culture procedure promoted precocious

germination of cotyledonary embryos of P. vulgaris Great Northern x P. acutifolius P.I.321637, without advancing the embryos to further developmental stages. These findings suggest that the developmental arrest of the interspecific hybrid embryos in vivo is due to intrinsic developmental controls.

Since growth regulators may be involved in the retardation of interspecific hybrid embryo growth, seed and embryo growth were measured after a pod culture period in the presence of 10^{-6}M of N^6-benzyladenine (BAP), gibberellic acid (GA_3) or abscisic acid. The growth regulators did not affect seed weight and embryo length in cultured P. vulgaris pods. However, the length of embryos in the largest class of P. vulgaris Great Northern x P. acutifolius P.I.321637 pods were significantly lower in the presence of BAP and GA_3 as compared to the control without growth regulators. Thus, at the concentration tested, growth regulators were not beneficial for further embryo development but BAP and GA_3 seemed to depress root elongation of the germinating embryos of the interspecific hybrid.

Although mature hybrid seed can not be generated in pod culture, hybrid plantlets can be obtained after a brief phase of culturing the germinating embryos. The germinating embryos from pod culture offer some advantage over embryos dissected at an earlier stage, since the presence of roots enhances nutrient uptake and hardening. Further studies leading to identification of the factors that cause developmental arrest of interspecific hybrid embryos may aid in devising the optimal pod culture medium for further growth of hybrid seeds and embryos.

SEED AND EMBRYO DEVELOPMENT IN POD CULTURE OF *PHASEOLUS VULGARIS* AND *P. VULGARIS* X *P. ACUTIFOLIUS*.

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LIST OF ABBREVIATIONS

ABA: abscisic acid

BAP: N^6-benzyladenine

2,4-D: 2,4-dichlorophenoxyacetic acid

GA_3: gibberellic acid

IAA: indole-3-acetic acid

NAA: α-naphthaleneacetic acid

SEED AND EMBRYO DEVELOPMENT IN POD CULTURE OF *PHASEOLUS VULGARIS* AND *P. VULGARIS* X *P. ACUTIFOLIUS*.

I. INTRODUCTION

Phaseolus vulgaris, the common bean, is widely cultivated and provides a major portion of the dietary protein in several Latin American countries. However, the common bean is generally susceptible to many viral, bacterial and fungal diseases. Resistance has been identified in related species, *P. coccineus* (runner bean), *P. acutifolius* (tepary bean) and *P. lunatus* (lima bean). For instance, *P. coccineus* contains resistance to bean yellow mosaic, bean common mosaic virus (Baggett, 1956) and anthracnose (Hubbeling, 1977), while *P. acutifolius* has resistance to common bean blight, caused by *Xanthomona phaseoli* (Coyne and Schuster, 1974), and halo bight, incited by *Pseudomonas phaseolicola* (Coyne *et al.*, 1971). Some varieties of *P. lunatus* are known to be resistant to the potato leaf hopper, *Empoasca fabalis* (Wolfenbarger and Sleesman, 1961a) and to some extent to the Mexican bean beetle, *Ephilachna varvivestis* (Wolfenbarger and Sleesman, 1961b). In addition, *P. acutifolius* is known to be resistant to drought, making it more adapted to dry land culture.

Transfer of desirable traits from the related *Phaseolus* species to *P. vulgaris* could greatly improve agronomic characters and yield of the common bean. Therefore, interspecific hybridization has been attempted to promote gene flow between *Phaseolus* species. Although fertilization takes place and embryos are formed, the interspecific hybrid embryos are

generally limited in their developmental potential. The particular stage at which the developmental arrest takes place depends on the interspecific combination as well as the direction of the cross (Mok et al., 1986). For instance, embryos of P. lunatus x P. vulgaris develop only to the four-celled stage, while those of the reciprocal cross attain the globular stage (about 0.2 mm). Embryos derived from crosses between P. vulgaris and P. acutifolius develop further and reach the cotyledonary stage. The most advanced development occurs in crosses between P. vulgaris and P. coccineus; either abnormal late cotyledonary embryos are formed (with P. coccineus as the female parent) or mature normal seeds (with P. vulgaris as the female parent). These immature embryos, with the exception of the four-celled embryos of P. lunatus x P. vulgaris, can be dissected and cultured in vitro and give rise to hybrid plants. However, the process is tedious, particularly in the case of the small P. vulgaris x P. lunatus embryos (Mok et al., 1978). Therefore, it is desirable to develop alternative or supplemental methods to embryo culture, such as pod culture, in order to expand and facilitate the efforts to transfer genes between Phaseolus species via interspecific hybridization. Such methods may also provide another approach to study the factors influencing embryo arrest which have been difficult to determine in vivo.

Plant hormones may be involved in the abnormal development of interspecific hybrid embryos of Phaseolus. Nesling and Morris (1979) reported that the levels of cytokinins in embryos of P. vulgaris x P. acutifolius were lower than in the parental embryos. In addition, while interspecific hybrid development of P. vulgaris x P. lunatus is usually arrested at the four-celled stage, pods formed when cytokinins were

added to the hydroponic culture medium in which the maternal plant was grown, often contained embryos at the globular stage. Although culturing of pods resulting from interspecific hybridization of large-seeded legumes has not been reported previously, the effects of growth regulators on the success of culturing selfed pods have been studied. Although Barratt (1986) obtained stimulatory effects of BAP and GA_3 on pea seed development, but the presence of growth regulators did not seem to be necessary for seed development of soybean (Obendorf et al., 1983). Thus, although the exact causes of interspecific hybrid embryo abortion are not known, there may be some involvement of plant hormones. Moreover, hormones have some effect on the development of embryos in cultured pods.

The present study focuses on cultures of immature pods of Phaseolus, initially using selfed pods of P. vulgaris to establish the protocols, and subsequently pods of P. vulgaris x P. acutifolius to test the feasibility of applying these procedures to enhance interspecific hybrid recovery. In addition, preliminary experiments to examining the effects of growth regulators on growth of selfed as well as hybrid seeds and embryos are presented.

The objectives of this study may be summarized as follows:

1) To devise methodologies to culture immature pods of P. vulgaris to obtain mature seeds;
2) To determine the developmental potential of seeds and embryos of P. vulgaris x P. acutifolius under the same conditions; and
3) To evaluate the effects of growth regulators in the pod culture medium on the growth of P. vulgaris as well as P. vulgaris x P. acutifolius seeds and embryos.

II. LITERATURE REVIEW

A. Ovary culture

Interspecific hybrids can not be obtained in many cases due to premature abortion of the hybrid embryos. In order to enhance wide hybridization, various *in vitro* techniques, such as embryo, ovule and ovary culture, have been employed (Bhojwani and Razdan, 1983). Although embryo culture of some interspecific hybrids has been successful, young embryos are not only difficult to excise but often require complex nutrient media for growth and differentiation (Bhojwani and Razdan, 1983). However, ovaries may be excised at very early stages of embryo development, thus offering an advantage over embryo culture. Moreover, they provide a maternal environment to the developing embryos (Srivastava *et al.*, 1980), which may promote embryo development.

La Rue (1942) was the first to report results of culturing immature ovaries, obtaining limited growth of ovaries as well as root formation on the pedicel. Subsequently, ovaries of several species have been cultured in attempts to study fruit growth and development (Nitsch, 1949; Jansen and Bonner, 1949; Nitsch, 1951; Makagawa *et al.*, 1983), to determine the influence of exogenous chemicals as well as endogenous physiological processes on fruit development (Wittembach and Bukovac, 1980; Barratt, 1986), and to obtain interspecific hybrid seeds (Inomata, 1982; Bajaj *et al.*, 1986). Ovary culture of maize, *Zea mays*, has been used to study the efficiency of *in vitro* pollination (Raman *et al.*, 1980; Gengenbach, 1984). Cultures of unpollinated ovaries of *Petunia axillaris* (De Verna and Collins, 1984), *Beta vulgaris* (Van Geyt *et al*

1987), Hordeum vulgare, and Gerbera (Yang and Zhou, 1982) were used successfully to obtain haploids.

Ovaries cultured in vitro before full elongation have displayed different responses depending on the species and the stage of development. Jansen and Bonner (1949) reported growth of ovaries of Lycopersicon from 1 mm to a final size of 4-8 mm in diameter after 1 to 2 months in culture. Nitsch (1951), using ovaries of Lycopersicon at various stages of growth, reported that ovaries cultured two days before full bloom or at full bloom did not grow appreciably. However, ovaries excised two days after full bloom increased two fold in diameter in a three-week period. Similarly, Cucumis ovaries cultured two days after full bloom enlarged from 5.8 mm in diameter to 11 mm after 24 days in culture Nitsch (1951). Also legume fruits seem to increase in size during culture. Barratt (1986) cultured pea pods of 40-50 mm length and observed an increase in pod length after 14 days in culture. However, pods cultured at a very early stage (10-13 mm pod length) displayed only a slight increase in length (Srivastava et al., 1980). In general, although the fruit sizes increased in culture, fruits were smaller than those formed under natural growth conditions (Nitsch, 1951; Baldev et al., 1965; Wittembach and Bukovac, 1980; Makagawa et al., 1983).

Young ovaries of Anethum graveolus, taken three days after pollination, produced seeds after a culture period of several weeks (Johri and Sehgal, 1966). Various report concerning legumes also mention success in seed development through ovary culture. Fully expanded fruits (pods) of soybean (with seeds of 50-60 mg in fresh weight) were cultured for 42 days and some mature seeds were obtained. The rate of germination of seed obtained through ovary culture was 75%

(Obendorf et al., 1983). Baldev et al. (1965) reported an increase in the fresh weight of pea seeds when the pedicels of detached fully elongated pods (13 days after pollination) were submerged in an agar nutrient medium for 10 days.

