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Optimisation of protoplast production in white lupin

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Abstract

The influence, was investigated, of abiotic parameters on the isolation of protoplasts from *in vitro* seedling cotyledons of white lupin. The protoplasts were found to be competent in withstanding a wide range of osmotic potentials of the enzyme medium, however, -2.25 MPa (0.5 M mannitol), resulted in the highest yield of protoplasts. The pH of the isolation medium also had a profound effect on protoplast production. Vacuum infiltration of the enzyme solution into the cotyledon tissue resulted in a progressive drop in the yield of protoplasts. The speed and duration of orbital agitation of the cotyledon tissue played a significant role in the release of protoplasts and a two step (stationary-gyratory) regime was found to be better than the gyratory-only system.

Additional key words: *Lupinus albus*, protoplast isolation, protoplast viability, protoplast yield.

Introduction

Protoplasts have diverse applications including the study of cell physiology and genetics, plasma membrane properties, cell-cell communication and particle uptake. In terms of its agricultural implications, the technology has played an important role in the study of host-parasite interactions, creation of synthetic amphidiploids and plant genetic transformation.

In the case of *Lupinus*, a cross incompatibility has been found to exist between species (Roy and Gladstones 1988, Busmann-Loock *et al.* 1992) which severely restricts hybridisation efforts. Protoplast technology is therefore now being explored (Wetten *et al.* 1999) with the aim of enabling the production of interspecific hybrids.

Several biotic and abiotic factors influence the isolation of protoplasts from a plant organ or cell suspension culture. These include some physio-chemical ones such as the osmotic potential and pH of the enzyme solution, vacuum infiltration and tissue agitation. Previous work on *Lupinus* (Schäfer-Menuhr 1987, 1988) evaluated only the osmoticum concentration but cross-referencing is not possible because the osmotic potential was not determined. Furthermore, the other parameters mentioned above have not been studied for *Lupinus* protoplast isolation. Moreover, to date, no protoplast work has been published on white lupin (*Lupinus albus* L.) which is not only an important component in many conventional crosses but also a potential candidate for somatic hybridisation.

The aim of this paper is to evaluate and establish the physio-chemical parameters for protoplast production from cotyledons of white lupin that can ultimately be used for studying lupin biology as well as for genetic enhancement *via* heterofusion.

Materials and methods

Protoplasts were isolated from the cotyledons of *in vitro* seedlings of white lupin (*Lupinus albus* L.) genotype CH304/70. The seeds of glasshouse grown plants were surface sterilised with 70 % (v/v) ethanol for 60 s

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Abbreviations: CPW - cell and protoplast wash medium; FDA - fluorescein diacetate; f.m. - fresh mass.

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followed by a treatment with 10 % (v/v) sodium hypochlorite and *Tween-20* for 15 min under a partial vacuum. After 3 washes in sterilised water, the seeds were imbibed for 2 h in sterilised water. The imbibed seeds were transferred onto moist filter paper in a sealed Petri dish and incubated in the dark at 25 ± 1 °C for 7 d. The seedlings were transferred to 175 cm^3 glass culture jars, containing 0.6 % (m/v) *Bacto* agar (*Difco Laboratories*, Detroit, USA), and stored at a temperature of 25 ± 1 °C and under 16-h photoperiod (cool white fluorescent lamps, irradiance of 80 $\mu mol\ m^{-2}\ s^{-1}$).

Protoplasts were isolated from cotyledons of 14-d-old *in vitro* seedlings. Individual cotyledons were sliced transversely into 1-mm-wide strips. After slicing, the tissues were transferred to a 60 × 15 mm *Nunclon* dish (*Nalge Nunc International*, Roskilde, Denmark) containing 5 cm^3 solution of 13 % m/v mannitol in cell and protoplast wash medium (CPW; Frearson *et al.* 1973), containing 27.2 mg dm^{-3} KH_2PO_4, 101 mg dm^{-3} KNO_3, 1480 mg dm^{-3} $CaCl_2 . 2 H_2O$, 246 mg dm^{-3} $MgSO_4 . 7 H_2O$, 0.16 mg dm^{-3} KI, 0.025 mg dm^{-3} $CuSO_4 . 5 H_2O$, pH 5.8, and were plasmolysed for 1 h. This followed the replacement of plasmolysis medium with the enzyme solution. The combination of enzymes used was 1 % (m/v) cellulase *Onozuka® R10* (*Yakult Pharmaceutical Industry Co.*, Tokyo, Japan) and 0.1 % (m/v) pectinase *Pectolyase Y23* (*Seishin Corporation*, Tokyo, Japan) – prepared following the procedure of McDonald *et al.* (1996).

The osmotic potential of the enzyme solution was varied using six mannitol concentrations (0, 0.2, 0.4, 0.6, 0.8 and 1.0 M). The osmotic potential of these six solutions were recorded before filter-sterilisation, using Wescor vapour pressure osmometer (*Wescor*, Logan, USA). The pH of enzyme medium was adjusted to 3.6, 4.2, 4.6, 5.2, 5.6, 6.2, 6.6 and 7.2 using either 1 M sodium hydroxide or 1 M hydrochloric acid.

