Efficient intergeneric fusion of pea (Pisum sativum L.) and grass pea (Lathyrus sativus L.) protoplasts

P. Durieu and S.J. Ochatt

INRA, Centre de Recherches de Dijon, Unité de Recherches en Génétique et Amélioration des Plantes, Laboratoire de Physiologie et Culture in Vitro, 21110 Bretenières, France

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Abstract

Large numbers of viable protoplasts of pea (Pisum sativum) and grass pea (Lathyrus sativus) were efficiently and reproducibly obtained and, for the first time, fused. Different procedures for fusion were compared, based either on electrofusion (750, 1000, 1250 or 1500 V cm$^{-1}$), or on the use of macro or micro-methods with a polyethylene glycol (PEG 6000 or PEG 1540), or a glycine/high pH solution. Over 10% of viable heterokaryons were obtained, with PEG as the most efficient and reproducible agent for protoplast fusion (>20% of viable heterokaryons). Both the division of heterokaryons and the formation of small calluses were observed.

Key words: Protoplast fusion, Pisum sativum L., Lathyrus sativus L., grain legumes.

Introduction

Pea (Pisum sativum L.) is an important protein-rich crop, and is increasingly used as an animal feed, with France as the main producer in Europe. New varieties are being developed to adapt this grain legume to various abiotic and biotic stresses. One way of increasing the genetic variability of peas is by somatic hybridization, which can help to introduce new characters from other species or genera. In this context, the grass pea (Lathyrus sativus L.) is a wild relative of pea that possesses several interesting agronomic traits that might be useful for P. sativum, especially in terms of disease resistance (Campbell, 1997). As these species are cross-incompatible, the only way to obtain such intergeneric hybrids is protoplast fusion and somatic hybridization. Several papers have been published on protoplast isolation from pea tissues (Lehminger-Mertens and Jacobsen, 1989, 1993; Puonti-Kaerlas et al., 1992; Ochatt et al., 2000a). Most authors reported callus regeneration, some obtained shoots (Puonti-Kaerlas and Eriksson, 1988; Lehminger-Mertens and Jacobsen, 1989; Böhmer et al., 1995) and, more rarely, whole plants were regenerated either via embryogenesis (Lehminger-Mertens and Jacobsen, 1993) or organogenesis and embryogenesis (Ochatt et al., 2000a). Conversely, for Lathyrus sp. calluses were obtained from L. odoratus L. protoplasts (Razdan et al., 1980), but not even protoplast isolation has been reported for L. sativus. On the other hand, polyethylene glycol was used for the fusion of Pisum protoplasts (Kao et al., 1974; Kao and Michayluk, 1974; Constabel and Kao, 1974), but not electrofusion. In this study, a range of chemical and electrical fusion methods has been tested, to select the most efficient and reproducible one.

Materials and methods

Plant material

Young (3–5 cm) shoots from embryo axes of Pisum sativum cv. Frisson and of a white-seeded Lathyrus sativus genotype were used as the source of material for protoplast isolation. Dry seeds were surface-sterilized and imbibed overnight as reported elsewhere (Ochatt et al., 2000b). Embryo axes were then excised as described previously (Lehminger-Mertens and Jacobsen, 1993) and cultured in hormone-free B5 medium (Gamborg et al., 1968) with 10 mM NH$_4$Cl, 3% sucrose and 0.6% agar (Böhmer et al., 1995). For germination, plates with 10 axes each were placed at 24/22°C, with a 16:8 h (light/dark) photoperiod of 90 μE m$^{-2}$ s$^{-1}$ from cool white fluorescent tubes.

Standard protoplast isolation

Protoplasts were isolated as described previously (Ochatt et al., 2000a). Briefly, epicotyls were finely chopped and plasmolysed

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1 To whom correspondence should be addressed. Fax: +33 3 8063 3263. E-mail: ochatt@epoisses.inra.fr

