

Protoplast, cell and tissue cultures for the biotechnological breeding of grass pea (*Lathyrus sativus* L.)

S. Ochatt¹, P. Durieu, L. Jacas and C. Pontécaille

Laboratoire de Physiologie et Culture in Vitro, URGAP, INRA, CR de Dijon, B.P. 86510, 21065 Dijon cedex, France

Email: (1) ochatt@epoisses.inra.fr

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Introduction

Grass pea consumption leads to neurolathyrism and a safer use of this environmentally adaptable crop needs cultivars with decreased neurotoxin levels. Were they cross-compatible, grass peas would be useful genetic resources for disease resistance breeding of peas (*Pisum sativum* L.). In vitro selection⁽⁹⁾ and gene transfer may provide grass peas better adapted for consumption, while interesting *Lathyrus* traits could be introduced into pea by somatic hybridisation^(2,4).

The main bottleneck in tissue cultures of *Lathyrus sativus* has been plant regeneration^(8, 9). Cell suspensions were established from which non-dividing protoplasts were isolated⁽⁴⁾, and the sustained division of leaf protoplasts and their fusion with pea protoplasts was reported⁽²⁾. Hyperhydricity (vitrification) has been observed in cultured tissues of many species. Their morphology was described and the influence of various physiological parameters reviewed⁽¹⁾, but information on the fundamental genetic mechanism(s) underlying the occurrence of hyperhydricity is practically non-existent. This article describes strategies for in vitro regeneration of fertile *L. sativus* plants, it examines the genetic background concomitant with the appearance of hyperhydricity and, also, reports the isolation and culture of grass pea leaf protoplasts, and their fusion with pea protoplasts to give somatic hybrid calluses.

Materials and Methods

Embryo axes from seeds of *L. sativus* genotypes LB, LIII and L12 were germinated on hormone-free medium and hypocotyl segments without any pre-existing meristem were used as initial explants as reported⁽⁶⁾.

Bud regeneration media were supplemented with BAP + NAA, with thidiazuron alone, or with zeatin + ABA, as used with pea⁽⁶⁾. Regenerated shoots were grown on the germination medium described above prior to rooting on half-strength hormone-free MS medium⁽⁵⁾. Rooted plants were weaned in the greenhouse⁽⁶⁾, and kept until they set seed. Experiments were repeated thrice (≥ 10 replicates per medium per genotype). Results, expressed as the mean regeneration percentage and number of shoots/explant, were statistically analysed with Newman-Keuls test

Regenerants were compared with seedlings in terms of phenotype, and they were analysed by flow cytometry⁽⁷⁾, with two independent DNA content analyses/sample, compared to leaf controls from seedlings of each genotype.

Protoplasts were isolated from leaves of all genotypes and cultured in the media tested for pea⁽⁷⁾. They were labelled with fluorescein diacetate (green), while rhodamine B isothiocyanate (red) was preferred for pea protoplasts⁽²⁾, and electrofusion (at 750, 1000, 1250, 1500 or 2000 V/cm) was compared with chemical fusion. The standard macro-method⁽³⁾ was compared with a micro-method we developed⁽²⁾, and both were tested with glycine, PEG 6000 and PEG 1540. Heterokaryon viability was assessed and they were cultured in various media.

Results

True-to-type plants were regenerated and the whole procedure, from hypocotyl explants to grains harvested from regenerants, took 4 (LB) to 5 months (LIII and L12). The optimum hormonal combination for regeneration was genotype-specific. LB (white-seeded) responded best on auxin-free medium with 5.0 mg/l BAP, while coloured-seeded genotypes responded best with 0.01 mg/l NAA, plus 5.0 mg/l BAP for LIII, but 3.0 mg/l BAP for L12 (Table 1). Rooting was optimum on half-strength hormone-free MS medium, but differences appeared within genotypes between shoots regenerated on different hormonal balances.

