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Organogenesis from 'Passe Crassane' and 'Old Home' pear (*Pyrus communis* L.) protoplasts and isoenzymatic trueness-to-type of the regenerated plants

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Summary. Large numbers of highly viable mesophyll protoplasts were isolated from shoot cultures of the scion cv 'Passe Crassane' and the rootstock genotype 'Old Home' of common pear (*Pyrus communis* L.). Protoplasts were cultured for both genotypes either as liquid layers or as liquid-over-agar cultures, in ammonium-free MS medium with 0.5 *M* mannitol, 50 mg/l casein enzymatic hydrolysate (CEH), 2.0 mg/l NAA and 1.0 mg/l BAP, plus either 0.5 mg/l IAA (for 'Old Home') or 2.0 mg/l IAA (for 'Passe Crassane'). Protoplast microcalli, obtained by day 60 ('Passe Crassane') or day 80 ('Old Home'), were transferred for further growth to ammonium-free MS medium with 2.0 mg/l NAA and 1.0 mg/l BAP. Shoot bud regeneration from the protoplast-derived callus was first attempted between 100 ('Passe Crassane') and 120 ('Old Home') days after protoplast isolation. For 'Passe Crassane', shoot buds were regenerated (day 130) on a half-strength MS medium with 0.1 mg/l IBA, 0.5 mg/l BAP, 50 mg/l CEH and 20 mg/l Ca-panthotenate. For 'Old Home', shoot but regeneration only occurred 30 days later and on the same medium as above, which was additionally supplemented with double the concentration of the group B vitamins found in the original MS formulation and 0.05 mg/l GA_3. Following micropropagation and in vitro rooting of shoots, the plants were transferred to soil following standard procedures. Trueness-to-type of the regenerated plants was assessed by analysing their leaf isozyme banding profiles (for EST, AP, PRX, SOD, ENP, LAP, PGI, AAT, ADH, MDH and PGM) and comparing them to those corresponding to the original shoots that provided the protoplasts. No differences between the mother shoots and the protoclones were observed for any one of the 11 isozyme systems studied.

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Key words: *Pyrus communis* L. – Protoplast culture – Plant regeneration – Isoenzymatic trueness-to-type

Introduction

In recent times, the use of protoplast technology for top-fruit tree breeding has received increasing attention, and protoplast-to-tree systems are nowadays available for several rosaceous fruit trees including various species and hybrids of *Prunus*, a number of apple scion and rootstocks and a few pear genotypes (see Ochatt et al. 1991 for review). Relative to other top-fruit trees, and despite their economic importance, tissue cultures of *Pyrus* genotypes have generally received little attention (Chevreau and Skirvin 1991), while protoplast studies with pear are few in number. However, the first example of whole plant regeneration from protoplasts of a top-fruit tree was for a form of wild pear, *Pyrus communis* var 'pyraster' L. (Ochatt and Caso 1986), and this was soon followed by the description of spontaneous protoclonal variation amongst those pear rootstock regenerants (Ochatt 1987). Protoplast studies of the common pear (*Pyrus communis* L.) were reported for the first time in 1988 with the scion cvs 'Conference' (Ochatt and Power 1988 a) and 'Williams Bon Chretien' (Ochatt and Power 1988 b) with the induction of rhizogenesis from protoplast-derived calli of the former, later extended to shoot bud regeneration from the roots thus obtained (Ochatt 1990), and complete plant regeneration from Williams' protoplasts. Other studies using protoplast technology for pears have included the development of a general strategy for the isolation of leaf protoplasts for a large number of top-fruit tree genotypes (Revilla et al. 1987), the effects of electroporation on the viability and competence for

growth of embryo-callus protoplasts of the cv 'Conference' (Rech et al. 1987), the recovery of somatic hybrid rootstocks after the fusion of *Pyrus* and *Prunus* protoplasts (Ochatt et al. 1989) and, more recently, the co-culture of leaf protoplasts of the pear cvs 'Williams', 'Passe Crassane' and 'Old Home' with *Erwinia amylovora*, the bacterium responsible for fire blight disease (Brisset et al. 1990). No other papers dealing with pear protoplasts have been published to date.

This paper describes the isolation, culture and plant regeneration of pear leaf mesophyll protoplasts of the scion cv 'Passe Crassane' and the rootstock cv 'Old Home'. In addition, the conformity of the regenerated protoplast-derived plants was assessed by studying the banding profiles of a large number of leaf isoenzymatic systems.

