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# The production of fertile, triploid somatic hybrid plants (*Nicotiana glutinosa* (n) + *N. tabacum* (2n)) via gametic: somatic protoplast fusion

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**Summary.** The fusion of gametic protoplasts with somatic protoplasts giving rise to gametosomatic hybrid plants was investigated. Gametosomatic hybrid plants were regenerated following the fusion of nitrate reductase deficient ($Nr^-$) *Nicotiana tabacum* Nia-130 leaf mesophyll protoplasts with *N. glutinosa* tetrad protoplasts. The resulting plants were confirmed as hybrids, based on leaf and floral morphology, chromosome number, leaf esterase and leaf callus peroxidase zymograms and Fraction-1-protein analysis. The five gametosomatic hybrid plants had the expected pentaploid, but functionally triploid chromosome number of $3n = 5x = 60$. The relevance of triploid gametosomatic hybrids in facilitating limited gene transfer, is discussed. The utilisation of tetrads as a generally available source of haploid protoplasts for fusion studies is proposed.

**Key words:** *Nicotiana* – Gametosomatic hybridisation – Tetrad protoplasts – Triploids

## Introduction

It has been proposed that the synthesis of triploid somatic hybrid plants by fusion of diploid protoplasts of a crop plant with haploid protoplasts of a wild type species could facilitate a limited gene transfer from the wild type species into the crop species (Pental and Cocking 1985). In triploid cells introgression could occur either by somatic crossing over, by transposition, or more probably, by trivalent formation at meiosis. The degree of trivalent formation would be dependent on the extent of homology between the haploid and diploid homologous chromosome complements. Unpaired wild type chromosomes might be lost at meiosis or randomly segregate to give gametes and ultimately offspring with a reduced wild type chromosome complement yet retaining the diploid genome intact. By repeated back-crossing to the diploid parent chromosomes of the wild type would be eliminated. Resulting plants and their progeny would need to be assessed for the introgression of novel genes, of wild type origin.

Protoplasts can be isolated from anther-derived haploid plants (Negrutiu 1981). However, the range of species in which anther culture is successful is limited (Maheshwari et al. 1980). An alternative source of haploid protoplasts are the microspores within a tetrad (Bhojwani and Cocking 1972). Tetrad protoplast isolation has been reported for a wide range of species including *Cajanus cajan, Lycopersicon esculentum, Nicotiana tabacum, Petunia hybrida, Triticum aestivum* and *Zea mays* (Bhojwani and Cocking 1972; Bajaj 1974; Deka et al. 1974) and can be extended to any fertile plant species. Tetrad protoplasts provide a readily available source of haploid protoplasts.

In the genus *Nicotiana* somatic hybrid plants have been recovered between nitrate reductase deficient ($Nr^-$) *Nicotiana tabacum* leaf mesophyll protoplasts ($2n = 4x = 48$) and *N. glutinosa* suspension protoplasts ($2n = 2x = 24$). The selection of hybrids was based on the restoration of nitrate reductase proficiency, green colour and plant regeneration capacity (Cooper-Bland et al. 1985). In order to assess the ease with which haploid tetrad protoplasts could be fused with diploid protoplasts, and triploid gametosomatic hybrid plants be recovered, this species combination was chosen. This report describes the somatic hybridisation of *N. tabacum* ($Nr^-$) (leaf mesophyll protoplasts) with *N. glutinosa* (wild type) (tetrad protoplasts). The implications of such hybridisation are discussed.

## Materials and methods

*1 Isolation of protoplasts*

Plants of *Nicotiana tabacum* Nia-130 ($Nr^-$) were grown in hydroponic tanks and mesophyll protoplasts isolated as previously described (Pental et al. 1982; Hamill et al. 1983).

