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Culture of plant somatic hybrids following electrical fusion

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Summary. Electrically-induced protoplast fusion has been used to produce somatic hybrids between *Nicotiana plumbaginifolia* and *Nicotiana tabacum*. Following fusion of suspension culture protoplasts (*N. plumbaginifolia*) with mesophyll protoplasts (*N. tabacum*) heterokaryons were identified visually and their development was followed in culture. Because electrical fusion is a microtechnique, procedures were developed for culturing the heterokaryons in small numbers and at low density. The fusion and culture procedures described are rapid, uncomplicated and repeatable. Good cell viabilities indicate that the fusion procedure is not cytotoxic. Fused material was cultured 1–2 days at high density in modified K3 medium (Nagy and Maliga 1976). The heterokaryons were isolated manually and grown, at low density in conditioned media. Calli have been regenerated. Esterase isozyme patterns confirm the hybrid character of calli and clonally-derived plantlets recovered from these fusions.

Key words: Electrical fusion – Protoplast regeneration – Isozymes – *Nicotiana*

Introduction

Cell fusion has developed into an extremely active field with a wide range of potential applications. Among plant biologists interest has centered on the production of nuclear or cytoplasmic hybrids between sexually incompatible species. The most popular fusion technique involves the use of concentrated polyethylene glycol (PEG) solutions and yields fusion rates in the range of 1–10%. Fusion rates can be enhanced by increasing the PEG concentration or extending the length of the treatment (Kao and Michayluk 1974), but this also reduces cell viability (Kao 1981). Indeed, severe cytotoxic responses to PEG have been reported for mesophyll protoplasts of several species (Kartha et al. 1974; Kao and Michayluk 1974; Constabel et al. 1975).

Recently, considerable attention has been attracted by demonstrations of cell and protoplast fusion using electric fields (Senda et al. 1979; Zimmermann and Vienken 1982). This technique avoids the use of potentially toxic chemicals, is extremely rapid and, under appropriate conditions, can produce fusion rates in excess of 50% (Zimmermann and Scheurich 1981; Bates et al. 1983; Watts and King 1984).

Despite these attributes there has been considerable concern over whether or not electrically fused protoplasts remain viable in culture. Indeed there has not, until now, been any demonstration of the successful recovery of a plant somatic hybrid produced by electrical fusion. However, extended viability has been demonstrated for mammalian cells (Bischoff et al. 1982; Finaz et al. 1984) and yeast protoplasts (Halfmann et al. 1983).

Cell fusion is a two-step process. First, intimate membrane contact is established then localized bilayer disruption is used to initiate fusion. In electrical fusion cell-to-cell contact is established by application of a high frequency AC electrical field. This creates charges on the protoplasts' surfaces which result in the protoplasts being collected in chains on the electrodes (see Pohl 1978 or Zimmermann and Vienken 1982 for reviews). With cell contact established fusion is initiated by the application of one or more short DC pulses of sufficient magnitude to cause reversible membrane breakdown (Zimmermann and Vienken 1982).

Two principal difficulties have hindered the practical application of electrical fusion to plant somatic hybridization. First, the technique, as it emerged from Zimmermann's laboratory, is essentially a microtechnique, thus the fusion products must be cultured in small numbers and at low

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densities. This, coupled with the general lack of readily available selectable markers in plants, makes hybrid identification and culture difficult. Second, electrical fusion has been technically demanding especially from the standpoint of the equipment involved which was often poorly suited to cell culture applications.

Bates et al. (1983) showed that *Vigna* protoplasts could be cultured after being subjected to the electrical fields necessary to induce fusion, but were unable to identify and follow the fate of the individual fusion products. Recently, Zachrisson and Bornman (1984) have obtained a similar result with *Brassica* protoplasts.

We have undertaken a systematic study of electrical fusion with the goal of making it a practical technique for somatic hybridization. In this paper we describe fusion procedures adapted to the constraints of plant tissue culture. The development of electrically fused protoplast heterokaryons is documented and the hybrid character of calli and regenerated material is confirmed by isozyme analysis. In a separate paper (Bates 1985) the quantitative aspects of the electrical fusion technique are discussed more fully.

Materials and methods

Plant materials

Seeds of *Nicotiana tabacum* L. var. 'Xanthi' and *N. plumbaginifolia* were obtained from the Tobacco Laboratory, Plant Genetics and Germplasm Institute, United States Department of Agriculture, Beltsville, MD. USA. Plants, grown either in the laboratory under a mixture of fluorescent and natural light (100–150 $\mu E/m^2 \cdot s$, 14 h light/10 h dark), or in the greenhouse, were watered regularly and fertilized biweekly with 6-6-6.