Interspecific hybrid seed formation in ovary culture has been accomplished in some genera. Interspecific hybrid seeds of Brassica campestris x B. oleracea were obtained by ovary culture but not in the reciprocal cross (Inomata, 1982). Bajaj et al. (1986), working with the hybrid of Brassica napus x B. juncea, used ovaries excised 2-9 days after pollination, and obtained mature seed after 35 days of culture. Seed formation varied with the size of the cultured ovaries. Aproximatelly 80% of large size ovaries showed seed formation, in contrast with 37.8% in the smaller size. De Verna et al. (1987) reported the production of hybrid seeds from ovary cultures of Nicotiana amplexicaulis x N. tabacum and N. benthamiana x N. tabacum. However, Richards and Rupert (1980), using excised ovaries of hybrids between T. ambiguum and Trifolium repens, only obtained partial embryo development.

B. Media used for ovary culture

1. Mineral nutrients

Different basal media have been adopted for the culture of ovaries of several species. Generally, the nutrient media have been based on either Nitsch's (1951), White's (1943) and Murashige and Skoog's (1962) inorganic salts. In early work on ovary culture Nitsch's medium (1951) was used. It contains a modification of Knop's salt mixture. Nitsch

(1951) obtained seeds after culturing ovaries of Cucumis and Lycopersicon on basal medium supplemented with thiamine, cysteine hydrocloride and tomato juice. However, ovary culture of Phaseolus vulgaris var. Red Kidney resulted in formation of young fruits (10-15 mm) but they did not grow further (Nitsch, 1951).

A modification of Murashige and Skoog's medium (1962) supplemented with different organic compounds proved to be effective for ovary culture of Glycine max (Obendorf et al., 1983; Hsu and Obendorf, 1982)) and intraspecific hybrids of Trifolium repens (Richards and Rupert, 1980). Modifications of Murashige and Skoog's medium (1962) were also beneficial for ovary culture of pea, Pisum sativum, (Barratt, 1986) and Prunus cerasus (Wittembach and Bukovac, 1980).

Modified White's medium (1943) has been succesfully used in Anethum and Foeniculum (Johri and Sehgal, 1966) and to support ovary culture of Pisum sativum (Srivastava et al., 1980). Similarly, Inomata (1982) obtained mature seed following interspecific hybridization of Brassica using White's medium.

The mineral nutrient composition did not seem to affect the development of immature cultured ovaries of Prunus cerasus (Wittembach and Bukovac, 1980). In both Nitsch's (1951) or modified Murashige and Skoog's medium (1962), fruits at early stages of development did not show growth during the culture period but older fruits (36 days) exhibited normal maturation and increase in weight. Sladky and Havel (1976) reported that there was no significant difference between White's medium (1943) and Murashige and Skoog's medium (1962) in the development of maize kernels. Both Murashige and Skoog's (1962) and White's (1943) medium supported the growth of older ovaries of Brassica's hybrids, but

were not able to stimulate significant growth of younger ovaries (Bajaj et al., 1986).

2. Sugars

Addition of carbohydrates has been found beneficial for the normal development in vitro of ovaries of many plant species. Generally, the concentration of sucrose in the medium is 3% to 5% (Srivastava et al., 1980; Hsu and Obendorf, 1982). On a simple basal medium containig mineral salts no growth was observed in cultured ovaries of Anethum, but growth was satisfactory when the medium was supplemented with 4 % sucrose (Johri and Sehgal, 1966). For Pisum sativum ovary culture, Barratt (1986) supplemented the medium with a low concentration (2 %) of sucrose. However, for Zea mays, Gengenbach (1984) has used as much as 15% sucrose, fructose or glucose, which proved to be effective for growth of developing kernels in vitro.

3. Vitamins

Vitamins have not been considered very important for ovary culture. In general the vitamins commonly used for tissue culture (e.g. thiamine.HCl, nicotinic acid, and pyridoxine.HCl) have been used also for ovary cultures (Obendorf et al., 1983; Barratt, 1986; Bajaj et al., 1986). Some modifications have been applied with varying levels of success. Nitsch (1949) added vitamin A, thiamine, riboflavin and niacin to the mineral salt medium but did not obtain good result. However, Jansen and Bonner (1949) achieved maximum growth and development of

Lycopersicon ovaries with media including eleven B-vitamins.

4. Amino acids and complex additives

Amino acids are usually added to ovary culture medium to provide an organic source of nitrogen. The development of maize kernels in vitro was highly influenced by the culture medium (Gengenbach, 1984). A source of reduced nitrogen such as glutamine or asparagine or a mixture of amino acids supported growth of kernels much better than nitrate. The amino acid mixture was based on the endosperm composition of the kernel. For several other species, the basal medium has been supplemented with casein hydrolysate (Barratt, 1986; Richards and Rupert, 1980). Casein hydrolysate also stimulated growth responses of ovaries of Anethum. The size of the fruits even surpassed that of naturally grown ovaries (Johri and Sehgal, 1966). Obendorf et al. (1983) indicated that glutamine was the only amino acid necessary for soybean ovary culture. Barratt (1986) reported growth and development of pea ovaries in a medium supplemented with asparagine, glutamine, glycine and casein hydrolysate. Srivastava et al. (1980) added glutamic acid to pod cultures of the same species. The importance of amino acids for ovary culture may be related to the finding that amino acids such as glutamine and glutamic acid serve as a source of nitrogen for protein synthesis in developing pea cotyledons (Millerd et al., 1975) and in maize kernel growth and development (Gengenbach, 1984).

Various natural extracts other than casein hydrolysate, i.e. coconut water and yeast extract, have been added to ovary culture medium (Inomata, 1982; Bajaj et al., 1986). These extracts may serve to supply

amino acids as well as other growth factors to the developing embryos. Nitsch (1941) emphasized the importance of sterile juice of tomato in the development of ovaries of Lycopersicon. Makagawa et al. (1983), using Nitsch's medium (1951) supplemented with malt extract, cucumber juice, fresh grape juice and vine sap, but found no marked improvement in growth of cultured ovaries of Vitis labruscana. Yeast extract was not favorable for cultures of Brassica campestris x B. oleracea ovaries, while casein hydrolysate and coconut water were stimulatory but not indispensable (Bajaj et al., 1986).

5. Plant growth regulators

The effects of exogenous growth regulators on growth of cultured ovaries seem to depend on the plant species. Nitsch (1951) observed benefical effect of 2,4-dichlorophenoxyacetic acid (2,4-D) on development of cultured ovaries of Lycopersicum, which ripened in the light without initiation of roots. Also, it was benefical in the induction of parthenogenesis of unpollinated tomato ovaries in vitro (Nitsch, 1949). Addition of indole-3-acetic acid (IAA) and 2,4-D to the basal medium did not stimulate the growth of endosperm or embryos of Anethum, but in the presence of exogenous gibberellic acid (GA_3) (1ppm) the ovules contained globular proembryos although further growth of embryos was arrested (Johri and Sehgal, 1966). The application of -naphthaleneacetic acid (NAA), N^6-benzyladenine (BAP) and GA_3, alone or in combination, stimulated parthenocarpic growth and coloring of berries of unpollinated ovaries of Vitis (Makagawa et al., 1983). In addition, while pollinated ovaries of Vitis grown in a medium supplied with

natural extracts and juices did not develop further, larger fruits were obtained in media supplemented with the cytokinin BAP (Makagawa et al., 1983). Barratt (1986) studied the effects of GA_3, NAA and BAP on pea seed development in ovary culture. Whereas NAA proved to be inhibitory, the presence of BAP at 10^{-4}M was the most effective concentration in promoting pod elongation but induced pod abnormalities. The addition of 10^{-5}M BAP alone or in combination with 10^{-6}M GA_3, significantly improved pod elongation and seed development. At 10^{-6}M, GA3 was most effective in promoting seed weight increase and germination after dehydration. T. ambiguum embryos in ovaries cultured in vitro did not grow unless BAP, GA_3 and IAA were added to the medium (Richards and Rupert, 1980). Application of kinetin had no significant effect on growth of ovaries of Prunus (Wittembach and Bukovac, 1980) and Anethum (Johri and Sehgal, 1966), and only stimulated callus formation at the basal end in ovaries of Brassica (Bajaj et al., 1986).

C. Types of support

Agar-solidifed media have been used by many authors because of the support provided to the developing ovaries (Nitsch, 1951; Gengenbach, 1977; Richards and Rupert, 1980; Gengenbach, 1984; Bajaj et al., 1986). In maize, agar-solidified medium proved to be more suitable than liquid medium for growth of ovaries or segments of cobs (Sladky and Havel, 1976). Generally, 0.8%-1.0% (w/v) of agar is included in the medium.

Liquid medium has been used by some investigators since it prevents the accumulation of toxic metabolites around the tissue (Schel and Kieft, 1986) and provides more direct transport of nutrients through the

pedicel (Barratt, 1986). Nitsch (1951) used liquid medium to avoid the effects of impurities in agar. Several kinds of support have been used to prevent submersion of tissue in the medium and death due to the lack of oxygen. Nitsch (1951) used glass wool or filter paper to support the ovaries during culture. A central hole in the filter paper allowed the pedicel to dip into the medium below. Schel and Kieft (1986) developed a complex culture system in which fresh medium was added continuously with a peristaltic pump to vessels containing the ovaries of maize which were placed on filters. In legumes, different types of support have been used. Barratt (1986) cultured pea pods by placing only the peduncle in the sterile medium. The container was sealed around the peduncle by silicone rubber. Srivatava et al. (1980) supported pea pods with glass beads. Culturing of pods in vertical position gave better result, particularly when the pedicel was removed and the cut end was immersed in the medium. Pods floating horizontally on a static liquid medium failed to grow. In contrast, Obendorf et al. (1983) observed the best result in growth and development of soybean seeds when pods were placed horizontally in an Erlenmeyer flask on a rotatory shaker. Experiments with only the pedicel submerged and supported with glass tubing gave variable results.

