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Improved protoplast culture and regeneration of shoots in pea (*Pisum sativum* L.)

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ABSTRACT

An improved system involving a modification of the bead culture system was developed for culturing pea protoplasts. Using this method, sustained divisions and callus growth could be obtained in all 10 cultivars tested. In the best responding cultivar division frequency could be raised from 17% in liquid culture to 80% in the bead system. Shoot regeneration with a reproducible frequency of about 1% could be obtained from protoplast-derived calli in two of the tested cultivars.

Abbreviations:

ABA	abscisic acid
BA	N^6-benzyladenine
NAA	α-naphthylacetic acid
2,4-D	2,4-dichlorophenoxy-acetic acid

INTRODUCTION

Legumes belong to the world's most important crops, the improvement of which is of great interest to plant breeders. The conventional breeding programs may be complemented with *in vitro* techniques where efficient protoplast and tissue culture systems allowing plant regeneration can be developed. Regeneration from various explants has been achieved in a number of legume species. Many forage legumes can be regenerated from protoplasts *in vitro*, while most grain legumes still appear to be recalcitrant (for reviews, see Dale 1983, Hammat et al. 1986). Organogenesis from protoplasts in grain legumes has so far been reported only in *Vigna* species (Davey et al. 1974, Shekhawat and Galston 1983, Krishnamurthy et al. 1984), *Psophocarpus tetragonolobus* (Wilson et al. 1985) and a wild relative of soybean, *Glycine canescens* (Newell and Luu 1985). In *Pisum sativum*, no organogenesis from protoplasts has been reported so far, although protoplast cultures can be established and induced to form callus (Constabel et al. 1973, Landgren and Torrey 1973, Gamborg et al. 1975, von Arnold and Eriksson 1976, 1977, Jia 1981). In this study we describe an improved method for culturing pea protoplasts and propose a regeneration scheme which gives reproducible regeneration of shoots from protoplast-derived callus.

MATERIALS AND METHODS

Plant Material

Ten different cultivars of *Pisum sativum*, cvs. Bello, Belman, Filby, Lotta, Petra, Proco, Simo, Stivo, Timo and Vreta were tested for their response in protoplast culture. Greenhouse-grown plants were kept at 12 h photoperiod at 25/20°C day/night temperature. From these the fourth leaf pair counted from the base of plants with 5 - 6 leaf pairs (von Arnold, pers. comm.) was used for protoplast isolation. The leaves were surface sterilized by soaking for 10 min in 10% Klorin (4.5% hypochlorite) with a few drops of TWEEN 20 and rinsed three times with sterile double distilled water.

Seeds were surface sterilized as described above except that 20% Klorin was used for 20 min, and germinated in the darkness at 20°C on 1.5% Bacto agar supplemented with 0.05 M $CaCl_2$. After 12 days, the epicotyls were excised and used for protoplast isolation. Shoot cultures were established from 12-day old seedlings on B5 medium (Gamborg et al. 1968) without hormones, supplemented with 2% sucrose and solidified with 0.4% SeaPlaque agarose (FMC corporation). They were subcultured monthly and maintained in culture chambers at 20°C in 16/8 h day/night regime with 25 $\mu E\ m^{-2}s^{-1}$ light intensity (Osram L 36W/30, warm white). These culture conditions were used for all subsequent experiments unless otherwise stated.

Protoplast Isolation

Protoplasts were isolated as described by Glimelius (1984) except that 0.4 M glucose was used instead of 0.4 M sucrose in the enzyme solution containing 1% Cellulysin and 0.1% Macerase (Calbiochem-Behring) in a modified K3 medium (Nagy and Maliga 1976). Briefly the method was as follows: Before the enzyme treatment, the tissue was cut into 0.5 mm thin strips and preplasmolysed for 1 h in

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0.3 M sorbitol and 0.05 M $CaCl_2$. The tissue was digested in the enzyme solution overnight and then filtered through a 53 µm nylon mesh. The protoplasts were floated on top of a 1:1 0.4 M sucrose:percoll cushion before washing twice by sedimentation in W5 medium (Menczel et al. 1981).