Buds measuring approximately 12 mm in length were selected from mature *N. glutinosa* plants. Following surface sterilisation of buds for 20 min in 10% v/v Domestos solution (Lever Bros. Ltd, UK) coupled with four washes in sterile tap water, one anther from each bud was removed and examined to determine the stage of pollen development. The remaining anthers, in those buds identified to contain mature tetrads, were gently crushed in enzyme solution which consisted of 2% w/v Driselase (Kyowa-Hakko, Kogyo Co. Ltd) in 9% w/v mannitol solution containing CPW inorganic salts (Frearson et al. 1973), pH 5.8. The tetrad suspension was passed through a 45 μ nylon sieve to remove debris and incubated at 25 °C, in the dark for 2 h. A 90–100% conversion of tetrads to protoplasts was achieved.

*2 Fusion and culture of protoplasts*

*N. tabacum* leaf mesophyll protoplasts ($5 \times 10^5$) in 6 ml CPW salts with 13% mannitol were mixed with an equal number of tetrad protoplasts still in enzyme solution. After centrifugation (80×g, 5 min) the supernatant was removed, and replaced with 10 ml of the high pH/calcium fusion solution (Keller and Melchers 1973) without disturbing the protoplast pellet. After incubation at 30 °C for 30 min, the supernatant was removed and replaced with 13% mannitol containing CPW salts, without disturbing the pellet. The pellet was finally resuspended in MS-1 medium (Pental et al. 1982) to give a final density of $5 \times 10^4$ mesophyll protoplasts/ml medium.

Fusion-treated protoplasts were cultured in 5 cm petri dishes (Gibco Europe Ltd.) (5 ml medium per dish) maintained at 25 °C in the dark. After 14 days, protoplasts had divided to give small colonies and were subsequently transferred to selection medium, MS-2 (Pental et al. 1982). Colonies were maintained at a density of $1 \times 10^3$/ml of MS-2 medium in a 9 cm petri dish (Sterilin Ltd.) (12 ml medium per dish) at 24 °C with a continuous illumination of 2,000 lux provided by cool white fluorescent tubes (Thorn, UK). Selected colonies were transferred to regeneration medium (M.S. inorganic salts (Murashige and Skoog 1962) with 3% w/v sucrose 1.0 mg/l 6-benzylamino purine and 2.0 mg/l indole acetic acid solidified with 0.8% agar, pH 5.8) and regenerated shoots subsequently rooted on MS salts with 3% sucrose solidified with 0.8% agar. Putative somatic hybrids were transferred to the open greenhouse and flowered.

*3 Analysis of regenerated plants*

*a) Somatic chromosome number.* Actively growing root tips, from side shoots of the putative somatic hybrids, pretreated in 0.05% w/v colchicine (Sigma) and 0.03% w/v 8-hydroxyquinoline (Sigma) for 6 h (15 °C) were squashed and stained in acetocarmine.

*b) Biochemical analysis.* (i) Soluble protein extracts were prepared from leaf tissue, or leaf callus initiated on M.S. medium supplemented with 3% w/v sucrose, 2.0 mg/l naphthalene acetic acid and 0.5 mg/l benzylamino purine solidified with 0.8% agar. Tissue was ground in ice cold buffer which consisted of 0.1 M, Tris HCl pH 6.8, 10 μm leupeptin, 0.1% w/v dithiothreitol and 15% v/v glycerol (1 ml buffer/g tissue).

Zymograms were obtained for putative somatic hybrids and the parent species by isoelectric focussing on precast polyacrylamide gels (L.K.B.) pH range 4–6.5 (leaf callus peroxidases) and pH range 3.5–9.5 (leaf esterases). Twenty–40 μl aliquots of extract per channel were applied to the appropriate gel.

Isoelectric focussing was for 4 h (1,400 v; 25 mA maximum) at 10 °C. Peroxidase isoenzymes were stained for in a solution of 50 mM Tris-HCl, pH 7.2, 200 mM NaCl, 0.05% w/v 4-chloro-1-naphthol (Sigma), 0.3% w/v $H_2O_2$ in 20% methanol. Esterase isoenzymes were stained for using the method of Smith (1970).

(ii) Fraction I protein analysis of putative hybrids and parent species was conducted following the method of Cammearts and Jacobs (1980).