III. MATERIALS AND METHODS

A. Plant Materials

Immature pods of Phaseolus vulgaris L. cv. Great Northern (GN) were obtained from plants grown in growth chambers or the greenhouse. Pods were selected for uniformity based on the size of seeds contained. They were grouped into three embryos size classes. They were 0.5, 0.6-1.0, and 1.5-2.5 mm in length, respectively. Interspecific hybrid pods of P. vulgaris cv. Great Northern x P. acutifolius L. Gray P.I. 321637 (Ac2) were collected 14 and 21 days after pollination. The embryos were about 2.0 and 2.5-3.0 mm, respectively, at these dates.

B. Culture methods

Pods were surface sterilized by immersion in 70% ethanol (with a few drops of Triton X100) for 30 sec. and in 35% Clorox (1.8% sodium hypochlorite) (with a few drops of Tween 20) for 10 min, rinsed twice in sterile distilled water, and dried with sterile filter paper. Pods were cultured in vitro by placing them in culture tubes (150 x 25 mm diam.) with their pedicels immersed in 10 ml of the liquid medium devised for pea pod culture by Barratt (1986) (see Table 1). The medium (pH 5.7) was autoclaved, with the exception of the amino acids and casein hydrolysate which were dissolved in double distilled water, sterilized by filtration through millipore filters (0.45 u), and added to the medium after autoclaving. For the experiment testing the effects of growth regulators, 10^{-6}M N^6-benzyladenine (BAP), 10^{-6}M gibberellic acid

Table 1. Medium used for pod culture of Phaseolus

Compound	Concentration mM	Concentration mg/l
Inorganic nutrients:		
KCl	10	746
K_2SO_4	1.5	261
$MgSO_4.7H_2O$	1.5	370
$MnSO_4.4H_2O$	0.1	22.3
$ZnSO_4.7H_2O$	0.03	8.6
$CuSO_4.5H_2O$	0.0001	0.025
$CaCl_2.2H_2O$	3	440
KI	0.005	0.83
KH_2PO_4	1.25	170
H_3BO_3	0.1	6.2
$Na_2MoO_4.2H_2O$	0.001	0.25
$CoCl_2$	0.0001	0.025
$FeSO_4.7H_2O$	0.05	13.9
Na_2EDTA	0.1	37.2
Organic nutrients:		
sucrose	58.2	20000
inositol	0.555	100
thiamine	0.03	10
nicotinic acid	0.008	1
pyridoxine	0.005	1
glycine	0.04	3
glutamine	40	5846
asparagine	20	2642
casein hydrolysate		1000

(GA_3), 10^{-6}M abscisic acid (ABA) or no growth regulators (0 control) were incorporated in the medium. These compounds were dissolved in small amounts of dimethyl sulfoxide and added to the sterile medium. Physical support was provided by glass wool (see Fig. 1). The cultures were kept at 27°C with a photoperiod of 16h (at photosynthetic photon flux density of 25 umol.s^{-1}.m^{-2}) for 21 and 16 days respectively for the P. vulgaris cv. Great Northern and P. vulgaris cv. Great Northern x P. acutifolius P.I. 321637 pods. The differences in culture periods reflected the differential onset of senescence for the selfed and hybrid pods.

C. Measurements

The length of pods, fresh weight of seeds and the length of embryos were measured at the beginning and the end of the culture period. Each experimental group consisted of 20 pods (with the exception of the growth regulator experiment with selfed pods in which 10 pods were used). Data of pod elongation, fresh weight, and embryo length before (control) and after culture were statistically analyzed using the t test at the 0.05 (**) and 0.01(*) levels. Significance in position effects and growth regulator effects were determined by the analysis of variance. Treatments which showed significant differences were compared using the LSD test (0.05 level).

Figure 1. The *Phaseolus* pod culture system used for the studies of seed and embryo growth.

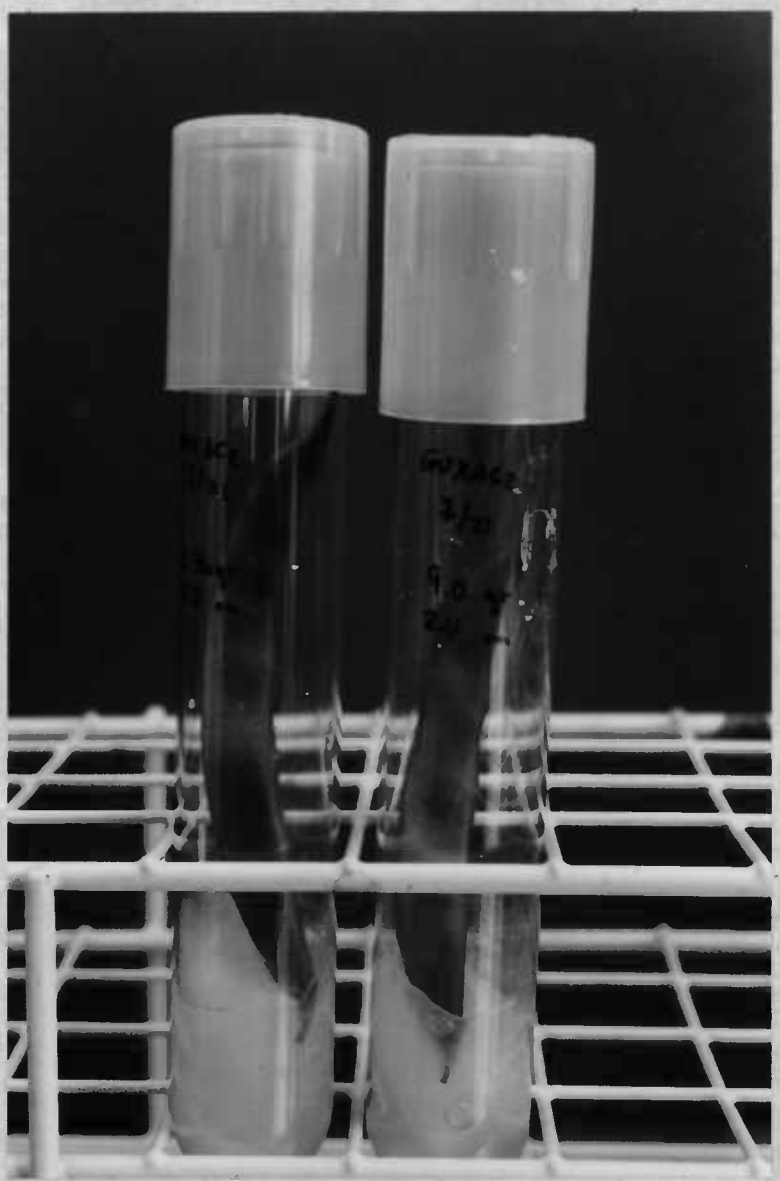

Figure 1

IV. RESULTS

A. Development of *P. vulgaris* cv. Great Northern and *P. vulgaris* cv. Great Northern x *P. acutifolius* P.I. 321637 embryos *in vivo*

At 14 days after pollination, selfed *P. vulgaris* cv. Great Northern and *P. vulgaris* cv. Great Northern x *P. acutifolius* P.I. 321637 pods were similar in length. The size of the hybrid seeds was slightly smaller than that of selfed seeds. However, hybrid embryos, characterized by their open and often uneven cotyledons (Mok *et al.*, 1978), were much smaller than the selfed embryos (Fig. 2). The size difference was even more pronounced for seeds (Fig. 3) and embryos (Fig. 4) collected 21 days after pollination. The hybrid embryos failed to grow further and pod abscission occurred soon thereafter.

B. Pod culture of *P. vulgaris* cv. Great Northern

Several methods for *in vitro* culturing of immature pods were explored. The pods were either placed flat in a liquid medium or upright on a support provided by a hollow glass rod or glass wool. The latter method proved to be the best, resulting in further development of seeds and embryos without browning of the pods, and was chosen for subsequent experiments.

Three different classes of pods were used for the experiment to determine seed and embryo growth. The pods were visually selected since this resulted in greater uniformity of pod and seed size than selection by age. The explanation for this may be that differences in growth

Figure 2. Embryos of *P. vulgaris* cv. Great Northern (large) and *P. vulgaris* cv. Great Northern x *P. acutifolius* P.I. 321637 (small) 14 days after pollination.