Callus initiated from *N. plumbaginifolia* stem internodes was grown on solidified MS medium (Murashige and Skoog 1962) containing 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) plus 0.2 mg/l kinetin and grown at 27 °C in the dark. Suspension cultures were initiated from this callus and maintained on MS medium containing 1 mg/l 2,4-D, at 25 °C, under dim fluorescent lights (5 $\mu E/m^2 \cdot s$, continuous illumination), with shaking at 125 rpm. Suspension cultures were subcultured every four days.

Protoplast isolations

Suspension culture cells were washed once with protoplasting salts (1 mM KNO_3 + 0.2 mM KH_2PO_4 + 1 mM $CaCl_2$ + 1 μM KI + 0.4 M mannitol + 3 mM morpholinoethanesulfonic acid (MES), pH 5.7). After centrifugation the cells were resuspended in protoplasting salts plus 1% Worthington CELF cellulase (Worthington Diagnostics, Freehold, New Jersey, USA) plus 0.1% Pectolyase Y-23 (Seishin Pharmaceutical CO. Ltd., Tokyo, Japan) which had been adjusted to pH 5.5 and filter sterilized. Digestion was carried out in the dark at 27 °C for 3–4 h with agitation at half-hour intervals. Protoplasts were purified by passage through a 62 μm mesh nylon screen, pelleted (100 g for 3 min), washed twice with 10 ml of protoplasting salts and once with 10 ml of 0.4 M mannitol. The final protoplast pellet was resuspended in 1 or 2 ml of 0.4 M mannitol and the protoplast density was determined with a hemacytometer.

N. tabacum mesophyll protoplasts were isolated as follows: half-expanded leaves from vegetative plants were surface sterilized in 10% chlorox + 0.1% Tween 80 for 6 min, rinsed three times with sterile water, and portions of the lower epidermis were removed with fine forceps. Peeled leaf portions were cut out and placed face down in the enzyme mixture. Digestion was carried out at 27 °C in the dark, with agitation every half-hour. Undigested leaf material was removed after 1 h and digestion was continued for another 1–2 h. Mesophyll protoplasts were purified as described above for the suspension culture protoplasts. Production of the two protoplast types was synchronized by starting digestion of the *N. tabacum* leaf material 1 h after the suspension culture cells.

Fusion equipment and procedure

Electrical fields were generated by a Zimmermann Cell Fusion Power Supply (GCA Corporation, Chicago, Illinois, USA). Fusions were carried out in a D.E.P. Systems Inc. (Metamora, Michigan, USA) "Open" Fusion Slide No. FCO 2050S which was sterilized by autoclaving. The electrical parameters for fusion were: AC field 600 kHz, 15 V; DC field, 50 V, 50 μs duration. Chain formation in an AC field requires a medium of low conductivity ($> 10^{-5}$ mho/cm), inclusion of even 1 mM KCl is inhibitory. For this reason the protoplasts are fused in a medium containing 0.4 M mannitol with no added salts. Protoplasts placed in the fusion chamber were exposed to an 8 V AC field for 60–90 s, followed by 15 V for 30 s. Then 2 DC pulses were given 9 s apart. After the second pulse the AC field was smoothly damped to 0 V over a period of 60 s. Fused material was then transferred to petri dishes using pasteur pipettes. All manipulations during fusion were carried out in a laminar flow hood.

Manual isolation of heterokaryons

Twenty four – 48 h after fusion, heterokaryons were manually picked out of the cultures using micropipettes. Glass capillary tubes (1.5–1.8 mm diameter, Kimax No. 51, Kimble Products) were drawn out by hand into micropipettes and their tips were fire polished to obtain an opening 100–200 μm in diameter. Heterokaryons were identified at 100× magnification with an inverted microscope (placed in the laminar flow hood). The micropipette was filled, by capillarity, with some culture medium. Then, controlling the micropipette's position by hand, heterokaryons were transferred by mouth suction through two drops of fresh culture medium. Parental mesophyll or suspension culture cells that had been inadvertently carried along were removed using the micropipette.

Post fusion culture

Fused material was cultured in a modification of Nagy and Maliga's K3 medium (1976) which contained 2% sucrose and 6.2% mannitol instead of 0.4 M glucose. This medium was filter sterilized and will henceforth be called K3S.

Conditioned culture medium was prepared by growing *N. plumbaginifolia* suspension culture protoplasts, at an initial density of 5×10^4 protoplasts/ml, in liquid K3S for 7 d. The cells were then removed by centrifugation leaving the supernatant as conditioned medium.

Regeneration of shoots from callus was achieved with Linsmaier and Skoog's RM medium (1965) containing 1 mg/l benzyladenine as the sole hormone. Rooting was carried out in P medium (RM salts and organics but with KNO_3, NH_4NO_3,

and $MgSO_4$ reduced to one-fifth, and with no hormones added; Menczel et al. 1982).