## Results

Following selection on MS-2 medium in which nitrate is the sole source of nitrogen, two colonies were recovered. One colony underwent regeneration giving rise to six separate shoots, five of which were ultimately transferred to the greenhouse. The other colony was lost due to bacterial contamination.

Cytological analysis of the five putative hybrid plants gave chromosome counts of 60 (Fig. 1) in all cases. This was the expected allopentaploid but functionally triploid somatic chromosome number since for *N. tabacum* (an allotetraploid) 2n=4x=48 and for *N. glutinosa* (a true diploid) 2n=2x=24.

Isoelectric focussing of leaf esterases and leaf callus peroxidases showed that the five putative hybrid plants

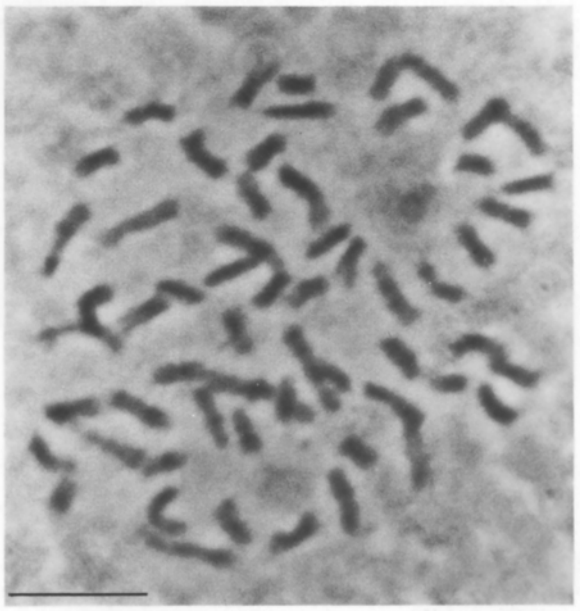

**Fig. 1.** Root tip squash showing the somatic chromosome complement (3n=5x=60) of a gametosomatic hybrid plant. All five plants recovered had the same chromosome number (bar = 10 μm)

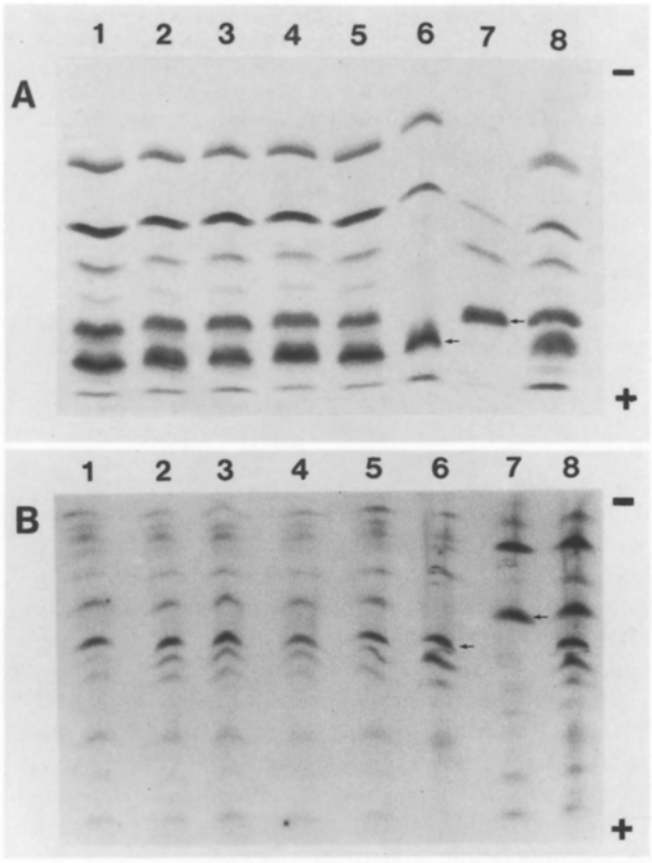


**Fig. 2.** Isoelectric focusing of leaf callus peroxidases (**A**) and leaf esterases (**B**), of gametosomatic hybrids and their parents. Channels from *left to right: 1–5*, triploid gametosomatic hybrids; *6, Nicotiana tabacum; 7, N. glutinosa* and *8,* physical mix (1 : 1) of proteins from *N. tabacum* and *N. glutinosa. Arrows* indicate unique parental bands present in the five hybrids