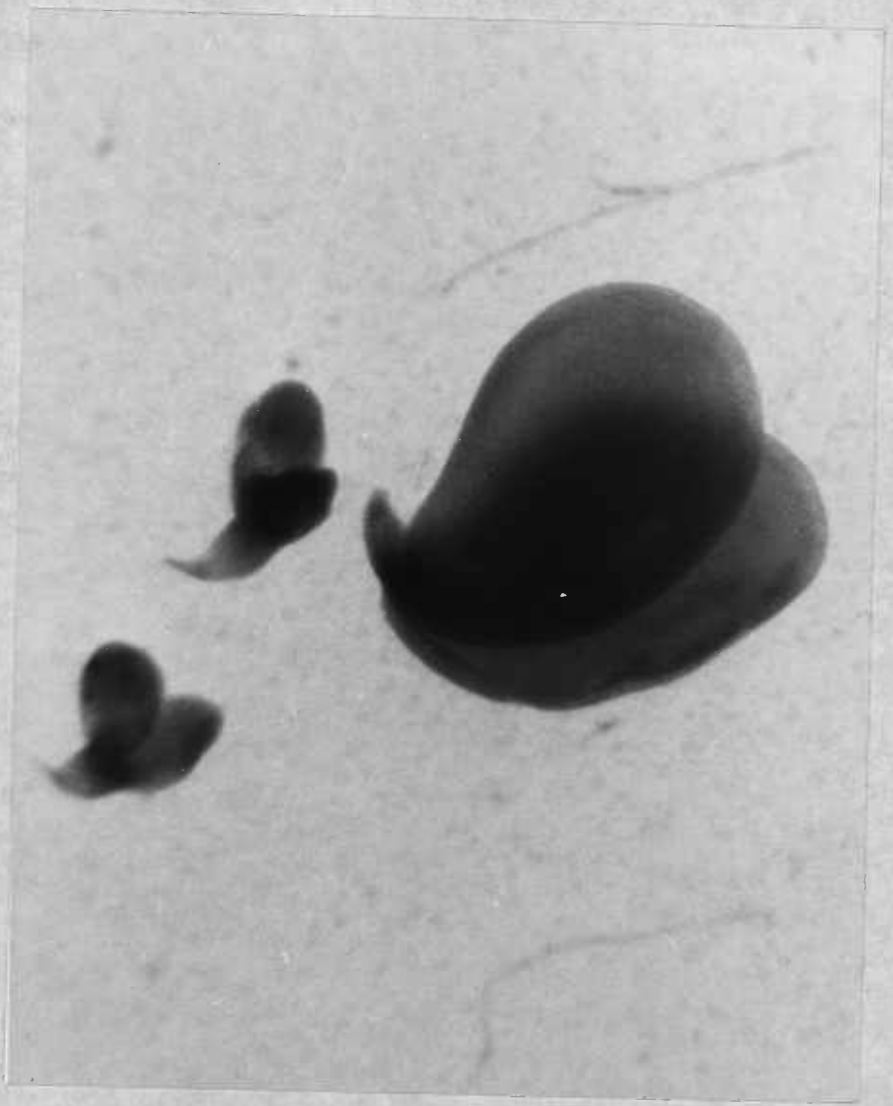

Figure 2

Figure 3. Pods with seeds of P. vulgaris cv. Great Northern (left) and P. vulgaris cv. Great Northern x P. acutifolius P.I. 321637 (right) 21 days after pollination.

Figure 3

Figure 4. Embryos of *P. vulgaris* cv. Great Northern (left) and *P. vulgaris* cv. Great Northern x *P. acutifolius* P.I. 321637 (right) 21 days after pollination.

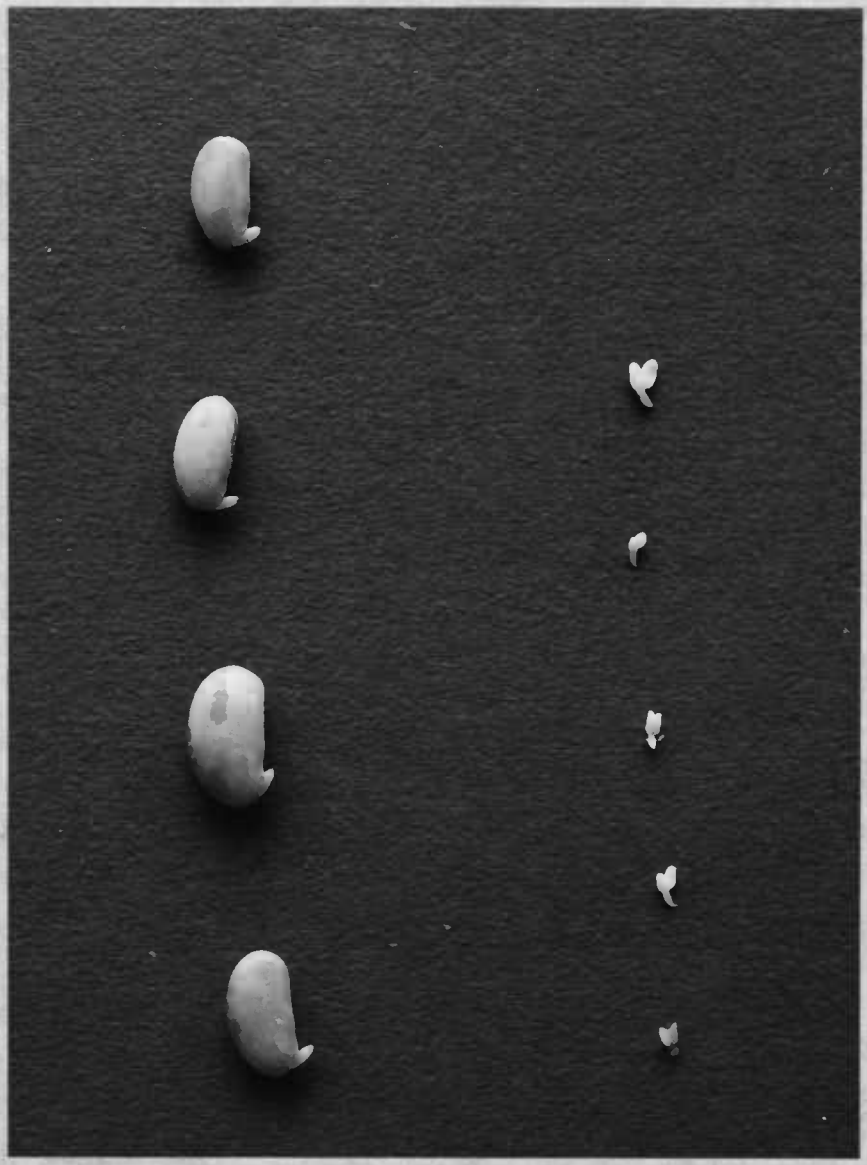

Figure 4

rates occurred depending on the location of the pod on the plant. The smallest size (6.0 cm) contained embryos (0.5 mm) at the heart or very early cotyledonary stage, while the middle (7.4 cm) and larger (9.2 cm) size pods had embryos (0.6-1.0 and 1.5-2.5, respectively) at the cotyledonary stage. During culture the two smaller size groups of pods increased in length (Table 2), although they did not reach the size generally attained *in vivo* (10.4 cm). The fresh weight of seeds (Fig. 5), and length of embryos (Fig. 6) increased greatly for all three classes of pods. The differences in the seed weight and embryo length between cultured and control pods were statistically significant (t-test) for almost all seed positions. (Results for the 6th position were omitted since many of the pods examined contained only five seeds.) Interestingly, usually only one seed in each pod showed advanced development. Several of these seeds developed to maturity, particularly in the middle and larger size pods, and were comparable in final weight (400-600 mg) reached by seed in pods left on the plants. The location of the embryo within the pod was an important factor influencing its further development. The growth of seeds and embryos located in the middle of the pods was most often enhanced. The positional differences (seed weight and embryo length) were significant for the small and large classes as determined by the analysis of variance. The results of the means separation by the LSD test are shown in Tables 3 and 4 respectively for fresh weight of seeds and length of embryos, which indicate that the best seed growth occurred at the second or third position (pedicel to tip). The difference between well developed and poorly developed embryos recovered from the same cultured pod is illustrated in Fig. 7. When pods were left in the culture medium for

Table 2. Length of three groups of pods (large, medium and small) of P. vulgaris cv. Great Northern before and after a culture period of 21 days.

Pod size	Length of pod (cm)	
	Before culture	After culture
Large	9.2	9.4 NS
Medium	7.4	7.8 **
Small	6.0	6.6 **

NS Non significant.
** Significant difference in size before and after culture at the 0.01 level (t test).

Figure 5. Seed weight at five seed positions before (control) and after culturing of *P. vulgaris* cv. Great Northern pods of three sizes, small (S), medium (M) and large (L). Significant differences between cultured and control pods are indicated for each position (* for 0.05 level and ** for 0.01 level by the t test.)