Abbreviations: MES, 2-N-morpholinoethanesulphonic acid; NAA, ω-naphthaleneacetic acid; PEG, polyethylene glycol; 2,4-D, 2,4-dichlorophenoxyacetic acid.
for 1 h in 10 cm³ CPW medium (Frearson et al., 1973) with 9% (Pisum) or 13% (Lathyrus) mannitol. Tissues were digested overnight on a continuous rotary shaker in an enzyme solution based on LP* medium (modified from Lehninger-Mertens and Jacobsen, 1989) with 72 g l⁻¹ of myo-inositol containing 2% Macerozyme R-10, 5% Fluka Cellulase (from Trichoderma viride) and 0.1% Pectolyase Y-23 for Pisum, but 3% Macerozyme R-10, 4% Cellulase Onozuka RS and 0.2% Pectolyase Y-23 for Lathyrus. Protoplasts were sieved (40 μm) and centrifuged successively at 35 g (5 min, 10 °C) and 70 g (5 min, 10 °C). Each pellet was resuspended in 250 mm³ of the appropriate plasmolyticm suscept. Pellets were mixed together and were finally layered on top of 7 cm³ of CPW solution plus 21% sucrose, and spun at 80 g (10 min, 10 °C). The protoplast density was determined and the viability evaluated with fluorescein diacetate (FDA) under UV light (B1 IF 420–485 filter) as described earlier (Widholm, 1972).

Isolation of protoplasts for fusion

For chemical fusion: Plasmolysis, digestion and the two first centrifugation steps were identical to those of the standard isolation protocol but, before the third centrifugation, protoplasts were stained with FDA (Lathyrus) or Rhodamine B isothiocyanate (Pisum). Both staining solutions were prepared by adding 150 mm³ from a stock (of 5 mg FDA or 30 mg Rhodamine per cm³ of acetone) to 7 cm³ of plasmolymic, from which five drops (approximately 150 mm³) were added to the pellets with Pisum or Lathyrus protoplasts, prior to floating them on a sucrose gradient as above. Under UV light, protoplasts stained with FDA fluoresced yellow-green while those stained with Rhodamine B fluoresced red. Density and viability were evaluated as described previously.

For electrofusion: All steps were identical to the isolation procedure for chemical fusion, but all solutions were devoid of salts, i.e. mannitol at 9% (w/v) for Pisum but at 13% (w/v) for Lathyrus, were used as plasmolyticm. The pellets were resuspended in an electroporation solution consisting of 6 mM MgCl₂, 200 mM MgSO₄, 0.5 M mannitol, and 3 mM MES modified from that of Rech et al. (Rech et al., 1987) and the protoplasts were floated on a 21% (w/v) sucrose-containing CPW solution (Power and Davey, 1990). Density and viability were evaluated as described above.

Protoplast fusion

Micromethod: Equal volumes of stained protoplasts of each species were mixed (1:1, v/v) with the fusion solution (Table 1) and spun at 100 g (10 min, 25 °C). The pellet was resuspended in a 2:1 vol. of washing solution prepared by adding 0.74% (w/v) CaCl₂·2H₂O to CPW 13 M (Power and Davey, 1990) and centrifuged as before. Fused and rinsed protoplasts were finally resuspended (1:1, v/v) in washing solution, and the percentage of heterokaryons formed was determined by counting the protoplasts that fluoresced both green and red under UV light.

Table 1. Composition of fusion medium

<table>
<thead>
<tr>
<th>Fusion medium</th>
<th>PEG 6000</th>
<th>PEG 1540</th>
<th>Glycine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusion agent (w/v)</td>
<td>30%</td>
<td>50%</td>
<td>3.75%</td>
</tr>
<tr>
<td>Osmoticum (w/v)</td>
<td>4% Sucrose</td>
<td>4% Glucose</td>
<td>9% Mannitol</td>
</tr>
<tr>
<td>CaCl₂·2H₂O (mM)</td>
<td>10</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>KH₂PO₄ (mM)</td>
<td>—</td>
<td>0.7</td>
<td>—</td>
</tr>
</tbody>
</table>

Results and discussion

Isolation and culture

With the isolation protocol used, more than 90% of viable protoplasts were obtained (Fig 6) for both pea and Lathyrus. Such a high viability is a prerequisite for success with their subsequent fusion. A density of 2.0 ± 0.2 × 10⁶ protoplasts g⁻¹ FW of digested tissues, sufficient for culture at the initial plating density of 1 × 10⁶ protoplasts cm⁻², was consistently obtained.

