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CULTURE OF VERY YOUNG *PHASEOLUS VULGARIS* L. PODS AND PLANTLET REGENERATION.

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Abstract

In order to rescue early aborting hybrids between *Phaseolus polyanthus* Greenm. (♀) x *Phaseolus vulgaris* L., a pod culture technique was developed on 2-day old *P. vulgaris* pods. Seven different culture techniques are described using a modified Phillips *et al.* (1982) medium for maturation. The application of high and variable osmolality conditions similar to what is observed *in vivo* during pod culture (1 wk) and before extracting the embryos, gave the best results in terms of pod, ovule and embryo development.

Our pod culture technique is a valuable way to rescue very immature embryos. High germination rates can be obtained, plantlet survival is higher than in any other technique used before and, for the first time, plantlet regeneration has been obtained from 2 days old *P. vulgaris* embryos. This technique could provide an alternative solution for crossing *Phaseolus* species when embryos abort at very early stages.

Key words: *Phaseolus vulgaris* L., osmotic pressure, sucrose, mannitol, Polyethylene glycol 8000, *in vitro*.

1. Introduction

Major production constraints of the common bean in Latin America and Africa are *Ascochyta* blight, Bean Golden Mosaic Virus (BGMV), and Bean Fly. A critical case study is the introgression of *Ascochyta* blight resistance from *Phaseolus polyanthus* Greenm. (PP) into the common bean *Phaseolus vulgaris* L. (PV) (Singh, 1999). Preliminary studies (data not shown) on the early abortion in PP (♀) x PV crosses showed that early nutritional barriers are related to a deficient endosperm development. In these experiments, the development of hybrid embryos was very poor when maintained *in vivo*. Most of the zygotes were still able to divide 3 to 4 days after pollination (DAP) which suggest that zygotes should maintain the ability to differentiate into hybrid embryos 2 days after cross-fertilization. Nevertheless, only 4 embryos out of 110 could continue their development until 6 DAP. Based on the above observations, we hypothesize that embryos of PP (♀) x PV should have the potential to be excised from expanded ovules or pods cultured at 2 to 3 DAP during several days. First results on the development of an *in vitro* pod culture technique for young pods of PV are reported by Geerts *et al.* (2000). Different culture techniques for 2-day old *Phaseolus* pods were described using a modified Phillips *et al.* (1982) medium for maturation. The authors showed that the application of high and variable osmolality conditions similar to those observed *in vivo*, during pod culture (1 wk) and before extracting the embryos, gave the best results in

terms of ovule and embryo development. A germination rate of 45.5 % was obtained and plantlet survival was higher than in any other technique used before (Lecomte, 1997). However, the plantlet survival remained very low (3 %) and no regeneration was described.

In this paper, we report advances in the development of *in vitro* pod culture technique described by Geerts *et al.* (2000). Different osmolality agents (sucrose, mannitol and Polyethylene glycol 8000) used in liquid and solid media were compared to confirm the interest to adjust *in vitro* conditions to those observed *in vivo*. Moreover, preliminary experiments were conducted in order to increase regeneration of plantlets. The use of a dehydration medium as proposed by Hu and Zanettini (1995) and of auxin to improve plantlet rooting are discussed.

2. Material and methods

2.1. Plant Material

A *P. vulgaris* genotype (NI 637) was grown in a growth chamber under controlled conditions: day/night temperature of 24/20°C, light intensity of 580 $\mu mol.m^{-2}s^{-1}$ and a day length of 11 h 30 min.

2.2. Pod culture

Pod culture technique was adapted from Geerts *et al.* (1999). Young pods, collected 2 DAP, are sterilized by alcohol and sodium hypochloride and directly cultivated under light (60 $\mu mol.m^{-2}.s^{-1}$ light intensity; 11 h 30 min day length) in Petri dishes during 1 wk at 26°C and 100% relative humidity. Seven pod culture techniques were compared. Techniques differed in osmoticum agent (sucrose, mannitol, Polyethylene glycol 8000 or PEG) and medium consistency (liquid or solid). Three of them involve liquid media (L1, L2 and L3 - no agar) and the other techniques involve solid media (S1, S2, S3 and S4 - 7 $g.l^{-1}$ Select Agar-Gibco BRL).