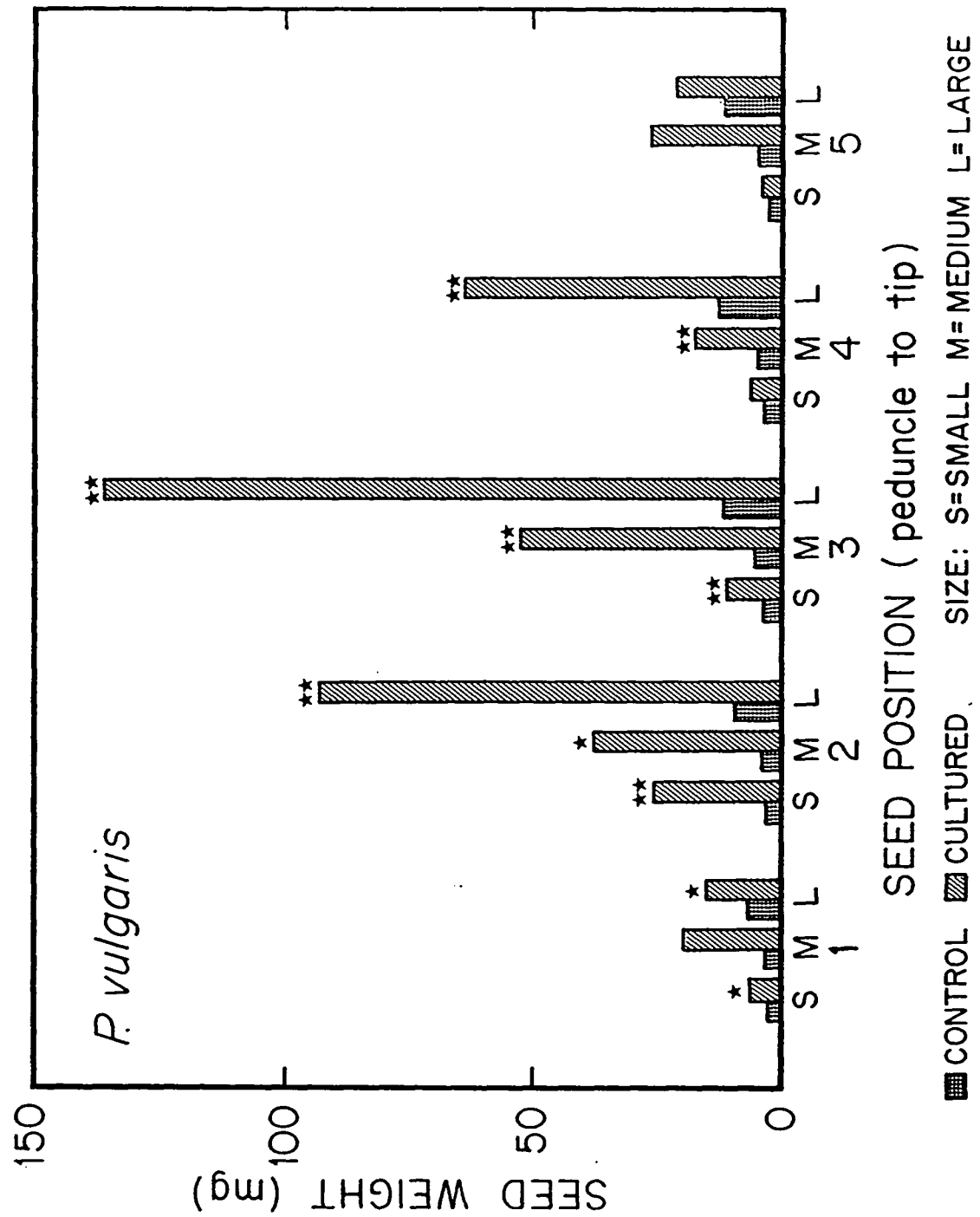
P. vulgaris
SEED WEIGHT (mg)
150
100
50
0
S M L S M L S M L S M L S M L
1 2 3 4 5
SEED POSITION (peduncle to tip)
CONTROL CULTURED SIZE: S=SMALL M=MEDIUM L=LARGE

Figure 5

Figure 6. Embryo length at five seed positions before (control) and after culturing of P. vulgaris cv. Great Northern pods of three sizes, small (S), medium (M) and large (L). Significant differences between cultured and control pods are indicated for each position (* for 0.05 level and ** for 0.01 level by the t test).

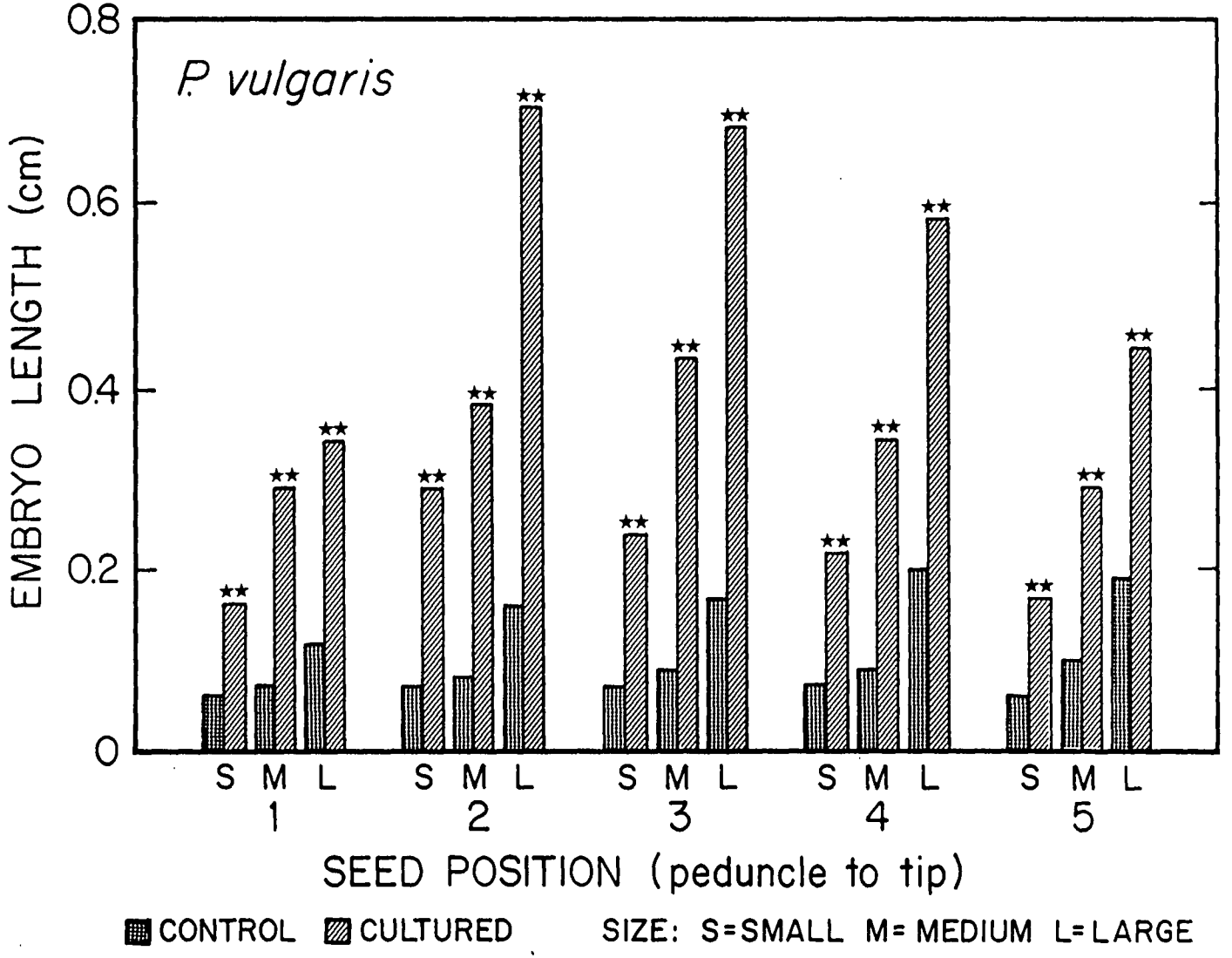
P. vulgaris
EMBRYO LENGTH (cm)
0.8
0.6
0.4
0.2
0
S M L S M L S M L S M L S M L
1 2 3 4 5
SEED POSITION (peduncle to tip)
CONTROL
CULTURED
SIZE: S=SMALL M= MEDIUM L=LARGE

Figure 6

Table 3. Means separation by the LSD test (0.05 level) of the fresh weight of seeds at 5 positions in pods of *P. vulgaris* cv. Great Northern cultured for 21 days. Separate analysis were performed for each of the three classes.

Pod size	Seed position (pedicel to tip)					
	1	2	3	4	5	p-value
Large	14.8 c	92.1 ab	136.9 a	63.7 bc	21.9 c	0.000
Medium	18.9	37.2	52.0 NS	17.5	26.7	0.322
Small	5.6 b	25.3 a	10.6 b	6.7 b	4.2 b	0.000

NS Non significant difference (F test).

Table 4. Means separation by the LSD test (0.05 level) of the length of embryos at 5 positions in pods of P. vulgaris cv. Great Northern cultured for 21 days. Separate analysis were performed for each of the three classes.

Pod size	Seed position (pedicel to tip)					
	1	2	3	4	5	p-value
Large	0.34 c	0.70 a	0.68 a	0.58 ab	0.44 bc	0.000
Medium	0.29	0.38	0.43 NS	0.34	0.29	0.476
Small	0.16 b	0.29 a	0.24 ab	0.22 ab	0.17 b	0.031

NS Non significant difference at (F test).

Figure 7. An immature and mature embryo of P. vulgaris cv. Great Northern obtained from the same pod (large size) after culturing.

Figure 7

longer than 21 days, some of the seeds which had reached maturity began to germinate within the pod (Fig. 8).

To determine whether the position effects may, at least in part, be related to differences in seed size at the start of the culture period, seed weight and embryo length of the control group (before culture) were analyzed statistically. There was a highly significant effect of the position only on seed weight of the smaller and medium groups. These were due to the lower weights at the first position (LSD test). Thus, it seems that at early developmental stages the seed size at the first position is lagging behind the other positions.

C. Pod culture of *P. vulgaris* cv. Great Northern x *P. acutifolius* P.I. 321637

Pods resulting from *P. vulgaris* cv. Great Northern x *P. acutifolius* P.I. 321637 crosses were cultured at 14 and 21 days after pollination. These particular ages were selected since the size of embryos fell in the same range as those in the earlier experiment with the larger *P. vulgaris* cv. Great Northern pods. In this case classification by age was preferable due to the large difference in size of pods and the absence of a clear correlation between pod size and embryo size. The culture period for the hybrid pods was shorter than that for selfed pods since the hybrid pods turned yellow at an earlier time than the selfed pods. Extending the culture period did not result in further growth of hybrid embryos.

During the culture period the pods did not increase in length (Table 5). In contrast to the selfed embryos, hybrid embryos in pod

Figure 8. Germinating seed in pod of P. vulgaris cv. Great Northern after culturing.