Materials and methods

Plant materials and protoplast isolation

Shoot cultures of *Pyrus communis* L. cvs 'Passe Crassane' and 'Old Home' were maintained by monthly subculturing on agar-solidified (0.7%, w/v) modified Lepoivre medium (Leblay et al. 1991) with 0.1 mg/l IBA (indole-3yl-butyric acid) and 0.5 mg/l BAP (6-benzylaminopurine) (pH 5.7), at 24 °C with a 16/8 h (light/dark) photoperiod of 2000 lux from cool white fluorescent tubes.

For protoplast islation, the most recently fully expanded leaves, taken from the shoot cultures, were chopped (1–2 mm thick), plasmolysed (1 h in CPW 13 *M* medium, Power and Davey 1990), and 1 g (f wt) of leaf tissues was digested (18 h) in 10 ml of an enzyme mixture that consisted of 1.0% Cellulase Onozuka R-10, 1.0% Hemicellulase and 0.1% Pectolyase Y-23 (for 'Old Home') or the same mixture with the further addition of 0.2% Macerozyme R-10 (for 'Passe Crassane'), both dissolved in CPW 13 *M* medium with 5 m*M* MES (2-*N*-morpholinoethane sulfonic acid) and 1.0% PVP-10 (polyvynilpyrrolidone, MW 10 000), pH 5.6, as previously described (Brisset et al. 1990). All subsequent manipulations, for the purification of protoplasts and assessments of protoplast size, yield and viability after isolation were as reported elsewhere (Ochatt and Power 1988 b; Ochatt and Power 1991).

Protoplast culture and plant regeneration

Protoplasts were cultured at an initial plating density of 0.5×10^5/ml medium as liquid layers or as liquid-over-agar cultures. For the latter method 1-ml aliquots of a liquid protoplasts medium suspension of each genotype (at 1×10^5 protoplasts/ml) was layered on top of 1 ml of a semisolid (0.625%, w/v, Seaplaque agarose) hormone-free, ammonium-free MS medium (Murashige and Skoog 1962) supplemented with 50 mg/l casein enzymatic hydrolysate (CEH). For both culture strategies, the medium composition of the liquid phase was optimized for each genotype. In both cases, this consisted of a basal NH_4NO_3-free MS salts medium, either with MS organics or with the complex organic mixture used for wild-pear protoplasts (Ochatt and Caso 1986), with 2.0 mg/l NAA (1-naphthaleneacetic acid), 1.0 mg/l BAP and IAA (4-indole-3yl-acetic acid) at 0.5 mg/l (for protoplasts of 'Old Home') or 2.0 mg/l (for those of 'Passe Crassane') and supplemented with 0.5 *M* mannitol as the osmoticum (pH 5.6), as described by Brisset et al. (1990).

For both genotypes, cultures were maintained statically, at 25 °C and under the light régime used for culture of the shoot cultures that provided the protoplasts. The liquid-over-agar culture strategy was adopted in order to avoid the need for the frequent additions of osmoticum-free medium that are generally required to reduce the osmotic pressure of the medium during protoplast growth. Therefore, except for observation purposes (twice a week during the first 15 days and once weekly thereafter), the cultures in liquid-over-agar were left undisturbed from day 0. Conversely, for protoplasts in liquid layers, the osmotic pressure of the medium was gradually reduced from day 15 by weekly additions of osmoticum-free medium in a 3:1 ratio (protoplast:mannitol-free medium) until the formation of microcallus 1–2 mm in diameter.

Results were assessed in terms of the mean ± SE of the percentage of the originally cultured protoplasts that: (1) divided at least once (day 15; IPE initial plating efficiency); (2) proliferated to reach the 10-cell colony stage (day 30; MPE intermediate plating efficiency); (3) proliferated to give microcalli of approximately 100 cells each, 1–2 mm in diameter (days 60–80; FPE final plating efficiency). All experiments involved a minimum of five replicated dishes per genotype and were repeated at least twice.