Protoplast Culture and Regeneration Experiments

The modified 8p medium (Glimelius 1984) with 0.4 M glucose, 4.5 µM 2,4-D, 2.2 µM BA and 2% sucrose was used in initial protoplast culture. Protoplasts were either grown in liquid culture and plated after 14 days as described by Glimelius (1984), or cultured using a modification of the bead culture system (Shillito et al. 1983). Protoplasts were initially suspended at a density of $1.5 \cdot 10^5$ protoplasts/ml. After 2 days they were diluted in liquid culture or embedded in 0.6% agarose in the bead culture system to a density of $7.5 \cdot 10^4$ protoplasts/ml. The agarose beads were transferred to liquid medium at day 6 after the isolation and subcultured weekly. At this time, the hormone content of the culture medium was reduced to one tenth of the original and a four-step serial dilution of glucose was started. After four weeks, when the level of glucose was zero, 2,4-D was replaced by 0.54 µM NAA.

When the colonies derived from protoplasts had reached a diameter of 0.5 - 1 mm they were transferred to B5 medium supplemented with 2% sucrose, 4 µM BA and 0.54 µM NAA and solidified with 0.4% agarose. At different time points the calli were transferred to regeneration medium (Kunakh et al. 1984) consisting of B5 with 2% sucrose, 22 µM BA, 23 µM kinetin and 3.8 µM ABA, and solidified with 0.4% agarose.

For the first six days after protoplast isolation the cultures were kept in low continuous light (0.5 µE $m^{-2}s^{-1}$, Osram L-fluora 40W/77) at 25°C after which they were transferred to culture chambers. The division frequency of the protoplasts expressed as percent of dividing protoplasts of all living protoplasts was determined six days after isolation by counting 600 protoplasts from each independent experiment.

From the initial experiments several cultivars were selected for comparing the different variables of the bead culture system. The effect of 4.6 µM zeatin riboside replacing BA was tested as well as the effect of embedding the protoplasts in 0.5 ml drops instead of whole discs. The optimal time for agarose embedding was determined by embedding the protoplasts at day 0, 2, 4 and 6 after isolation. Greenhouse-grown plants, axenic shoot cultures and epicotyls were tested to determine the effect of donor tissue on the growth of protoplast-derived calli.

RESULTS AND DISCUSSION

Optimizing the Culture System

Agarose-embedding compared to the liquid culture improved division frequency in all the tested cultivars (table 1). In the best responding cultivar, Filby, the division rate increased from 17% to 80% and division rates up to 91% could be obtained using this method (figure 1). In those cultivars where no divisions were observed in liquid culture (Belman, Lotta, Simo and Timo) sustained divisions could also be obtained by using the bead culture system. The survival of Filby protoplasts after two days of culture was 67%. The first divisions in culture took place on day 2 - 3 after protoplast isolation.

The effect of timing of protoplast embedding was tested in cv. Filby (table 2). Highest division frequencies were obtained when protoplasts were embedded 2 days after isolation, while embedding at day six resulted in division rates comparable to those obtained in liquid culture. It thus appears that the embedding affects the early phases of colony formation positively, but has little effect when applied after this critical stage.

TABLE 1

Division frequency of protoplasts of pea cultivars in liquid and bead cultures.

cultivar	division frequency ($\bar{x}$ ± SD%) bead culture		liquid culture	
Bello	3.8±3.4	(n=7)[a]	1.3±0.5	(n=4)
Belman	36.0±6.0	(n=2)	0	(n=2)
Filby	80.0±6.7	(n=28)	17.2±8.6	(n=5)
Lotta	0.8±0.1	(n=2)	0	(n=2)
Petra	57.0±8.0	(n=10)	3.2±2.6	(n=5)
Proco	10.0±1.0	(n=2)	1.7±0.7	(n=2)
Simo	9.0±1.0	(n=2)	0	(n=2)
Stivo	5.0±1.6	(n=4)	0.9±0.5	(n=3)
Timo	0.6±0.1	(n=2)	0	(n=2)
Vreta	1.9±0.3	(n=7)	0.6±0.2	(n=3)

a) n = number of independent experiments

TABLE 2

Effect of timing of agarose-embedding on division frequency of Filby protoplasts.