Figure 8

Table 5. Length of two groups of pods (21 days and 14 days after pollination at start of culture period) of *P. vulgaris* cv. Great Northern x *P. acutifolius* P.I. 321637 before and after a culture period of 16 days.

Pod age	Length of pod (cm)	
	Before culture	After culture
21 days	8.7	8.7 NS
14 days	9.2	9.3 NS

NS Non significant difference in size before and after culture (t test).

culture did not reach maturity, but germinated precociously (Fig. 9). The slight increases in hybrid seed weight (Fig. 10) and embryo size (Fig. 11) during the culture period were not due to further embryo development but rather to the formation of roots. Moreover, the weight and size increases were small relative to the increases observed in the selfed seeds and embryos of P. vulgaris cv. Great Northern, even though the initial hybrid seed weights were comparable to those of the largest class of selfed pods used. The absence of seed position effects (analysis of variance), both in seed weight and embryo length, also distinguished hybrids from selfs.

D. The effects of growth regulators on pod culture of P. vulgaris cv. Great Northern.

The responses of cultured pods to 10^{-6}M BAP, GA_3 and ABA in the medium were tested. The results of these experiments with regard to the fresh weight of seeds of P. vulgaris cv. Great Northern are presented in Tables 6, 7 and 8 respectively for the large, medium and small sizes of pods. Again, there were significant increases in the fresh weights of seeds in the cultured pods as compared with the control group (before culture). The effects of the growth regulators, the seed positions and their interactions were determined by the analysis of variance. These analyses showed that the interactions between growth regulator and position effects were highly significant for the large and medium size classes. This indicates that there are shifts in optimal seed position between the growth regulator treatments. The data suggest that while the middle positions were optimal for the ABA and 0 (control)

Figure 9. Embryos of P. vulgaris cv. Great Northern x P. acutifolius P.I. 321637 after culturing of a 21-day old pod.

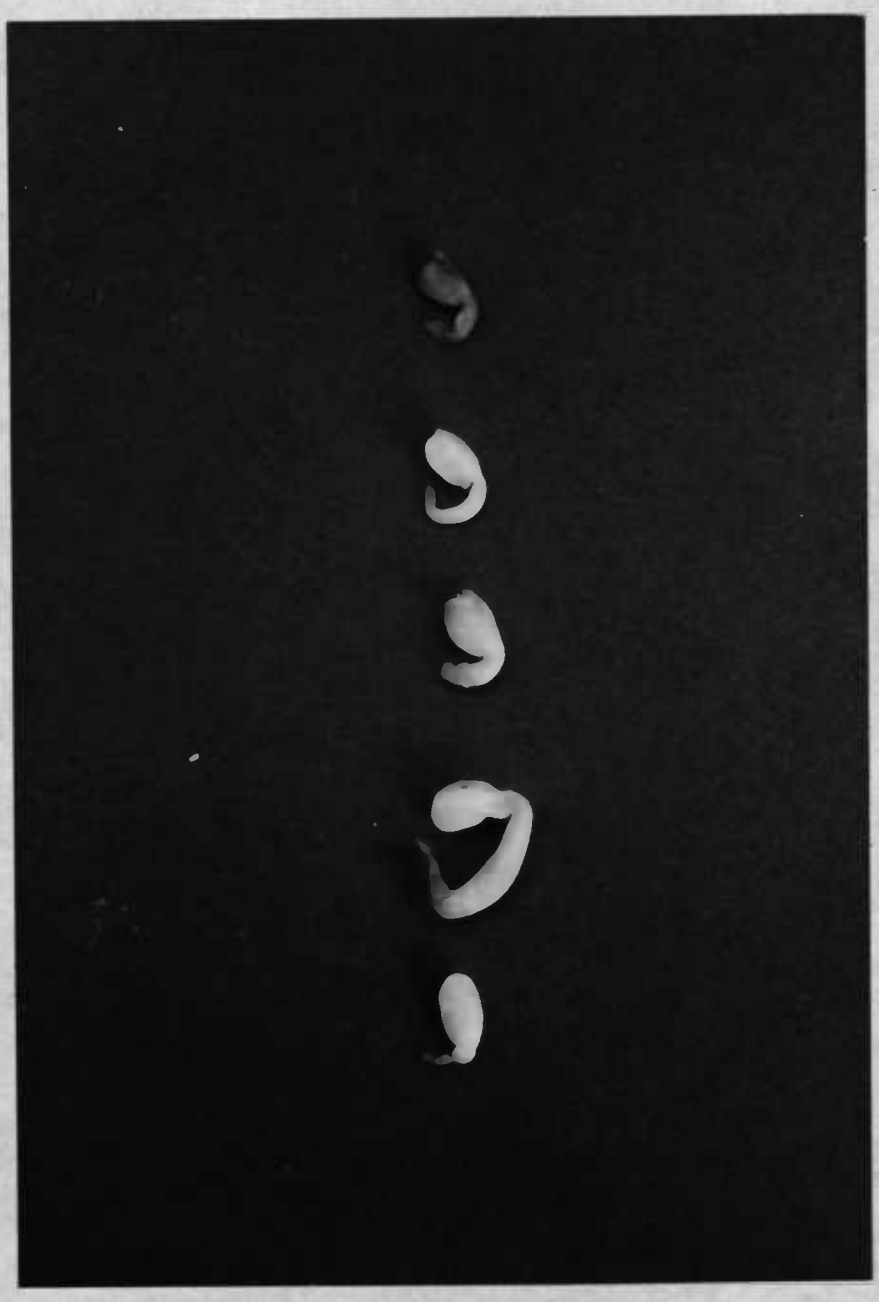

Figure 9

Figure 10. Seed weight at five seed positions before (control) and after culturing of P. vulgaris cv. Great Northern x P. acutifolius P.I. 321637 pods of two ages, 14 and 21 days after pollination. Significant differences between cultured and control pods are indicated for each position (* for 0.05 level and ** for 0.01 level by the t test).

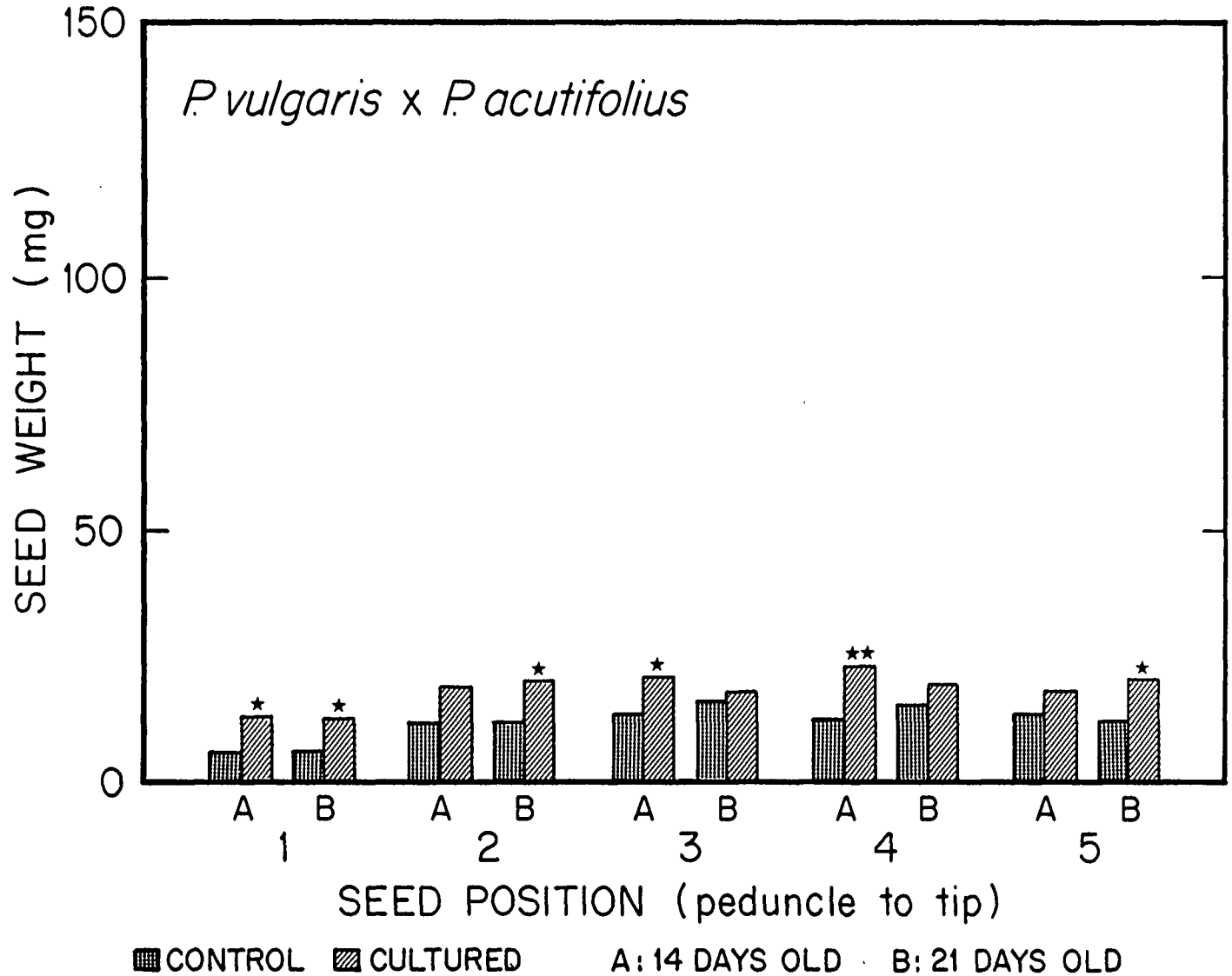
P vulgaris x P acutifolius
SEED WEIGHT (mg)
150
100
50
0
A B A B A B A B A B
1 2 3 4 5
SEED POSITION (peduncle to tip)
CONTROL
CULTURED
A: 14 DAYS OLD
B: 21 DAYS OLD

Figure 10

Figure 11. Embryo length at five seed positions before (control) and after culturing of P. vulgaris cv. Great Northern x P. acutifolius P.I. 321637 pods of two ages, 14 and 21 days after pollination. Significant differences between cultured and control pods are indicated for each position (* for 0.05 level and ** for 0.01 level by the t test).