Protoplast microcalli (1–2 mm in diameter; approximately 60 days from isolation for 'Passe Crassane' or 80 days from isolation for 'Old Home') were transferred for further growth (two 3-week successive subculture passages) to a semisolid (0.8%, w/v, agar) ammonium-free MS medium with 2.0 mg/l NAA and 1.0 mg/l BAP (pH 5.8). Between 100 ('Passe Crassane') and 120 ('Old home') days after protoplast isolation, approximately 125-mm^3 (200 mg f wt) callus portions were transferred to the media to be tested for shoot bud regeneration. These media were based on full- or half-strength MS medium (with or without NH_4NO_3) and were supplemented with a range of combinations of IBA, BAP and/or GA_3 (gibberellic acid) at 0.0, 0.05, 0.1, 0.5 or 1.0 mg/l. The effect on shoot bud regeneration of additionally supplementing these media with CEH (50, 100, 200 mg/l), Ca-panthotenate (5, 10, 20 mg/l) and/or double the concentration of the group B vitamins found in the original MS formulation (i.e. 0.2 mg/l thiamine-HCl, 1.0 mg/l pyridoxine-HCl, 1.0 mg/l nicotinic acid) was also assessed. The percentage of regenerating calli and the number of regenerated shoot buds per callus were recorded. All such media assessments were replicated at least 25 times, with all experiments being repeated twice.

For multiplication and internode elongation (a requisite for successful rooting), the regenerated shoots of both pear genotypes were transferred, for three successive (4 week) subculture passages, to a full-strength MS medium (with NH_4NO_3) supplemented with 0.1 mg/l IBA and 1.0 mg/l BAP, and with 0.0 mg/l (for 'Passe Crassane') or 0.1 mg/l GA_3 (for 'Old Home'), pH 5.8. Shoots (greater than 3.0 cm in height) were rooted after 1 week in half-strength MS medium with 3.0 mg/l IBA and 0.5 mg/l NAA followed by 3 weeks in half-strength, hormone-free MS medium (Patat-Ochatt and Power 1990). Finally, the pear protoplast-derived plants were transferred ex vitro and acclimatized (Ochatt and Power 1988 b).

Isoenzymatic assessments

For electrophoresis of enzymes, 200 mg f wt of leaf tissues taken from each protoclone studied as well as from the original shoots that provided protoplasts were ground in 1 ml of buffer. This extraction buffer was adapted from Bousquet et al. (1987) by adding 2 m*M* phenyl methyl sulfonide fluoride and adjusting the pH to 8.0. After 1 h of centrifugation at 35 000 *g*, the supernatants were stored for a few days at −20 °C until used for elec-

Table 1. Results of the isolation and culture of leaf mesophyll protoplasts of 'Passe Crassane' and 'Old home' pear. Mean ± SE from three successive experiments with five replicates each

Genotype	Protoplast isolation			Protoplast culture		
	Yield ($\times 10^7$/g fwt)	Viability (%)	Size (μm)	IPE (%) (day 15)	MPE (%) (day 30)	FPE (%)[a]
P. Crassane	0.48 ± 0.09	92 ± 4	12 ± 4	17.66 ± 0.85	2.55 ± 0.19	1.41 ± 0.11
Old Home	0.75 ± 0.19	95 ± 3	22 ± 5	20.51 ± 1.11	4.78 ± 0.33	1.96 ± 0.23

IPE, Initial plating efficiency; MPE, intermediate plating efficiency; FPE, final plating efficiency
[a] FPE was assessed by day 60 for 'Passe Crassane' and by day 80 for 'Old Home'

trophoresis. Migration was performed in polyacrylamide gels for esterase (EST), acid phosphatase (AP), leucine amino peptidase (LAP), endopeptidase (ENP), superoxide dismutase (SOD), peroxidase (PRX) and aspartate aminotransferase (AAT), while starch gels were used for alcohol dehydrogenase (ADH), malate dehydrogenase (MDH), phosphoglucoisomerase (PGI) and phosphoglucomutase (PGM). The procedures and conditions for migration were as described by Chevreau and Laurens (1987). Thereafter, the gels were stained following standard procedures (Vallejos 1983).

Results

The results for the isolation and culture (in terms of IPE, MPE and FPE) of the protoplasts of both pear genotypes studied are summarized in Table 1.