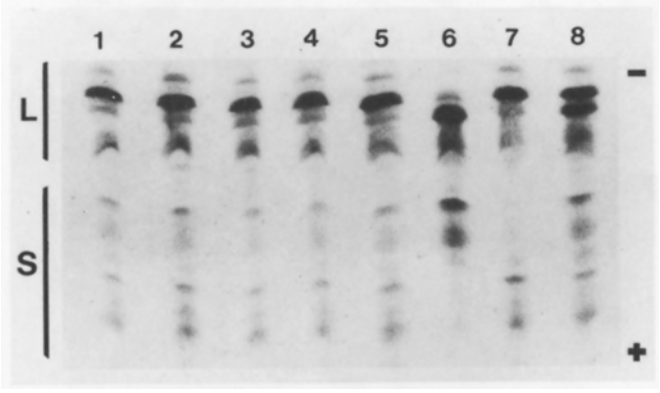


**Fig. 3.** Isoelectric focusing of Fraction I protein polypeptides from gametosomatic hybrids and their parents. Channels from *left to right: 1–5* triploid gametosomatic hybrids; *6, Nicotiana glutinosa; 7, N. tabacum* and *8,* physical mix (1 : 1) of proteins from *N. tabacum* and *N. glutinosa.* Hybrids have the large subunit (L) of *N. tabacum,* and small subunits (S) of both *N. tabacum* and *N. glutinosa*

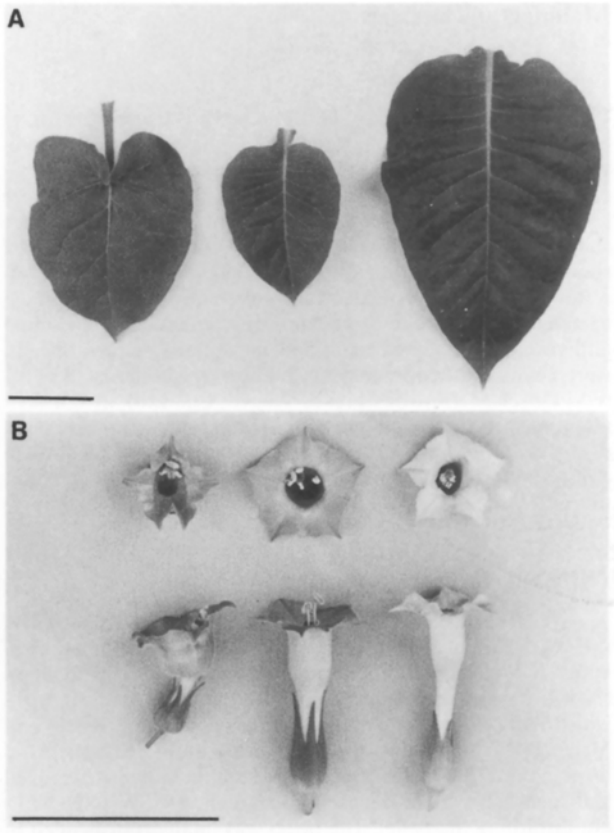


**Fig. 4.** Leaf (**A**) and floral (**B**) morphology of *N. tabacum* cv 'Gatersleben' (*right*); a triploid gametosomatic hybrid (*centre*) and *N. glutinosa* (*left*). The flower colour of *N. tabacum* corresponds to red group 52C, of *N. glutinosa* to red orange group 35C and the hybrid to red group 48B, of the Royal Horticultural Society colour chart (bar = 5 cm)

had banding patterns which were the summation of the two parents (Fig. 2 A, B).

Isoelectric focusing of Fraction 1 protein polypeptides showed that the regenerated plants had the large subunit (chloroplast encoded) of *N. tabacum,* and small subunits (nuclear encoded) of both *N. tabacum* and *N. glutinosa* (Fig. 3), thus confirming their hybridity at the nuclear level.