For each of the solid cultures S1, S2 and S3, a sequence of three solid media (Medium I, Medium II and basal medium) were used. Medium I and Medium II are described in Table I. Basal medium contains Phillips *et al.* (1982) mineral salts, 80 $g.l^{-1}$ sucrose 1 $mg.l^{-1}$ thiamin HCl, 5 $mg.l^{-1}$ nicotinic acid, 0.5 $mg.l^{-1}$ pyridoxine, 100 $mg.l^{-1}$ myoinositol, 1,000 $mg.l^{-1}$ L-glutamine, 1,000 $mg.l^{-1}$ casein hydrolysate, 0.1 µM NAA, 10 µM adenine and 1.0 µM Benzylaminopurine. Pods were cultured on Medium I during 1 day, transferred to Medium II for 2 days more, and lastly transferred to basal medium for 4 days more. For S4 technique, pods were directly transferred on basal medium for one week.

For the culture on liquid media, pods were supported on sterilized glass beads (3 mm ϕ) in a Petri dish (15 cm x 4 cm) containing 100 ml of liquid Medium I (Table II). Petri dish was connected on the top-center by a peristaltic pump to a 1 liter bottle (Fig. 1b') containing the basal liquid culture medium without complementary addition of osmoticum. The composition of the basal liquid medium was similar to the composition of the basal solid medium excepted for the content in agar. During the first five days of culture, 100 ml per day were dripped from the 1 liter bottle in the Petri dish, permitting a constant evolution of the culture medium osmolality. After 5 days the osmolality of the

liquid medium in the Petri dish reached 350 mosm. A lateral aperture of the Petri dish discharges the excess of liquid, maintaining constant the volume of liquid in the Petri dish. During our experiments, a Millipore filter was added to prevent contamination from the 1 liter bottle (Fig. 1b) and Plant Preservative Mixture (PPM) was used to control pod contamination.

After one wk of pod culture, embryos were extracted from ovules reaching at least 2 mm and transferred to basal solid medium for 2 wk in order to continue maturation processes. Mature embryos were transferred to a dehydration medium for 2 wk (Hu and Zanettini, 1995) before culturing them on a pre-germination medium containing the salts of Gamborg *et al.* (1968) and 1 µM Acid Indol Acetic (AIA) for 1 wk. Eventually, embryos were transferred to a germination medium containing the salts of Gamborg *et al.* (1968) but without AIA.

The pod growth (PG) was assessed using the following parameter: PG = (Pf - Pi) / Pi x 100 where Pf is the final length (measured after 1 wk of culture) and Pi, the initial length of pod. At extraction, the number and the length (in mm) of developed ovules and the number of extracted embryos per pod were noted. After 14 days of *in vitro* culture, embryo growth and development on the maturation medium was assessed using the following parameters: (i) the germination rate or rate of embryo showing normal development of root axis, shoot primordia and at least one cotyledon; (ii) the embryo growth rate (LE) calculated as LE = (Lf - Li) / Li x 100 where Lf is the final length and Li, the initial length of embryo axis. After 50 days of *in vitro* culture, acclimatization rate was recorded as the number of plantlets still surviving after 50 days of culture out of the total number of germinated embryos. The results are discussed on the basis of variance analysis using one classification criteria (culture conditions) and *t* tests considering each pod as a replication.

3. Results

Figure 1 illustrates the different steps to regenerate plant from an *in vitro* pod culture technique. Table III presents the results concerning the influence of the seven culture techniques on *Phaseolus* pod, ovule and embryo growth and development. Significant differences between media were observed, mainly for pod growth (P = 0.019), number of ovules (P = 0.040) and of extracted embryos (P = 0.013) per pod. In general, pod growth was higher on liquid media compared to solid media, reaching 90.1 % on L1 media. However, pod growth was not correlated to the development of ovules, nor of embryos. Indeed, the number of pods containing at least one developed ovule was not significantly higher (55 % of the pods on L1 compared to 48 % on S1). Moreover, the number of ovules longer than 2 mm per pod was the highest on S1 media with a mean of 3.7 developed ovules per pod compared to a mean of 2 developed ovules on L1 media. Finally, the number of extracted embryos was also lower on L1 media with only 1.9 extracted embryos per pod compared to 3.8 embryos on S1 media.