This is the first report of the successful isolation of large numbers of highly viable protoplasts from tissues of L. sativus. In the past, only L. odoratus had been studied in this context (Razdan et al., 1980). The enzyme mixture used was close to one already employed for pea (Lehninger-Mertens and Jacobsen, 1989), but the cellulase used in the current study was weaker, Onozuka RS instead of Onozuka YC. Previous work in this laboratory has shown the latter to cause reduced viability with only...
For both species, the best rate of division and microcallus formation was observed from non-fused, stained protoplasts on KP medium. KP medium did not sustain proliferation of a large number of colonies with Lathyrus but could, nevertheless, be used with protoplasts of both genotypes. Medium LP*60 seems adequate for Pisum protoplasts although the number of microcalli produced was less important than with medium KP. However, LP*60 medium caused bursting and death of Lathyrus protoplasts within a few days. Thidiazuron-containing media were comparable to medium LP*60 for pea protoplasts, but the Lathyrus protoplasts were strongly plasmolysed in such media, and extensive bursting occurred resulting in death.

These results differ from those reported for leaf protoplasts of L. odoratus, that were cultured at a lower density on a B5 modified medium (Razdan et al., 1980). Likewise, for pea protoplasts, only Puonti-Kaarlas and Eriksson (Puonti-Kaarlas and Eriksson, 1988) and the authors (Ochatt et al., 2000a) had used a KM-based medium, while all other reports dealing with pea protoplasts generally preferred various modifications of LP* medium as described previously (Lehminger-Mertens and Jacobsen, 1989).

KP medium was chosen for culture of the heterokaryons as based on responses from non-fused protoplasts, it gave the best results for both parents. Within 3 d, the division of heterokaryon-derived cells was observed, for both chemical and electrical fusion methods and, 2 weeks later, small cell colonies were formed.

Chemical fusion

Three different agents were used for chemical fusion of protoplasts (glycine, PEG 6000, PEG 1540; see Table 1). Fusion was possible with all three agents (Figs 1, 2), but glycine was statistically the least efficient, with about 10% of heterokaryons produced. In addition, with glycine, the formation of crystals and agglomerations of debris that entrapped the heterokaryons and curtailed their subsequent development was always observed. Rinsing the fused protoplasts twice or using a fresh glycine solution each time failed to prevent these phenomena. No difference was detected between the macro and micro-method. Used alone, this agent (High pH/Ca$$^{2+}$$) did not appear to be very efficient, but it had been noticed (Kao and Michayluk, 1974; Kao et al., 1974) that, coupled with a PEG-treatment, glycine permitted an increased heterokaryon formation.

Fusion with PEG was most efficient, with about 20% of heterokaryons produced (Figs 1, 3). Statistically, no differences were detected between macro and micro-methods. Also, PEG 6000 was as efficient as PEG 1540. Chandra et al. (Chandra et al., 1988) with Solanum viarum (+) S. dulcamara, and Kao and Michayluk (Kao and Michayluk, 1974) with Vicia hajastana (+) Pisum sativum observed similar results. However, the use of PEG 1540 involves a second rinsing and could thereby decrease the density of protoplasts. For both PEG solutions, micro-methods seemed technically more suitable, because of the absence of centrifugation, which consistently damaged the protoplasts and decreased their density. Viability was not measured after fusion, but the division of heterokaryons was apparent for micro-methods using PEG solutions (Fig. 5), showing them not to be toxic. In this respect, the lack of division for protoplast-derived cells fused using macro-methods is likely to be due to a reduced cell density following the repeated centrifugations needed for rinsing the fused protoplasts. The non-toxic nature of PEG at the concentration and duration used had already been observed (Kao and Michayluk, 1974).

Electrofusion

Although protoplast fusion was possible for all voltages tested (Figs 1, 4), and was coupled with an efficiency of heterokaryon formation that increased with the voltage applied, no statistical differences appeared between the various electrofusion treatments. Thus, the production of heterokaryons at 1500 V cm$$^{-1}$$ was best, but large variations between successive experiments have been observed, probably reflecting the difference of protoplast quality between several independent isolations. Electrofusion induces heavy mechanical shocks (due to micro-pore formation) and, although the Ca$$^{2+}$$ ions present in the solution for electrofusion protect the membranes, protoplasts can explode after fusion. Interest was therefore attached to the re-evaluation of protoplast (and heterokaryon) viability following electrofusion, as shown in Fig. 6. This figure clearly shows the efficiency and
reproducibility of the isolation protocol, with nearly negligible variations in the viability of freshly isolated protoplasts for both genotypes. In this context, the isolation protocols for each genotype were identical during all successive experiments, but small differences (age of material, exact duration of digestion, time between two centrifugations, etc.) still exist and cannot be entirely suppressed. These factors could influence protoplast quality and can thus explain the variations observed in the efficiency of electrofusion and in the subsequent viability of the fused protoplasts. Consequently, despite having observed sustained proliferation from electrofused proto-

Fig. 2. Typical microscopic field under direct (a) and UV light (b). Arrows show heterokaryons fluorescing red/green.