days after isolation	division frequency ($\bar{x}$ ± SD%)	
0	30.0±9.6	(n=3)[a]
2	80.0±6.7	(n=28)
4	50.3±2.5	(n=3)
6	16.7±11.0	(n=3)

a) n = number of independent experiments

Embedding distributed the protoplasts evenly in the culture medium, while in liquid medium they tended to aggregate on the rim of the Petri dishes. This led to higher local densities of protoplasts, which might have become inhibitory to the further growth of the colonies later on. Diluting the culture medium, which may lower the concentrations of inhibitory substances, has been shown to be essential for sustained divisions of pea protoplasts (von Arnold and Eriksson 1976, Jia 1982). The frequent replacement of the medium surrounding the beads as proposed by Shillito et al. (1983) and Webb et al. (1987) seems to be more efficient in removing inhibitory substances than dilution of the liquid culture at day 2. Embedding in small drops, which should facilitate the exchange with the medium did not improve the division rate of protoplasts further (table 3).

TABLE 3

The effect of BA, zeatin riboside and embedding as drops or discs on division frequency of Filby and Petra protoplasts.

cultivar	embedding	division frequency ($\bar{x}$ ± SD%)		
		BA 4 µM	zeatin riboside 4.6 µM	
Filby	drops	84±2	47±32	(n=4)[a]
	discs	81±7	56±24	(n=4)
Petra	drops	55±5	2±2	(n=4)
	discs	53±2	2±2	(n=4)

a) n = number of independent experiments

The superiority of agarose embedding and the bead system has already been established for several other plant species, including legumes (Shillito et al. 1983, Lörz et al. 1983, Gilmour et al. 1987, Webb et al. 1987). Further reasons suggested for this superiority are that the agarose stabilizes membranes or prevents cell wall precursors from leaking from the surface of the cell (Shillito et al. 1983, Thor et al. 1987). In this study these seem to be of minor importance, since the best division rates were obtained when the protoplasts were embedded after cell wall regeneration, which was complete by day 2 after isolation as seen by calcofluor white staining.

The effect of Donor Tissue

The effect of donor tissue was tested in cv. Filby using the bead culture system. The choice of donor tissue has been shown to play an important role for in vitro competence in many plant species, and in pea Jia (1982) found that only the two youngest leaf pairs produced proliferating protoplasts. In this study various tissues could be used as protoplast donors, and no large differences were detected between epicotyl, leaf and shoot culture derived protoplasts (table 4).

TABLE 4

Effect of donor tissue on division rates of Filby protoplasts.

donor tissue	division rate ($\bar{x}$ ± SD%)	
leaf	78.7±7.2	(n=18)[a]
shoot cultures	78.5±5.5	(n=8)
epicotyls	84.8±2.1	(n=12)

a) n = number of independent experiments

Choice of cultivar

The effect of the cultivar on protoplast behavior in culture (von Arnold and Eriksson 1977, Jia 1982) as well as on regenerative competence from explants (Malmberg 1979, Hussey and Gunn 1984, Rubluo et al. 1984) is well documented in pea. In this study, too, great differences were observed between the cultivars used (table 1). In addition, the culture requirements of various pea lines also varied with respect to the hormones used. In both Filby and Petra the use of zeatin riboside instead of BA lowered the division rate (table 3). In contrast, Stivo and Vreta protoplasts gave about twofold higher division rates on zeatin riboside compared to BA, while in Bello no preferences could be observed in this respect. Similar differences have been obtained with Medicago varieties in their ability to respond to hormones by forming somatic embryos in suspension culture (Kao and Michayluk 1981).