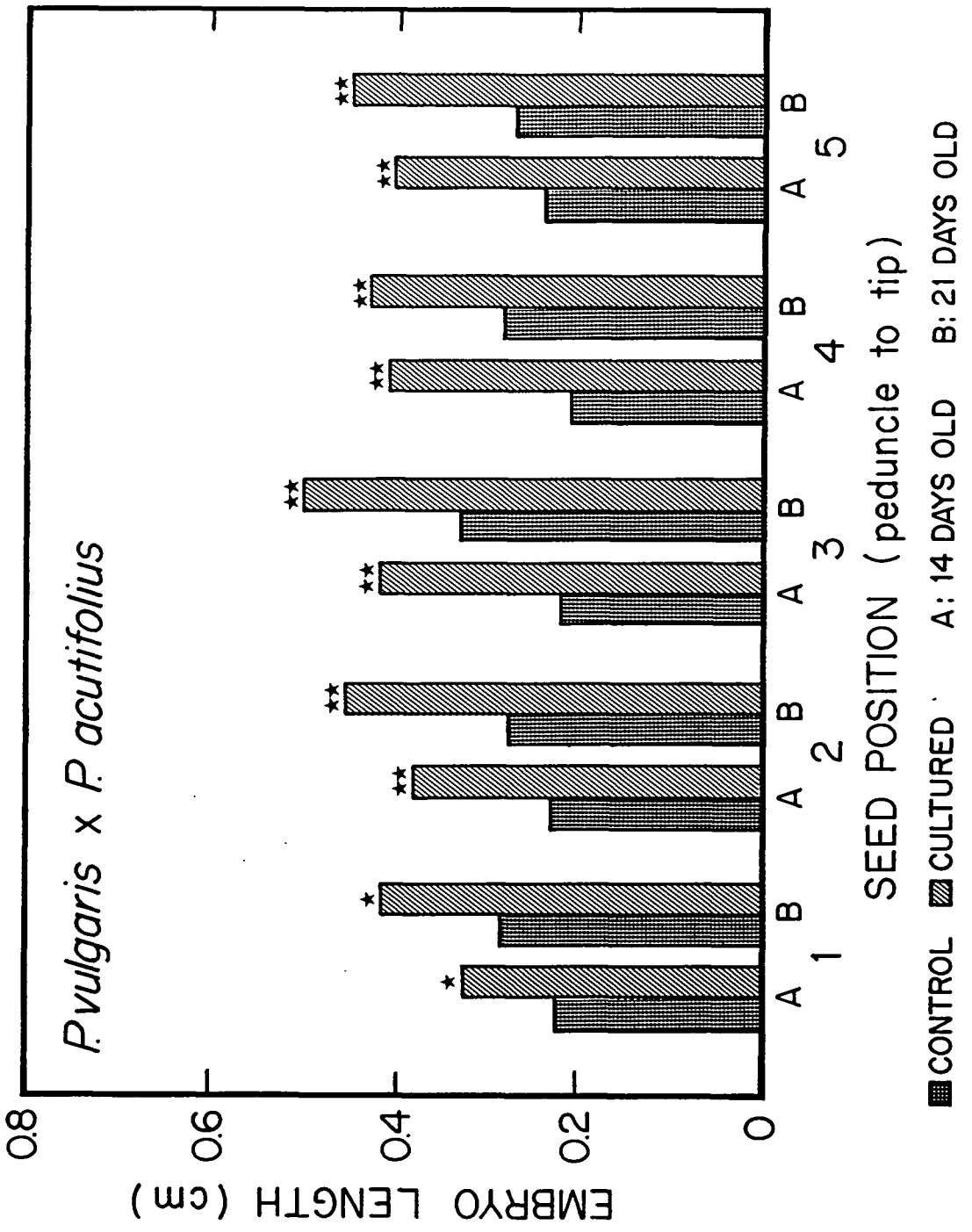
P.vulgaris x P. acutifolius
EMBRYO LENGTH (cm)
0
0.2
0.4
0.6
0.8
SEED POSITION (peduncle to tip)
1 2 3 4 5
A B A B A B A B A B
CONTROL CULTURED A: 14 DAYS OLD B: 21 DAYS OLD

Figure 11

Table 6. Effects of growth regulators[1] and seed position on the fresh weight of seeds of P. vulgaris cv. Great Northern in pods (large size) cultured for 21 days.

Treatment	Seed position (pedicel to tip) 1	2	3	4	5
	Fresh weight (mg)				
Before culture	5.6	7.6	8.9	10.8	8.6
After culture					
0	7.6 c[2]	12.6 c	135.5 a	92.3 ab	61.3 bc
BAP	89.3 ab	78.2 abc	47.7 bc	38.8 bc	51.1 bc
GA3	19.1 bc	72.9 abc	47.1 bc	42.8 bc	45.1 bc
ABA	10.0 c	26.7 bc	145.8 a	34.1 bc	49.0 bc

[1] Growth regulator concentration: 10^{-6} M
[2] Means separation by the LSD test (0.05 level)

Table 7. Effects of growth regulators[1] and seed position on the fresh weight of seeds of *P. vulgaris* cv. Great Northern in pods (medium size) cultured for 21 days.

Treatment	Seed position (pedicel to tip) 1	2	3	4	5
	Fresh weight (mg)				
Before culture	3.1	4.6	5.2	5.6	4.8
After culture					
0	6.7 cd[2]	33.6 ab	5.6 cd	20.6 abcd	5.7 cd
BAP	41.4 a	21.6 abcd	12.5 bcd	3.2 cd	1.4 d
GA3	11.9 bcd	5.0 cd	23.3 abcd	27.4 abc	26.2 abcd
ABA	17.4 abcd	23.5 abcd	13.9 bcd	8.3 cd	4.8 cd

[1] Growth regulator concentration: 10^{-6} M

[2] Means separation by the LSD test (0.05 level)

Table 8. Effects of growth regulators[1] and seed position on the fresh weight of seeds of P. vulgaris cv. Great Northern in pods (small size) cultured for 21 days.

Treatment	Seed position (pedicel to tip)				
	1	2	3	4	5
	Fresh weight (mg)				
Before culture	2.2	2.8	3.1	3.3	3.5
After culture					
0	4.7	9.3	15.8	5.8	2.7
BAP	10.9	14.0	4.5	2.6	0.7
GA3	2.8	3.6	7.4	3.9	2.7
ABA	5.6	7.8	4.2	2.3	1.7
			NS[2]		

[1] Growth regulator concentration: 10^{-6} M
[2] NS, Non significant difference (F test)

treatments, the greatest increases in seed weight in the presence of BAP were at the first position. However, although there were highly significant effects of the seed position for all three size classes, there were no significant differences between the growth regulator treatments.

The effects of growth regulators in the pod culture medium on the length of embryos of P. vulgaris cv. Great Northern are presented in Tables 9, 10 and 11 respectively for the large, medium and small sizes of pods. Generally, the embryo length displayed response patterns similar to the seed weight. However, the interaction between the growth regulator and position effects was significant only for the large pod class. Again, the effects of growth regulators were not significant. The position effect was highly significant for the large pod class, but not significant for the medium and small classes.

E. The effects of growth regulators on pod culture of P. vulgaris cv. Great Northern x P. acutifolius P.I. 321637

The effects of growth regulators in the medium on the growth of seeds and embryos were also determined for the interpecific hybrids. The results of these experiments with regard to seed weight are presented in Tables 12 and 13 respectively for the pods cultured 21 days and 14 days after pollination. Addition of growth regulators at 10^{-6}M did not stimulate seed growth and development over the 0 control. Results of the analysis of variance indicated that the growth regulator treatments were not significant. Although the interactions between growth regulator treatments and the positions were not significant,

Table 9. Effects of growth regulators[1] and seed position on the length of embryos of P. vulgaris cv. Great Northern in pods (large size) cultured for 21 days.

Treatment	Seed position (pedicel to tip) 1	2	3	4	5
	Embryo length (cm)				
Before culture	0.12	0.13	0.17	0.17	0.15
After culture					
0	0.26 e[2]	0.31 de	0.67 ab	0.67 ab	0.49 bcde
BAP	0.52 bcd	0.61 abc	0.49 bcde	0.44 bcde	0.51 bcde
GA3	0.29 de	0.53 abcd	0.49 bcde	0.46 bcde	0.46 bcde
ABA	0.26 e	0.39 cde	0.79 a	0.48 bcde	0.55 abcd

[1] Growth regulator concentration: 10^{-6} M
[2] Means separation by the LSD test (0.05 level)

Table 10. Effects of growth regulators[1] and seed position on the length of embryos of P. vulgaris cv. Great Northern in pods (medium size) cultured for 21 days.