Interestingly, 'Passe Crassane' protoplasts proliferated in a medium with MS organics, while those of 'Old Home' required an organics-richer medium, so that a complex organic mixture (see Materials and methods) had to be added. For both genotypes, cell-wall regeneration was completed after 12–13 days, with first divisions occurring 24–48 h later. These, as well as the results for IPE, MPE and FPE, were similar for both culture strategies assessed. However, leaf protoplasts of 'Passe Crassane' grew faster in the liquid-over-agar cultures, reaching the microcallus stage (more than 100 cells each, approximately 1–2 mm in diameter) as many as 20 days earlier (i.e. by day 60) than protoplasts cultured as liquid layers or than 'Old Home' protoplasts cultured in either way, where microcallus formation could only be verified by day 80 of culture.

Two successive 3-week-long subculture passages on the callusing medium were required before the microcalli produced attained the minimum size for transfer to the media to be tested for shoot bud regeneration. The transfer of microcalli smaller than 125 mm^3/(i.e. approximately 200 mg fwt) to such media always resulted in tissue browning and callus death. Conversely, once the minimum critical size mentioned above was reached, 45% (for 'Old Home') to 60% (for 'Passe Crassane') of the protoplast-derived callus portions survived and either continued proliferating or differentiated shoot buds following transfer to the regeneration media. These responses applied to both genotypes, irrespective of the protoplast culture strategy adopted.

In line with results for the first culture stage, microcalli of 'Passe Crassane' deriving from protoplasts that had been cultured in liquid-over-agar medium grew faster, and by day 100 shoot bud regeneration from them was first attempted. The best regeneration responses were obtained using a half-strength MS medium supplemented with 0.1 mg/l IBA, 0.5 mg/l BAP, 50 mg/l CEH and 20 mg/l Ca-panthotenate, where typically 35% of the calli underwent caulogenesis, with an average of two shoot buds per callus portion by day 130 of culture. On the other hand, shoot bud regeneration was more efficient for 'Old Home' (50% of the calli differentiating with an average of five to six shoot buds each), but it only occurred 30 days later, and on the same medium as above that was additionally supplemented with 0.05 mg/l GA_3 and double the concentration of the group B vitamins as in the original MS formulation. Media with MS salts at full-strength always failed to support organogenesis.

Noteworthy was the fact that for 'Passe Crassane' only those calli deriving from the liquid-over-agar cultures were capable of undergoing organogenesis. In this respect, calli recovered from the liquid protoplast cultures proliferated as green, compact, nodular tissues, but never regenerated any shoots buds. No such differences due to the initial strategy employed for the culturing of protoplasts were apparent with 'Old Home' cultures. For both genotypes, regenerating calli were friable and yellowish in colour at the time of transfer; they turned reddish within a week, brownish a week later, and smooth, green areas (subsequently developing into shoots buds) appeared thereafter.

For 'Passe Crassane', protoplast-derived calli retained the competence for shoot bud regeneration over three successive subcultures, and then lost it. However, it could be restored by adding GA_3 (0.005 mg/l) to the regeneration medium, and the calli remained caulogenic for a further five (4-week) subculture passages. Converse-