After the pre-plasmolysis, the plasmolyticum was replaced with the enzyme medium and the incubate was subjected to vacuum. The unsealed *Nunclon* dishes were placed in a bell jar that was attached to a vacuum-line. The vacuum treatment was applied for 0, 10, 20 and 30 min and the dishes were then sealed with *Nescofilm®* (*Nippon Shoji Kaisha*, Osaka, Japan) and stored in the dark in the growth room for overnight digestion. No vacuum was applied in other experiments.

Two similar gyratory shakers, placed side by side, were used to investigate the effect of tissue agitation on protoplast release. These were set at 30 and 50 rpm. Each speed treatment was split into 3 duration treatments: 30 min (G30), 60 min (G60) and overnight (Go) shaking. For Go, the replicate digestions were stored in dark boxes and were maintained on the shakers overnight (14 h) under growth room conditions. For shorter duration treatments, the incubates were maintained stationary overnight in dark boxes on a growth room shelf. After 14 h, they were transferred to the boxes already on the shakers, for 60 min but the G30 set was removed 30 min earlier than the rest. The total incubation time was the same for all the treatments.

The data were collected in terms of protoplast yield g^{-1}(f.m.) of the cotyledon tissue. Where necessary, the data were transformed to square root values before carrying out the analyses of variance and fitting orthogonal polynomials on the computer package *SAS*. The viability of protoplasts was ascertained with fluorescein diacetate (FDA) vital stain using a modification of the method of Widholm (1972), after resuspending them in a known final volume of CPW9M (9 % m/v mannitol in CPW medium).

Results and discussion

Osmotic potential of the enzyme solution: The results showed that the protoplast yield was significantly ($P < 0.001$) dependent on the mannitol concentration in the enzyme medium, with the exception of non-significant difference between concentrations of 0.8 and 1.0 M. The fact that intact protoplasts were observed in all mannitol concentrations indicates that *Lupinus* protoplasts were capable of withstanding a wide range of osmotic potentials. However, for future experiments, the mannitol concentration in the enzyme solution was kept at an intermediate between the two peaks (Fig. 1) at 0.5 M corresponding to an osmotic potential of -2.25 MPa. This concentration also conforms to previous suggestions that the concentration of osmoticum should leave the medium slightly hypertonic so that the released protoplasts remain slightly plasmolysed (Lindsey and Jones 1989). Mannitol was used in the present investigation to alter the osmotic potential of the enzyme medium, as was done in some other studies (Patnaik *et al.* 1981, Tegeder *et al.* 1995), because mannitol is a metabolically inert sugar alcohol (Evans 1976, Warren 1991). In general, the osmotic potential requirements of the medium for isolation of *Lupinus* cotyledonary protoplasts appeared to be slightly higher than those of the cotyledonary protoplasts of some other legumes (Larkin *et al.* 1988, Zhongyi *et al.* 1990, McDonald *et al.* 1996). As mentioned earlier, a comparison of our results with those of Schäfer-Menuhr (1987) on *L. angustifolius* is not possible because the osmotic potential of the enzyme solution used for protoplast isolation was not reported by the author.

Enzyme solution pH: The pH of the enzyme medium played an important role in protoplast production, as

evident by highly significant differences ($P < 0.001$) between the treatments (Fig. 2), supporting the earlier findings of Saxena *et al.* (1986). In the present study, no protoplasts could be isolated at pH 3.6 and the maximum was at pH 5.2 and again at 6.6. A similar observation

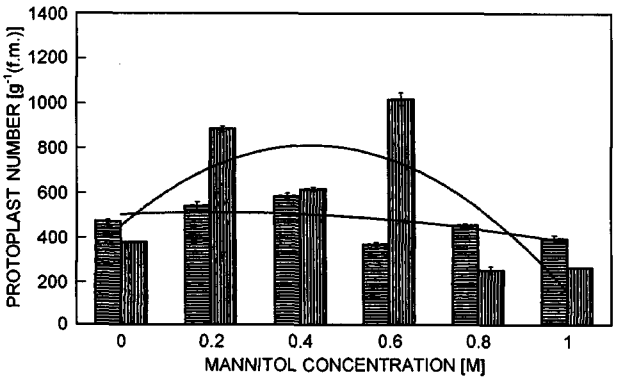

Fig. 1. The relationship between mannitol concentrations in the enzyme solution and the corresponding yield of protoplasts. The experiment was repeated and data collected on two occasions separated by an interval of 5 weeks. The data were transformed to square root values. Fitted polynomials (quadratic) are shown.