While the development stage of extracted embryo was quite variable ranging from globular to cotyledonar, the mean embryo length was however higher when extracted from pods cultured on liquid media. Despite these data, no significant differences were noted during the development of embryos on basal solid medium whatever the technique used. A mean embryo growth of 96 % was obtained. Mean values of germination rate were quite different between media but with high standard deviations. Therefore, statistical analysis could not point out differences between media (P = 0.214). Also, the

mean acclimatization rate was close to 90 % without significant differences between media (P = 0.529).

4. Discussion

Contamination and use of PPM biocide have limited the number of observations. In our study, differences are mainly related to pod development. The following considerations can however be made. First, when comparing S1 to S4 techniques, we noticed the importance of using a medium containing a high sucrose content during the first days of culture. Second, the use of PEG affected negatively the ovule development. On S3 media, only 13 % of the pods contained at least one developed ovule. On L3 and S3 media, we observed curvature of the pods and the presence of superficial necrosis stopping pod growth. Also, hypertrophy of pod integument conducted to pods in which only one or two ovules developed well. Embryos extracted from these ovules reached very often cotyledonar stage but no further development was noted. Third, the use of mannitol permitted to obtain an intermediate pod growth of 65 % with a relatively high number of extracted embryos. At extraction, embryo stage and length were similar to those developed on sucrose media. Although contamination problems appeared during the germination of these embryos in liquid culture, all germinated embryos could survive during the first 50 days of culture. Fourth, the use of high concentrations of sucrose at the beginning of pod culture permitted to reach a germination rate of 60.7 %. This value is to be compared with the 45.5 % presented in our previous results (Geerts *et al.*, 2000) and confirmed the importance of osmotic adjustments when culturing young pods. Fifth, modifications of regeneration protocol, i.e., transfer of the embryos on the dehydration medium as proposed by Hu and Zanettini (1995) during two wk instead of one, and the further transfer during one week on a medium containing AIA, have increased significantly the acclimatization rate from 3 % in our previous results (Geerts *et al.*, 2000) to 90 %. Particularly, the addition of AIA at low concentration permitted to obtain a more regular plantlet rooting.

Despite this result, one factor responsible of the loss of embryos during the *in vitro* culture was the development of callus on root apex accompanied by vitrification. Callus appeared between the 25^{th} and 30^{th} day of culture at the end of the dehydration phase in more than 85 % of the developed embryos. Nevertheless, for the first time, regeneration of some plantlets from two days old PV embryos has been achieved (Fig. 1).

5. Conclusion

The pod culture techniques we developed allowed for the first time the regeneration of PV plantlets from two-day old embryos. A variation of the osmolality during the culture of the pods had a very positive effect on the development of the ovules and the embryos.

Although no significant differences could be observed between the sequential and the continuous osmolality modification techniques regarding the final regeneration rate of adult plants from the same number of cultivated pods, the follow-up of the culture was much easier when using our device to obtain a progressive decrease of the osmotic pressure of the medium.

Compared to the previous technique used in our laboratory (Geerts *et al.* 2000) where after one week of culture on the basal maturation medium the embryos were cultivated

only during one week on a dehydration medium before being transferred on a germination medium, better results in embryo germination and plantlet acclimatisation were obtained by cultivating successively the embryos for two wk on the dehydration medium and for one wk on pre-germination medium containing 1 µM AIA before transferring them on the final germination medium.

In order to increase the regeneration rate we obtained for very immature PV embryos, progress have to be made in the control of pod contamination during cultivation and in the development of a more adequate hormonal balance enhancing the rooting and the development of germinated embryos.

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Table I. Description of the culture technique on solid media: type and quantity of osmoticum added to the basal medium respectively for S1, S2 and S3.