Table 1. Mean caulogenesis and hyperhydricity related to the regeneration medium

Genotype	Cytokinin (mg/l)	% regeneration ^c	buds/explant ^c (mean number)	hyperhydric shoots ^c (%)
LB	BAP (1.0) ^a	0 f	0 f	0 f
	BAP (3.0) ^b	20.8 b	1.9 c	44.8 c
	BAP (5.0) ^b	45.83 a	3.25 a	74.8 b
	TDZ (2.2)	10.4 c	2.5 b	84 b
	Zeatin (10.0)	0 f	0 f	0 f
L III	BAP (1.0) ^a	0 f	0 f	0 f
	BAP (3.0) ^b	6.125 d	2.0 c	0 f
	BAP (5.0) ^b	12.25 c	1.25 d	0 f
	TDZ (2.2)	1.02 e	1.0 e	0 f
	Zeatin (10.0)	0 f	0 f	0 f
L 12	BAP (1.0) ^a	16 bc	1.625 cd	7.74 e
	BAP (3.0) ^b	52 a	1.345 d	20 d
	BAP (5.0) ^b	14 bc	3.0 a	14.3 d
	TDZ (2.2)	2 e	1.0 e	100 a
	Zeatin (10.0)	2 e	1.0 e	100 a

a, containing 0.0, 0.01 or 0.05 mg/l NAA

b, containing 0.0, 0.01, 0.05, 0.1 or 0.5 mg/l NAA

c, figures within a column followed by different letters differed significantly ($P < 0.05$)

Shoots with severe hyperhydricity symptoms were produced on media with low NAA (0.01 or 0.05 mg/l) plus high (5.0 mg/l) BAP, or when TDZ or zeatin and no auxin was added. Such shoots were non-rootable, while non-hyperhydric shoots rooted readily, giving fertile plants. Hyperhydricity never occurred with LIII, whatever the hormones used for regeneration, while regenerants of LB and L12 were very prone to hyperhydricity (Table 1). When regenerated shoots were analysed by flow cytometry, hyperhydricity was associated with abnormal profiles, with three peaks corresponding to 2C, 4C and 8 C DNA levels (Fig. 1). Conversely, all phenotypically normal regenerants had a diploid DNA content (profiles with 2C and 4C DNA peaks), comparable to seedling tissues. The time in culture did not modify DNA content, nor did it affect the occurrence of hyperhydricity. Auxins had a stronger effect than cytokinins, an increase in BAP having no effect on hyperhydricity or DNA content. The sole addition of NAA to a medium with hitherto high BAP levels sufficed to induce hyperhydricity and increased the DNA content of regenerants. The only

clear effect of cytokinins was when BAP was compared to TDZ, the latter provoking more hyperhydricity.

Large numbers of viable protoplasts were obtained from the leaves of all genotypes, and they proliferated to give calluses, but plants have not been regenerated to date. In terms of fusion with pea protoplasts, glycine was the least effective agent (~10 % heterokaryons), while PEG was best (>20 % heterokaryons). With electrofusion, heterokaryon formation was increased from 750 to 1500 V/cm, but fell drastically beyond this threshold. In all, electrofusion permitted the largest heterokaryon formation but with a large variability, and PEG 6000 appeared as the most efficient and reproducible fusion agent (Table 2). Heterokaryons fluoresced both green and red under UV light, and divisions could be monitored during several days (up to 4-6 celled colonies). Several hundreds of calluses have been obtained from the fused protoplasts and regeneration experiments are under way.