Treatment	Seed position (pedicel to tip)				
	1	2	3	4	5
	Embryo length (cm)				
Before culture	0.08	0.09	0.10	0.10	0.10
After culture					
0	0.16	0.37	0.20	0.30	0.21
BAP	0.31	0.37	0.27	0.20	0.18
GA3	0.23	0.21	0.39	0.45	0.29
ABA	0.23	0.26	0.29	0.24	0.14
			NS[2]		

[1] Growth regulator concentration: 10^{-6} M
[2] NS, Non significant difference (F test)

Table 11. Effects of growth regulators[1] and seed position on the length of embryos of *P. vulgaris* cv. Great Northern in pods (small size) cultured for 21 days.

Treatment	Seed position (pedicel to tip)				
	1	2	3	4	5
	Embryo length (cm)				
Before culture	0.06	0.06	0.07	0.07	0.09
After culture					
0	0.12	0.16	0.21	0.21	0.14
BAP	0.19	0.24	0.11	0.15	0.05
GA3	0.14	0.16	0.19	0.13	0.10
ABA	0.13	0.22	0.18	0.17	0.18
			NS[2]		

[1] Growth regulator concentration: 10^{-6} M
[2] NS, Non significant difference (F test)

Table 12. Effects of growth regulators[1] and seed position on the fresh weight of seeds of P. vulgaris cv. Great Northern x P. acutifolis P.I. 321637 in pods (21 days after pollination at start of culture period) cultured for 16 days.

Treatment	Seed position (pedicel to tip)				
	1	2	3	4	5
	Fresh weight (mg)				
Before culture	6.5	11.9	16.6	16.3	10.9
After culture					
0	12.5	19.7	17.4	19.0	20.3
BAP	9.5	11.5	17.0	22.8	14.8
GA3	12.1	14.9	13.6	18.9	13.6
ABA	14.1	21.6	22.2	18.0	16.8
	b[2]	a	a	a	ab

[1] Growth regulator concentration: 10^{-6} M
[2] Mean separation by the LSD test (0.05 level)

Table 13. Effects of growth regulators[1] and seed position on the fresh weight of seeds of P. vulgaris cv. Great Northern x P. acutifolis P.I. 321637 in pods (14 days after pollination at start of culture period) cultured for 16 days.

Treatment	Seed position (pedicel to tip)				
	1	2	3	4	5
	Fresh weight (mg)				
Before culture	6.0	12.1	13.3	12.3	13.4
After culture					
0	13.0	18.6	21.0	22.5	18.2
BAP	16.1	26.2	25.6	22.3	24.0
GA3	13.0	17.4	22.0	17.4	17.5
ABA	12.0	17.9	22.1	23.5	19.0
Mean	13.5	20.0	22.7	21.4	19.6
	b[2]	a	a	a	a

[1] Growth regulator concentration: 10^{-6} M
[2] Mean separation by the LSD test (0.05 level)

there were highly significant position effects for both classes. These position effects were due to the significantly lower weight of the seeds at the first position (LSD test, 0.05 level).

The length of embryos in the same experiment are presented in Tables 14 and 15 respectively for the pods cultured 21 days and 14 days after pollination. Interestingly, the effects of growth regulators on embryo length were highly significant in the analysis of variance of the older pods, while neither the position effects nor the interactions were significant. The growth regulator effects were due to significant depression of growth by BAP and GA_3 as compared to the 0 control (Table 16).

Table 14. Effects of growth regulators[1] and seed position on the length of embryos of P. vulgaris cv. Great Northern x P. acutifolis P.I. 321637 in pods (21 days after pollination at start of culture period) cultured for 16 days.

Treatment	Seed position (pedicel to tip)				
	1	2	3	4	5
	Embryo length (cm)				
Before culture	0.29	0.28	0.33	0.28	0.27
After culture					
0	0.42	0.46	0.50	0.43	0.45
BAP	0.36	0.36	0.34	0.42	0.39
GA3	0.36	0.38	0.36	0.38	0.34
ABA	0.39	0.35	0.44	0.44	0.53
	NS[2]				

[1] Growth regulator concentration: 10^{-6} M
[2] NS, Non significant difference (F test)

Table 15. Effects of growth regulators[1] and seed position on the length of embryos of P. vulgaris cv. Great Northern x P. acutifolis P.I. 321637 in pods (14 days after pollination at start of culture period) cultured for 16 days.

Treatment	Seed position (pedicel to tip)				
	1	2	3	4	5
	Embryo length (cm)				
Before culture	0.23	0.23	0.22	0.21	0.24
After culture					
0	0.33	0.38	0.42	0.41	0.41
BAP	0.42	0.42	0.40	0.36	0.38
GA3	0.33	0.36	0.40	0.39	0.38
ABA	0.33	0.35	0.38	0.40	0.43
			NS[2]		

[1] Growth regulator concentration: 10^{-6} M
[2] NS, Non significant difference (F test)

Table 16. Means separation by the LSD test (0.05 level) of the length of embryos of *P. vulgaris* cv. Great Northern x *P. acutifolius* P.I. 321637, as influenced by the growth regulators, in pods (21 days after pollination at start of culture period) cultured for 16 days.

Pod stage	Growth regulators			
	0	BAP	GA_3	ABA
21 days old	0.45 a	0.37 bc	0.36 c	0.43 ab

V. DISCUSSION

The data presented here show that pod elongation as well as seed and embryo growth can be stimulated by the pod culture protocol. Pod elongation was significant for the small and medium groups, but not for the larger group, since at that point about the maximum pod length was attained. Pod elongation preceeds the phase of rapid weight increase of seeds in Phaseolus (Walbot et al., 1972). The significant enhancement of selfed seed and embryo growth of P. vulgaris by pod culture indicates that the procedure can partly replace in vivo conditions. Seeds weighing about 5 mg and containing embryos of less than 1 mm initially, could be nurtured to maturity. The same methodology was successfully applied to obtain mature seeds (over 400 mg) of P. acutifolius P.I. 321637, starting with pods containing the same size embryos as for P. vulgaris cv. Great Northern (unpublished results).

Only one or at most two selfed seeds in a pod could be stimulated to reach maturity in culture, while the other seeds remained small or increased only slightly in size. This indicates that a rapidly developing embryo may become an active sink for nutrients, limiting the growth of the other embryos. The optimal position may be determined by the interaction of several factors. One may be the initial size of the seeds, which seems to be smaller at the first position particularly. This may be due to the order of fertilization, which progresses from the tip of the ovary to the pedicel end. Another factor which may play a role could be the nutrient gradient in the cultured pod, possibly favoring the positions closer to the pedicel. In the experiments described here the developing embryo was more often located in the

middle of the pod than at the ends, which could be the result of the two opposing forces. Similar observations were made by Obendorf et al. (1983) for soybean pod cultures in which a higher percentage of the middle seeds were able to develop. However, in their experiments, the position effect was dependent 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 pedicel end. Optimal seed growth occurred when the pods were placed flat in the culture medium. We have attempted to enhance seed growth by varying our culture system, but found that submersion of pods or laying pods flat in a shallow layer of medium reduced embryo growth. It should be noted, however, that the initial seed weight in the experiments with soybeans was approximately 50 mg, while we used pods with seed weights ranging from 1 to 15 mg.

In contrast to the selfed seeds, interspecific hybrid seeds did not reach maturity after pod culture. Instead, the embryos germinated precociously. Previous studies of seed development in vivo have shown that in many of the interspecific crosses of Phaseolus, endosperm divisions were delayed, while the growth rate of embryos at early stages was also lower (Mok et al., 1978; Rabakoarihanta et al., 1979; Thomas, 1964). These observations suggest that interspecific hybrid embryos are intrinsically limited in their developmental potential. The results obtained from in vitro pod culture seem to confirm these conclusions since selfed seeds were able to grow to maturity.

The physiological basis of hybrid embryo arrest is not known. The finding by Nesling and Morris (1979) that P. vulgaris x P. acutifolius embryos contain lower levels of extractable cytokinins than those of the

parental species indicate 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 x P. vulgaris embryos from the four-celled stage to the globular stage (Mok et al., 1986). However, addition of BAP to the pod culture medium in this study did not promote further development of the interspecific P. vulgaris x P. acutifolius embryos, but had a slight inhibitory effect on the root elongation of the precociously germinating embryos. Also GA_3 depressed root elongation, while ABA did not have any effect. The growth regulators neither promoted nor inhibited seed growth in the selfed pods. The only effect of growth regulators was the shift in the optimal position in the pod, which was most obvious in the presence of BAP.

It may not be easy to explain the results obtained here concerning the effects of growth regulators on hybrid embryo growth. Since only one growth regulator concentration was tested, it is not known what the effects over a range of concentrations would be. Moreover, it is difficult to predict the levels of growth regulators reaching the embryos due to the presence of the pod tissue. Also, it seems that the endogenous hormone levels in the interspecific hybrid embryos are quite different from those in normal embryos. Thus, these results may not be compared with those obtained in cultures of isolated normal embryos (Walbot et al., 1972; Yeung and Sussex, 1979).

The ability to substitute embryo rescue with pod culture to obtain interspecific hybrids is of practical value. Pod culture is much simpler than embryo culture, because dissection of embryos at early stages can be avoided. Although mature hybrid seeds have not been generated in pod culture, plants can be obtained either by directly

placing the germinating embryos in soil or after a brief phase of culturing the germinating embryos in culture medium. The germinating embryos offer some advantage over embryos dissected directly from immature pods. For example, the already developed roots promote nutrient uptake and hardening. However, the plantlets are not as strong as those obtained from mature selfed seed and special care must be taken to prevent wilting. Further studies of interspecific hybrid embryos may lead to identification of the factors involved in the developmental arrest and precocious germination, which may also benefit the design of optimal pod culture medium leading to formation of more mature interspecific hybrid embryos.

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