All five plants were grown to maturity and exhibited an intermediate morphology when compared to the parents (Fig. 4). For comparative purposes the variety Gatersleben was used since this is representative of the nitrate reductase proficient wild type counterpart to the Nia-130 *N. tabacum* mutant.

Pollen viability as assessed using acetocarmine stain was 45–74% for the triploid somatic hybrids, 95% for *N. tabacum* and 99% for *N. glutinosa.* Seed set was possible following self pollination of the hybrids and also in reciprocal crosses with *N. tabacum* cv 'Gatersleben'.

## Discussion

Following the fusion of allotetraploid (functionally diploid) *Nicotiana tabacum* ($Nr^-$) protoplasts with haploid *N. glutinosa* tetrad protoplasts, which are incapable of division, it was anticipated that only somatic hybrid colonies would be capable of growth on a selection medium which contained nitrate as sole nitrogen source. The five plants, regenerated from one of the selected colonies, have been shown to be somatic hybrids as judged by morphological, cytological and biochemical analysis. The plants also have the expected allopentaploid, but functionally triploid chromosome number of $3n=5x=60$. Tetrad protoplasts have not previously been used in successful attempts at somatic hybridisation largely due to the fact that they are recalcitrant in culture. This study shows that they can provide an ideal source of haploid protoplasts. Hybrids produced by the fusion of a somatic protoplast with a gametic protoplast should not strictly be described as somatic hybrids since this describes the fusion of two somatic protoplasts. We propose that such hybrids should be called gametosomatic hybrids to indicate the contribution from a gametic protoplast. Somatic hybrids of *N. tabacum* and *N. glutinosa* have been reported previously (Nagao 1979; Uchimaya 1982; Horn et al. 1983; Cooper-Bland et al. 1985) but in all cases normal diploid protoplasts were used. It is interesting to note that the triploid gametosomatic hybrids reported here were very similar in morphology to the amphiploid somatic hybrids of *N. tabacum* and *N. glutinosa*.

Where the somatic hybrids previously reported, have had the expected amphiploid chromosome number of 72 they were also found to be fertile (Uchimaya et al. 1982). The inheritence of nuclear and cytoplasmic coded characteristics has been followed in several generations of backcrossing to the parents (Uchimaya et al. 1984). Similarly the triploid gametosomatic hybrids have a high pollen viability and a high capacity for seed set. The triploid gametosomatic hybrids and their offspring are currently being assessed in this respect.

It has been suggested that limited gene transfer could be mediated by haploid-diploid fusion of protoplasts and the regeneration of triploid hybrid plants. Triploid somatic hybrid production may also encourage fertility since a diploid component is retained. This could bypass the inevitable problem of sterility in somatic hybrids that are of sexually incompatible parents whether limited gene transfer or novel hybrid production was the objective.

Previous reports of limited gene transfer via cell fusion have included *Parthenocissus tricuspidata* with *Petunia hybrida* (Power et al. 1975) where cell lines were obtained which had chromosomes solely of *Parthenocissus* yet exhibited isoperoxidase activity of both parents. Similarly the correction of nuclear albinism in *Daucus carota* was possible by fusion with *Aegopodium podgaria* protoplasts (Dudits et al. 1979). In this case only *Daucus* chromosomes were retained. In an attempt to not only direct chromosome loss but also overcome somatic incompatibility *Daucus* protoplasts have also been fused with $\gamma$-irradiated *Petroselinum hortense* protoplasts. In this case cytological analysis suggested incomplete loss of *Petroselinum* genome with a single chromosome being retained (Dudits et al. 1979).

The ability to now produce triploid fertile hybrids may provide a more controlled alternative for the study of limited gene transfer which combines both somatic and sexual hybridisation; such an assessment is now underway.

As a first step this study has shown that tetrad protoplasts provide an ideal system for the delivery of a haploid chromosome complement into a fertile diploid background. Tetrad protoplasts readily isolated from a broad spectrum of species are incapable of division thus eliminating the need to develop counter selection for the haploid parent. Anther-derived haploids exist for a few species and their exploitation in somatic hybridisation studies would almost certainly require the concomitant development of a counter selection directed at the elimination of unfused of homokaryon protoplasts. This simplification of selection may even be extended to fusion studies where the allotetraploid product is required, in this case, a tetraploid plant would be used as a source of 2n tetrad protoplasts for use in fusion studies.