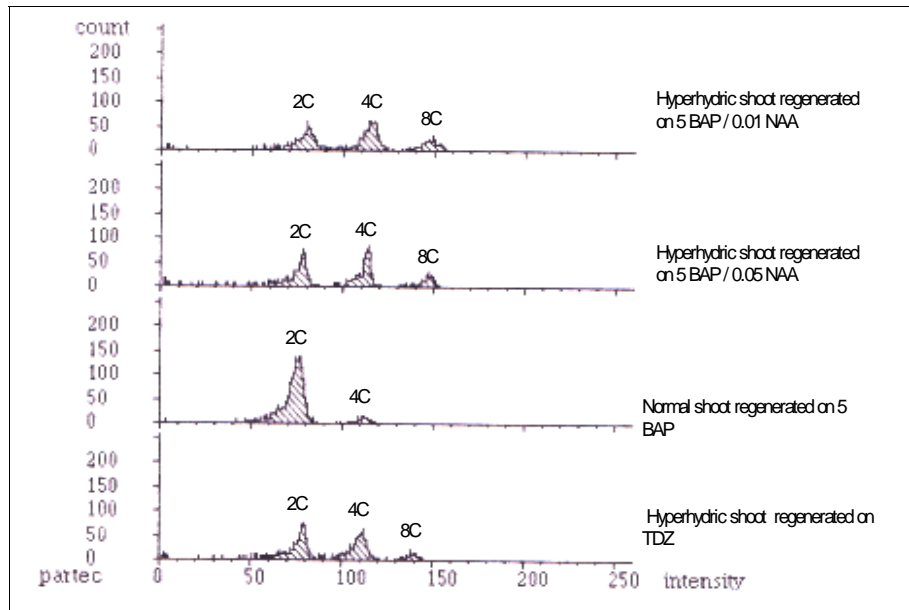


Figure 1. Flow cytometric profiles of LB regenerants on different regeneration media

Table 2. Plating efficiency of *Pisum (+) Lathyrus* heterokaryons (mean data from ≥ 200 heterokaryons/treatment and 3 independent experiments)

Fusing agent	Fusion method	Heterokaryon formation (%)	IPE (%)	FPE (%)
Non-fused LB protoplasts	NA	NA	63.28	2.15
Non-fused LIII protoplasts	NA	NA	37.55	1.44
Non-fused L12 protoplasts	NA	NA	29.22	0.58
Glycine	Micro	11.4	16.12	0.0
	Macro	9.2	19.25	0.0
PEG 1540	Micro	20.6	23.5	2.15
	Macro	19.7	4.65	0.22
PEG 6000	Micro	21.3	28.0	2.75
	Macro	22.5	7.4	0.42
Electrofusion (V/cm)	750	10.1	21.81	1.86
	1000	13.9	19.46	1.42
	1250	18.8	22.3	2.85
	1500	22.1	25.0	3.11

NA: not applicable; IPE: % heterokaryons dividing once; FPE: % heterokaryons undergoing sustained division

Discussion

Plant regeneration competence was strongly genotype-dependent, with LIII more recalcitrant than LB, and L.12 intermediate in responses. Production of rootable shoots before was rare^(8,9), with a very specific and narrow growth regulator requirement⁽⁸⁾. However, when our regenerants had a normal phenotype and a normal DNA content, rooted and fertile plants were consistently and reproducibly obtained on hormone-free medium.

A clear relationship had been established in the past between the hormones in regeneration media and hyperhydricity of shoots recovered⁽¹⁾, in line with our results. Additionally, here, flow cytometry permitted correlation of an abnormal DNA content with the hyperhydricity of regenerants and with a reduced competence of such shoots for subsequent rooting and fertile plant production. A similar correlation was observed between the media used and the regeneration of fertile plants from pea hypocotyls⁽⁶⁾, while the regeneration of fertile protoplast-derived plants has been correlated with the absence of endoreduplication in calluses derived from the cultured protoplasts⁽⁷⁾.

Hyperhydricity was more strongly linked to the presence of auxin and to the auxin/cytokinin balance, than to the cytokinin level or the number of subcultures, contrasting claims that a prolonged period of culture may result in hyperhydricity⁽¹⁾. Roy et al⁽⁸⁾ also underlined the paramount influence of hormone interactions on the regeneration of rootable shoots of *Lathyrus*, but provided no explanation for the fundamental mechanism underlying this phenomenon. In addition, the difference observed in susceptibility to hyperhydricity depended on the type of cytokinin used and on the genotype studied, such genotype-specific responses being commonplace in legumes^(6,7).

The availability of a reliable regeneration technique for the production of fertile plants may help in the breeding of *Lathyrus sativus* itself, while regenerating somatic hybrids might yield genotypes with the disease resistance from grass pea coupled with the grain quality from protein pea. These results, added to those obtained with pea⁽⁷⁾, stress the interest of flow cytometry as an early screening strategy to avoid hyperhydricity in cultured tissues, and for the optimisation of plant regeneration in grain legumes.

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