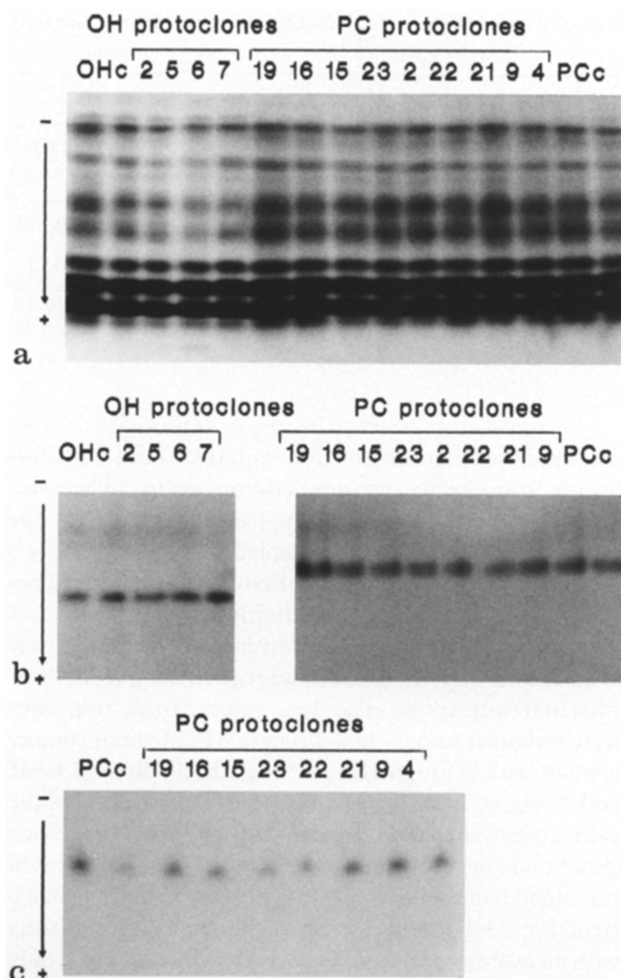

Plate 1. In vitro leaf zymograms for **a** EST, **b** ENP and **c** ADH. *OH* 'Old Home', *PC* 'Passe Crassane', *OHc* control 'Old Home' (micropropagated plant), *PCc* control 'Passe Crassane' (micropropagated plant)

ly, for 'Old Home', regeneration ability by protoplast-derived calli has been retained on the original regeneration medium to date, over ten subculture passages.

The shoots thus obtained were detached from the callus and transferred to the media devised for shoot multiplication and internode elongation. On such media, an average of 45% of 'Old Home' shoots and 65% of 'Passe Crassane' shoots were capable of sustained growth and proliferation 4 weeks after transfer, while the rest failed to grow, became chlorotic, then browned and finally died. The propagation medium only supported a doubling of shoot number for 'Passe Crassane', but was coupled with the production of tall (longer than 3.0 cm), rootable shoots by the end of the first subculture period. For 'Old Home', the protoplast-derived shoots were more leafy and proliferated more rapidly, with a monthly multiplication ratio of four to five shoots per original bud transferred, although a minimum of three successive passages on the multiplication medium were required before rootable shoots (i.e. with long internodes) were obtained. In turn, 'Old Home' shoots were easier to root than those of 'Passe Crassane', and they were also less prone to dehydration following transfer to soil. Thus, 35% of the 'Passe Crassane' shoots were successfully rooted, of which 40% survived ex vitro transfer, while for 'Old Home', these percentages were of 45% and 65%, respectively. Table 2 gives data on the various stages of culture, from the callusing stage, relative to the number of initially cultivated protoplasts. A projection of such data indicates that an average of about 1300 (for 'Passe Crassane') to about 2900 (for 'Old Home') complete, autotrophic plants in soil could theoretically be produced from each millilitre of leaf protoplasts initially cultured. However, only a reduced sample of such protoplast-derived trees of 'Passe Crassane' and 'Old Home' pear are being conserved in soil at present.

Table 2. Percentage culture efficiency of 'Passe Crassane' and 'Old Home' pear protoplast-derived callus as related to the initial number of cultured protoplasts. Mean data from three successive experiments with 25 replicates per culture stage per genotype

Percentage initially cultured protoplasts producing	Genotype	
	Passe Crassane	Old Home
Fast-growing callus	0.846	0.882
Shoot buds	0.296	0.441
Proliferating shoots	0.192	0.198
Rooted shoots	0.067	0.089
Weaned plants	0.026	0.058

Of relevance to this study was the assessment of four 'Old Home' and nine 'Passe Crassane' randomly selected protoplast-derived shoots (protoclones) for their trueness-to-type relative to the original shoots that provided the protoplasts. Therefore, and in the absence of any evident phenotypic deviation for such protoclones, biochemical studies involving the analysis of the banding profiles of a large number of leaf isozymes were subsequently undertaken. No differences between the banding profiles of the mother shoots and the protoclones were observed for either of the 11 leaf isoenzymatic systems studied. It was noteworthy, also, that the buffer as developed proved suitable for the extraction of a wide array of proteins, and their activity could, in turn, be evidenced by using both polyacrylamide and starch gels. As an example, the figures (a–c) in Plate 1 show the leaf isozyme banding profiles of both genotypes for EST, ENP and ADH. Further studies at the whole plant level and in the field will be necessary before a fully conform nature of the regenerated plants of such protoclones, as

compared to conventionally propagated plants of both 'Passe Crassane' and 'Old Home' pear, can be ascertained.