Techniques	*Osmoticum added*	*Medium* I* (1) *580 mosm* (+)	*Medium* II* (2) *450 mosm*
S1	Sucrose	63 $g.l^{-1}$	22 $g.l^{-1}$
S2	Mannitol	36 $g.l^{-1}$	16 $g.l^{-1}$
S3	Polyethylene glycol 8000	128 $g.l^{-1}$	55 $g.l^{-1}$

*Medium containing Phillips *et al.* (1982) mineral salts and 80 $g.l^{-1}$ sucrose. (1) contains 0.25 $mg.l^{-1}$ thiamin HCl, 1.25 $mg.l^{-1}$ nicotinic acid, 0.125 $mg.l^{-1}$ pyridoxine, 25 $mg.l^{-1}$ myoinositol, 250 $mg.l^{-1}$ L-glutamine, 250 $mg.l^{-1}$ casein hydrolysate, 0.095 µM abscisic acid, 5.5 µM Tryptophan and 0.1 µM NAA; (2) contains 1 $mg.l^{-1}$ thiamin HCl, 5 $mg.l^{-1}$ nicotinic acid, 0.5 $mg.l^{-1}$ pyridoxine, 100 $mg.l^{-1}$ myoinositol, 1,000 $mg.l^{-1}$ L-glutamine, 1,000 $mg.l^{-1}$ casein hydrolysate, 0.1 µM NAA, 10 µM adenine and 1.0 µM Benzylaminopurine, (+) osmolalities of media.

Table II. Description of the culture technique on liquid media: type and quantity of osmoticum added to the basal medium respectively for L1, L2 and L3.

Techniques	*Osmoticum*	*Medium* I* (1) *580 mosm* (+)
L1	Sucrose	64 $g.l^{-1}$
L2	Mannitol	36 $g.l^{-1}$
L3	Polyethylene glycol 8000	128 $g.l^{-1}$

*Medium containing Phillips *et al.* (1982) mineral salts and 80 $g.l^{-1}$ sucrose, 0.25 $mg.l^{-1}$ thiamin HCl, 1.25 $mg.l^{-1}$ nicotinic acid, 0.125 $mg.l^{-1}$ pyridoxine, 25 $mg.l^{-1}$ myoinositol, 250 $mg.l^{-1}$ L-glutamine, 250 $mg.l^{-1}$ casein hydrolysate, 0.095 µM abscisic acid, 5.5 µM Tryptophan and 0.1 µM NAA. (+) osmolality of medium.

Table III. Evaluation of the influence of seven culture conditions on *Phaseolus* pod, ovule and embryo growth and development.

Parameters	*Medium*						
	L1	*L2*	*L3*	*S1*	*S2*	*S3*	*S4*
Total number of pods	87	46	28	181	24	16	67
Pod growth (in %)	90.1 ± 4.3	67.6 ± 6.2	73.8 ± 7.9	43.9 ± 3.3	63.6 ± 4.8	28.1 ± 6.8	37.1 ± 5.6
Number of pods containing at least one developed ovule	48 (55 %)	20 (44 %)	12 (43 %)	87 (48 %)	15 (63 %)	2 (13 %)	29 (43 %)
Mean number of developed ovules per pod	2.0 ± 0.2	3.5 ± 0.4	1.8 ± 0.5	3.7 ± 0.2	2.1 ± 0.3	1.0 ± 0.0	2.6 ± 0.2
Mean ovule length after 7 days pod culture (in mm)	1.7 ± 0.1	1.6 ± 0.1	2.1 ± 0.1	1.6 ± 0.0	1.5 ± 0.1	1.7 ± 0.2	1.6 ± 0.1
Mean number of extracted embryos per pod	1.9 ± 0.2	3.0 ± 0.3	1.7 ± 0.3	3.8 ± 0.2	2.2 ± 0.3	1.0 ± 0.0	2.7 ± 0.2
Mean embryo length at extraction (in µm)	17.4 ± 2.7	17.5 ± 3.7	18.8 ± 5.8	16.9 ± 2.3	14.3 ± 3.8	13.8 ± 12.5	16.5 ± 4.1
and corresponding stages(*)	G to C	LG to C	EHS to C	G to C	G to HS	G to EHS	G to C
Embryo growth (in %)	98.8 ± 0.3	96.4 ± 0.2	-	94.2 ± 1.1	87.4 ± 3.8	99.0 ± 1.6	99.1 ± 1.2
Total number of cultured embryos	35	12	-	82	12	2	24
Germination rate (in %)	30.8 ± 7.5	7.5 ± 5.1	-	60.7 ± 4.0	38.2 ± 12.1	50.0 ± 50.0	50.1 ± 8.5
Acclimatization rate (in %)	91.7 ± 6.0	100.0 ± 0.0	-	77.6 ± 4.3	100.0 ± 0.0	100.0 ± 0.0	76.0 ± 9.0

Values are given with their standard error (± SE);(*) G = globular, LG = late globular, EHS = early heart-shaped, HS = heart-shaped, C = cotyledonar.