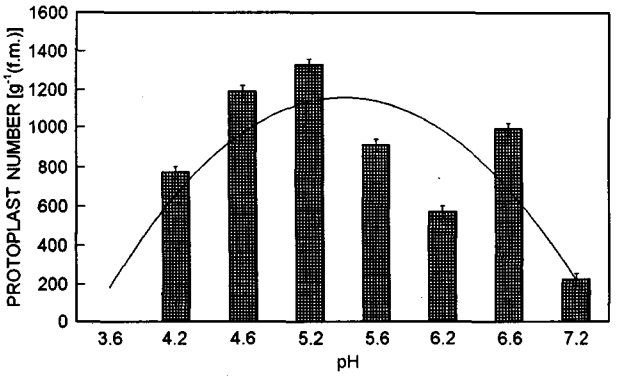

Fig. 2. The effect of pH of the enzyme solution on the yield of isolated protoplasts. Second degree polynomial function is shown.

was also made by Uchimiya and Murashige (1974) who suggested that this could be due to the presence of two isozymes of cellulase. When the polynomials were fitted to our data, a second degree polynomial function was found to be highly significant ($P < 0.001$) but other higher degree polynomials were also significant suggesting a complex relationship between the pH of the enzyme medium and the yield of protoplasts. Although earlier work on *Lupinus* mesophyll protoplasts found a pH 6.8 to be applicable (Schäfer-Menuhr 1987, Schäfer-Menuhr and Stürmer 1987) the enzyme combination used for protoplast isolation was different and is therefore likely to cause differences in optima. The optimum pH range for cellulase activity is between 4.0 and 5.0 whereas for most of the pectinases, it is between 5.0 and 6.0 (Chaplin and Bucke 1990). The present study pointed to a combination of 1 % (m/v) cellulase *Onozuka® R10* and 0.1 % (m/v) pectinase *Pectolyase Y23*, with a pH between 5.2 and 5.6.

Vacuum infiltration of the enzyme solution: The protoplast yield dropped progressively with increase in the duration of vacuum infiltration from 10 to 30 min and significantly ($P < 0.05$) higher number of protoplasts were produced when no vacuum was applied. The drop in the protoplast yields from 1.1×10^6 g^{-1}(f.m.) (no vacuum) to 5.7×10^5 g^{-1}(f.m.) (30 min vacuum) appeared to arise as a result of rupturing of the protoplasts that were released into the enzyme medium soon after the commencement of vacuum treatment. The orthogonal analysis of our results indicated a highly significant ($P < 0.01$) linear relationship between the yield of intact protoplasts g^{-1}(cotyledon f.m.) and the vacuum treatments. Thus, our findings are in contrast to some other studies that found the application of vacuum infiltration useful to the digestion of cotyledon (Zafar *et al.* 1995) as well as other tissues (Lin 1983, Myers *et al.* 1989, Koch *et al.* 1996).

Tissue agitation by gyration: Gyratory shaking proved to be a crucial final step in liberating the protoplasts from the digested tissue. Two regimes, gyratory-only and stationary-gyratory, were compared for their relative efficacy for protoplast liberation and the effect on protoplast viability. The results suggest that the latter was more efficient as evident by high yield (Fig. 3) and viability (Fig. 4) of the isolated protoplasts. The G60 treatment was significantly ($P < 0.05$) superior to G30 and Go treatments. Such two-step regimes have also been found useful in other studies (Bhojwani and White 1982, Shekhawat and Galston 1983a,b, Radionenko *et al.*

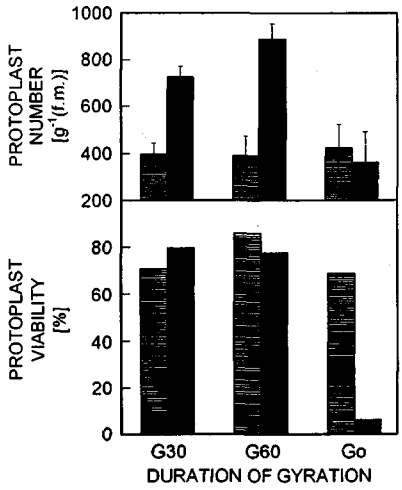

Fig. 3. Effect of the speed and duration of gyration on the protoplast yield and the viability of extracted protoplasts as determined by FDA vital stain. The incubation was carried out for 30 min (G30), 60 min (G60) and overnight (Go) on orbital shakers set at 30 and 60 rpm.

1994). In terms of the speed of gyration, it was apparent that 50 rpm orbital speed was capable of improving protoplast yield substantially over that produced at 30 rpm. At 30 rpm gyratory shaking, it was not critical whether a one-step or a two-step system was followed because protoplast yield (Fig. 3) and the proportion of viable protoplasts (Fig. 4) remained much the same across treatments. On the contrary, a gyratory shaking at 50 rpm resulted in significant ($P < 0.05$) improvements in protoplast yield as well as proportional viability following the two-step system. A similar procedure was employed in other legume protoplast isolations (Landgren 1981, Arya *et al.* 1990).

Conclusion: The optimum condition for *Lupinus* cotyledonary protoplast release included osmotic potential of the enzyme medium adjusted to -2.25 MPa with 0.5 M mannitol, pH 5.6, and an overnight stationary incubation of the tissue in enzyme medium followed by an application of gyratory agitation for 60 min at 50 rpm speed. The protocol developed allowed the production of a reproducible yield of protoplasts with more than 70 % viability, as shown by FDA vital staining, and which re-synthesised cell walls within four days. This thus opens the way for further investigations of the later behaviour of these protoplasts in terms of regeneration and hybridisation.

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