Discussion

As had been the case for all of the *Pyrus* genotypes studied so far (Ochatt and Caso 1986; Ochatt and Power 1988 a, b; Ochatt 1990, and references therein), ammonium ions proved inhibitory for the growth of 'Passe Crassane' and 'Old Home' leaf protoplasts. A similar result was observed during recent experiments on the co-culture of such protoplasts with the bacterium *Erwinia amylovora* (Brisset et al. 1990), but this study was not concerned with the culture of protoplasts beyond the initial developmental stages. Against this background, the results presented in this article provide a reliable strategy for the efficient regeneration of complete, autotrophic plants from the cultured mesophyll protoplasts of both of the pear genotypes 'Passe Crassane' and 'Old Home'. In turn, 'Old Home' becomes the first pear rootstock belonging to the species *P. communis* L (common pear) for which a protoplast-to-tree system is available.

A difference in response during protoplast culture was evident between both genotypes and, in this respect, the adoption of the liquid-over-agar culture method proved most favourable for the growth of 'Passe Crassane' protoplasts. Such a double-phase culture strategy, which was originally devised to control vitrification during the micropropagation of pear shoots (Viseur 1987), has since found wide application for the in vitro propagation of numerous plant genotypes, and has also been employed successfully for the culture, to the microcallus stage, of leaf protoplasts of 'Greensleeves' apple (Doughty and Power 1988).

The requirement for a medium rich in organic components displayed by 'Old Home' protoplasts, as compared to those of the scion cv 'Passe Crassane', concurs with results obtained for other fruit tree genotypes where protoplasts of pear (Ochatt and Caso 1986) and apple (Patat-Ochatt et al., 1988) rootstocks required media rich in organic components as compared to protoplasts of scion varieties for both *Pyrus* (Ochatt and Power 1988 a, b; Ochatt 1990) and *Malus* (Patat-Ochatt et al. 1988; Patat-Ochatt and Power 1990). Casein enzymatic hydrolysate included in the semisolid phase, however, was needed for successful responses of 'Passe Crassane' protoplasts cultured in the liquid-over-agar media; this is in agreement with previous results for 'Williams' pear protoplasts (Ochatt and Power 1988 b).

In line with results for shoot bud regeneration from protoplast-derived tissues of other *Pyrus* systems (Ochatt and Caso 1986; Ochatt and Power 1988 b), IBA and BAP provided optimum responses. In this respect, it is interesting to highlight the progressive change in the phenotype of calli leading to shoot bud regeneration. This coincides, both in timing and in the appearance of tissues, with the developmental sequence that occurs during caulogenesis in many woody fruit trees (Ochatt et al. 1991), but clearly contrasts with the responses observed for most herbaceous species protoplast systems studied to date (Ochatt and Power 1991).

The generalized weakness of the plants regenerated in these experiments and the relatively large losses due to dehydration upon soil transfer are in agreement with the results reported for protoplast-derived plants of other woody plant genotypes (Ochatt et al. 1991).

The leaf isoenzymatic assessments in this study, performed on several protoclones selected at random amongst the regenerated shoots, have shown that at least within the timeframe of this work no detectable spontaneous protoclonal variation occurred. This contrasts with results for wild pear protoplast-derived trees, where a significant proportion of the regenerated trees were spontaneous variants (Ochatt 1987). On the other hand, these results are in agreement with those observed for *Prunus* protoplast-derived tissues and plants, where only after a lengthy direct recurrent selection strategy was soma/protoclonal variation (for an increased salt and drought tolerance) successfully established (Ochatt and Power 1989). In addition, it is interesting to point out that the analysis of banding profiles of leaf isozymes has been used only rarely to assess the trueness-to-type of protoplast regenerants. As far as woody species are concerned, this has only been reported for the sweet orange, *Citrus sinensis* (Kobayashi 1987). In this same context, an additional and significant contribution of our studies was the development of an efficient method for isozyme analysis from in vitro leaf extracts of *Pyrus communis* L.. To date, pear isozymes have only been studied once for wild pear (Ochatt et al. 1989) and twice with common pear: one study reported the analysis of shoot extracts for EST, PRX and AP in a gradient polyacrylamide gel (Menendez and Daley 1986), while the other concerned pollen extracts analysed in starch gels for 7 isozymes (Cerezo and Socias i Company 1989). The methodology as developed in this study is, therefore, likely to be applicable for a wide spectrum of experiments concerned with the breeding of pears, either by conventional means or through the use of biotechnological approaches.

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