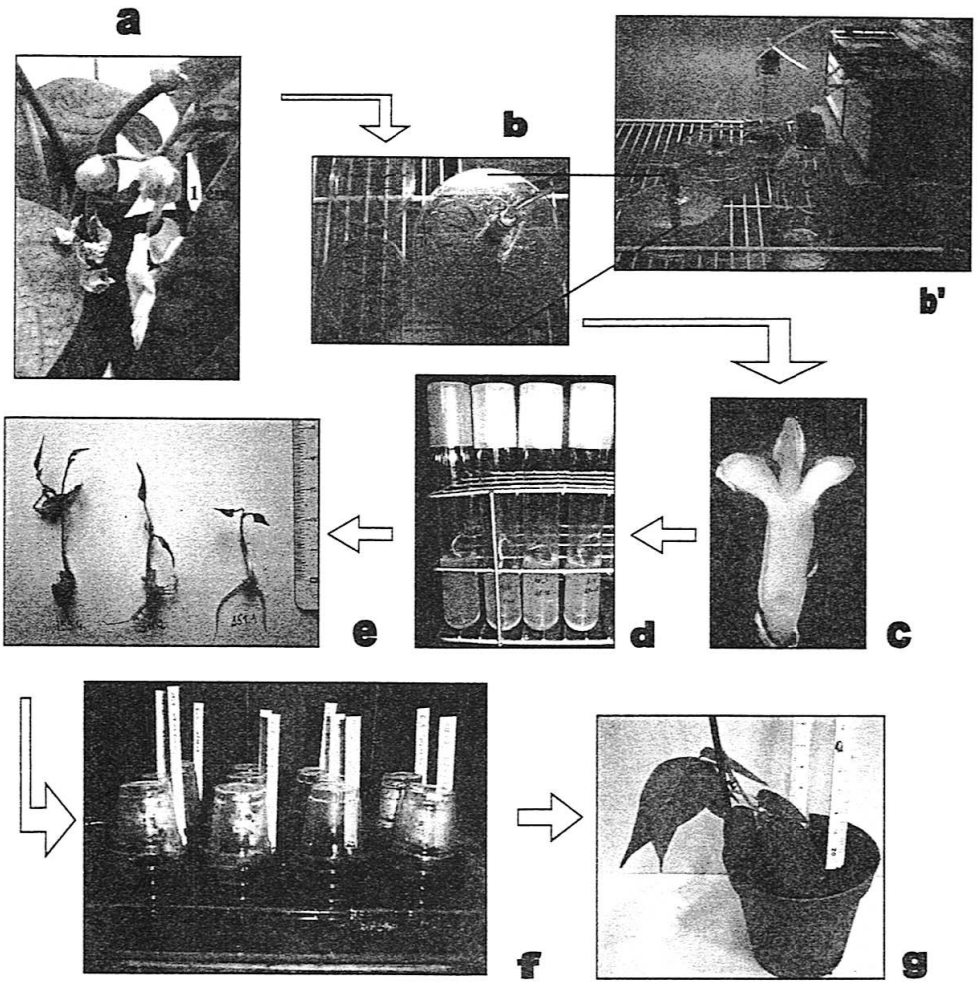

Figure 1. Steps of plant regeneration using an *in vitro* pod culture technique adapted for 2 days old *Phaseolus* pods. Pods are collected 2 DAP (a) and cultured on solid medium (b at left) or on liquid medium (b at right and b'). After one wk, embryos are extracted and cultured on solid media for maturation (c). After a dehydration period, germinated embryos are transferred in tubes for rooting in presence of AIA (d and e). After rooting and a period of *in vitro* growth, plantlets are transplanted in Jiffy pots for acclimatization (f). Eight wk later, plantlets are regenerated (g).