Fig. 3. Typical microscopic field after a chemical fusion observed under UV light. Heterokaryons fluoresce in red/green.

Fig. 4. Typical microscopic field after an electrofusion observed under UV light. Heterokaryons fluoresce in red/green.

Fig. 5. Division of a heterokaryon-derived cell (arrow) under UV light.
plants, the use of a chemical micro-method with PEG 6000 as fusing agent should be preferred. This is in line with data by Chand et al. (Chand et al., 1988), with protoplasts of Nicotiana tabacum and Solanum dulcamara, but contrasts those observed by Bates (Bates, 1985) with N. tabacum and N. plumbaginifolia, who obtained 19% of heterokaryons with electrofusion, versus 10% with PEG 8000 treatment.

This is the first report on the isolation and culture of viable protoplasts of Lathyrus sativus L. Also, for the first time the fusion of protoplasts of pea and grass pea is described. In addition, although chemical versus electrofusion have been frequently contrasted in the past, this has not been the case for comparisons of macro- and micro-methods for chemical fusion, nor had electrofusion ever been tested with Pisum protoplasts before. The strategies detailed here allowed the development of heterokaryons which reproducibly underwent cell division to give small colonies (Table 2).

Whole plant regeneration from such, heterokaryon-derived microcalluses will take at least 12–15 months (Böhmer et al., 1995; Ochatt et al., 2000a; Puonti-Kaerlas and Eriksson, 1988), but will permit the creation of genetic novelties including interesting agronomic traits, in terms of stress tolerance and rusticity from Lathyrus, and with respect to grain quality from Pisum.

Note added in proof

While this manuscript was in litteris, data were reported on the isolation of protoplasts from leaves and cell suspensions of one grass pea accession (McCutchan et al., 1999), but these failed to undergo sustained division during culture.

Acknowledgements

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References

McCUTCahan JS, Larkin PJ, Stoutjesdijk PA, Morgan ER, Taylor WJ. 1999. Establishment of shoot and suspension cultures

Table 2. Plating efficiency* of the cultured Pism (+) Lathyrus heterokaryons fused with different methods

Data are the mean from at least 200 counted heterokaryons per treatment and three independent experiments.

<table>
<thead>
<tr>
<th>Fusing agent</th>
<th>Fusion method</th>
<th>Heterokaryon formation (%)</th>
<th>IPE (%)</th>
<th>FPE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>Micro</td>
<td>11.4</td>
<td>16.12</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Macro</td>
<td>9.2</td>
<td>19.25</td>
<td>0.0</td>
</tr>
<tr>
<td>PEG 1540</td>
<td>Micro</td>
<td>20.6</td>
<td>23.5</td>
<td>2.15</td>
</tr>
<tr>
<td></td>
<td>Macro</td>
<td>19.7</td>
<td>4.65</td>
<td>0.22</td>
</tr>
<tr>
<td>PEG 6000</td>
<td>Micro</td>
<td>21.3</td>
<td>28.0</td>
<td>2.75</td>
</tr>
<tr>
<td></td>
<td>Macro</td>
<td>22.5</td>
<td>7.4</td>
<td>0.42</td>
</tr>
<tr>
<td>Electrofusion</td>
<td>750</td>
<td>10.1</td>
<td>21.81</td>
<td>1.86</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>13.9</td>
<td>19.46</td>
<td>1.42</td>
</tr>
<tr>
<td></td>
<td>1250</td>
<td>18.8</td>
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<tr>
<td></td>
<td>1500</td>
<td>22.1</td>
<td>25.0</td>
<td>3.11</td>
</tr>
</tbody>
</table>

*IPE=percentage of heterokaryons observed that divided at least once; FPE=percentage of dividing heterokaryons undergoing sustained proliferation.