Tetrad protoplasts and their utilisation in triploid gametosomatic hybrid plant production therefore provides a novel and alternative approach for the genetic manipulation of plants.

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## References

Bajaj YPS (1974) Isolation and culture studies on pollen and pollen mother cell-protoplasts. Plant Sci Lett 3:93–99

Bhojwani SS, Cocking EC (1972) Isolation of protoplasts from pollen tetrads. Nature New Biol 239:29–30

Cammearts D, Jacobs M (1980) A simple electrophoretic procedure for the determination of the polypeptide composition of the subunits of Fraction I protein. Anal Biochem 97:317–320

Cooper-Bland S, Pental D, Cocking EC (1985) Somatic hybridisation between nitrate reductase deficient *N. tabacum* and *N. glutinosa*. (in prep)

Deka PC, Mehra AK, Pathak NN, Seu SK (1977) Isolation and fusion studies on protoplasts from pollen tetrads. Experientia 33:182–184

Dudits D, Hadlaczky GY, Bajszar GY, Koncz CS, Lazar G, Horvath G (1979) Plant regeneration from intergeneric cell hybrids. Plant Sci Lett 15:101–112

Dudits D, Fejer O, Hadlaczky GY, Koncz CS, Lazar GB, Horvath G (1980) Intergeneric gene transfer mediated by plant protoplast fusion. Mol Gen Genet 179:283–288

Frearson EM, Power JB, Cocking EC (1973) The isolation, culture and regeneration of *Petunia* leaf protoplasts. Dev Biol 33:130–137

Hamill JD, Pental D, Cocking EC, Muller AJ (1983) Production of a nitrate reductase deficient streptomycin resistant mutant of *Nicotiana tabacum* for somatic hybridisation studies. Heredity 50:197–200

Keller WA, Melchers G (1973) The effect of high pH and calcium on tobacco leaf protoplast fusion. Z Naturforsch 28c:737–741

Maheshwari SC, Tyagi AK, Malhotra K, Sopary SK (1980) Induction of haploidy from pollen grains in Angiosperms – the current status. Theor Appl Genet 58:193–206

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–479

Nagao T (1979) Somatic hybridisation of protoplasts. 2. The combinations of *Nicotiana tabacum* and *N. glutinosa* and *N. tabacum* and *N. alata*. Jpn J Crop Sci 48(3):385–392

Negrutiu I (1980) Nutritional requirements of protoplast-derived haploid tobacco cells grown at low cell densities in liquid medium. Planta 149:7–18

Pental D, Cooper-Bland S, Harding K, Cocking EC, Muller AJ (1982) Cultural studies on nitrate reductase deficient *Nicotiana tabacum* mutant protoplasts. Z Pflanzenphysiol 105:219–227

Pental D, Cocking EC (1985) Some theoretical and practical possibilities of plant genetic manipulation using protoplasts. Hereditas (Suppl) 3:83–92

Power JB, Frearson EM, Hayward C, Cocking EC (1975) Some consequences on the fusion and selective culture of *Petunia* and *Parthenocissus* protoplasts. Plant Sci Lett 5: 197–207

Smith HH, Hamill DE, Weaver EA, Thompson KH (1970) Multiple molecular forms of peroxidases and esterases among *Nicotiana* species and amphiploids. J Hered 61: 203–212

Uchimaya H (1982) Somatic hybridisation between male sterile *Nicotiana tabacum* and *N. glutinosa* through protoplast fusion. Theor Appl Genet 61:69–72

Uchimaya H, Kobayashi S, Ono M, Brar DS, Harada N (1984) Characterisation of nuclear and cytoplasmic information in the progeny of a somatic hybrid between male sterile *Nicotiana tabacum* and *N. glutinosa*. Theor Appl Genet 68: 95–100