Regeneration Experiments

2,4-D has been shown to be obligatory for sustained divisions in pea protoplasts, while additional NAA reduced the division rate (Gamborg et al. 1975, von Arnold and Eriksson 1977, Jia 1982). However, as 2,4-D has been shown to inhibit organogenesis in explant-derived callus (Rubluo et al. 1984), it was replaced by NAA in the later culture stages. Protoplast-derived calli survived the transfer to the regeneration medium only after they had reached a diameter of 1.5 - 2 mm, i.e. after four to six weeks culturing on solid medium. Differences were detected in the growth rate of the cultivars when cultured on the regeneration medium. Most of Vreta calli died when placed on this medium, while Bello and Filby calli developed into green, relatively rapidly growing calli (doubling time about 2 months) with dark green, globular structures.

After six to nine months of culture small shoot-like structures could be observed in Petra and Stivo calli. The shoot regenerating calli were derived from protoplasts of 12-day old epicotyls (Petra), or of leaves (Stivo). So far in Petra regenerants have been obtained from 10 calli out of 700, while in Stivo 6 calli out of 750 developed shoot - like structures. These were excised from the calli and cultured further on the shoot culture medium. After several subcultures the shoots have developed a more normal appearance, but no roots have formed on these shoots so far (figure 2). Once induced the calli have retained the capacity to produce new shoots for at least one year.

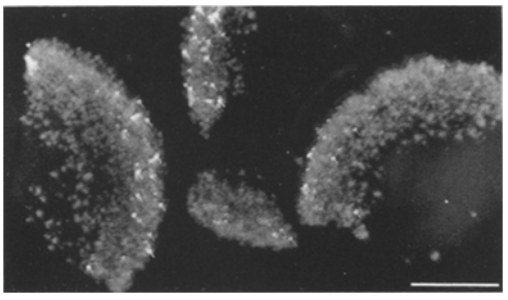

Figure 1: 2-week old Petra protoplast-derived colonies in bead culture (bar = 10 mm).

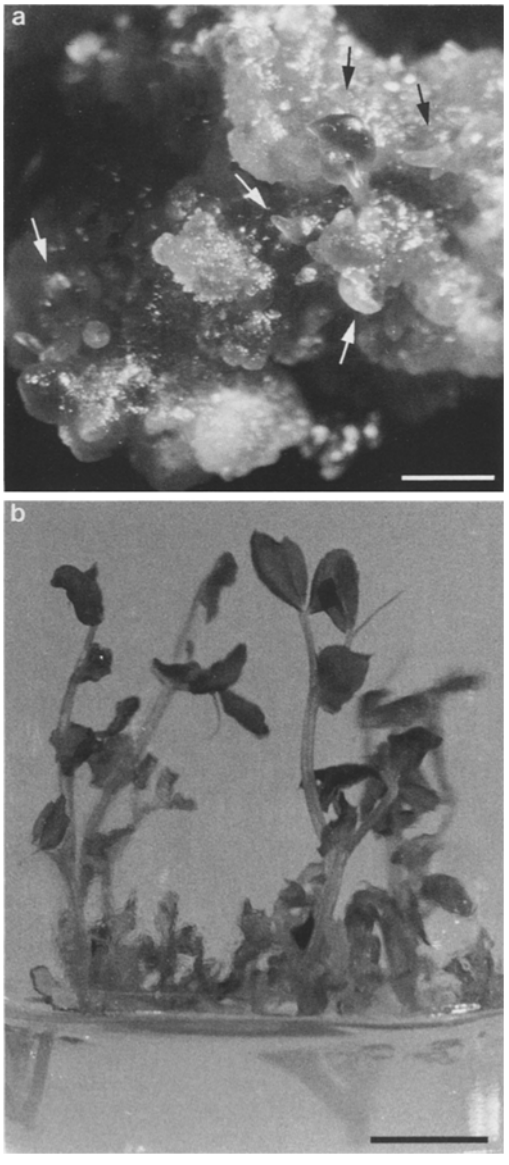

Figure 2a: Stivo protoplast-derived callus regenerating shoots (bar = 10 mm).
b: shoots regenerated from Petra protoplasts (bar = 10 mm).

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