All cultures were grown at 27 °C, 70% relative humidity, in continuous fluorescent light (20 μE/m² · s for protoplasts, 50 μE/m² · s for all other cultures).

Electrophoresis

Proteins were separated on nondenaturing polyacrylamide slab gels. The gels (10% separating gel + 3% stacking gel) were prepared as indicated in the Sigma Technical Bulletin No. MKR-137. 50–100 μg of protein was added to each lane for electrophoresis.

Enzyme extraction

Plants and calli were placed in the dark for 16 h before use. Samples of tissue (0.2 g for leaves, 1 g for callus) were homogenized on ice in 1 ml of 0.5 M Tris (pH 6.8) containing 80 mM dithiothreitol and 20% glycerol. The homogenate was centrifuged (12,000 g for 10 min) and tracking dye was added to the supernatant. Extracts were used immediately.

Esterase staining

Esterase isozymes were visualized using the procedure described by Brewbaker et al. (1968).

Results

Parameters of electrical fusion

The extent of fusion depends on the values selected for the AC and DC electrical fields which are influenced in turn by the size and design of the fusion chamber and to some extent by cell density and probably cell type as well. The electrical fields used here (see "Materials and methods") were chosen through a series of experiments (Bates 1985) in which the optimum production of *N. tabacum* – *N. plumbaginifolia* heterokaryons was sought.

Adaptation of electrical fusion to protoplast culture. Heterokaryon production and recovery are optimal when the protoplasts are fused at high cell densities. Prior to fusion the two protoplast types, suspended in 0.4 M mannitol, were adjusted to a density of 5×10^5–10^6 cells/ml and were mixed together in equal amounts. Individual drops of this suspension were fused and transferred to plastic petri dishes. Since pelleting the protoplasts and readjusting their density would result in substantial losses a method had to be found for adding an appropriate amount of medium to the suspension of fused protoplasts.

In control experiments we found that both types of parental protoplasts grew well (individually and in combination) in K3S at 5×10^4 protoplasts/ml. Therefore, we initially attempted to culture the fused protoplasts by combining them with an equal volume of double strength (2×) K3S containing 0.4 M mannitol, (yielding a final medium concentration of approximately full strength (1×) K3S + 0.4 M mannitol). Using this approach we never obtained more than a few cell divisions; most of the cells simply budded and became swollen. Even cells which clearly were not fusion products responded in this way. The problem was solved by mixing the protoplast suspension after fusion with 1× rather than 2× K3S (osmoticum maintained at 0.4 M). This diluted medium gives good growth and cell divisions in both the heterokaryons and the parental cells. Further experiments revealed that an interaction between medium concentration and cell density controls development in such drop cultures. Figure 1 shows that the protoplasts are much more tolerant of high cell densities when grown in diluted culture media. The efficacy of using diluted K3S is also apparent when the time course for the initiation of cell divisions in high density cultures is examined (Fig. 2). Suspension cell protoplasts, in half-strength (½×) K3S, begin division on day 3 and are followed, after a 1 day lag, by the mesophyll protoplasts. In contrast protoplasts cultured at high density in full-strength K3S never grow well. The mesophyll protoplasts, in ½× K3S, probably reach plating efficiencies approaching 50% after 7–8 days, however this is difficult to quantify

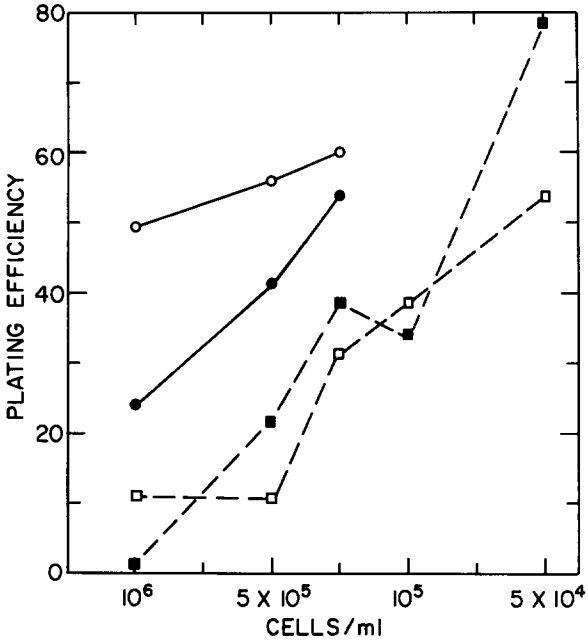

Fig. 1. Effect of cell density and medium concentration on protoplast division. Plating efficiency is defined here as the percentage of live protoplasts which had divided at least once during the first 6 days in culture. Suspension culture protoplasts (*open symbols*) and mesophyll protoplasts (*filled symbols*) were suspended at the cell densities indicated in either 1×K3S (*dashed lines*) or 1/2×K3S (*solid lines*)

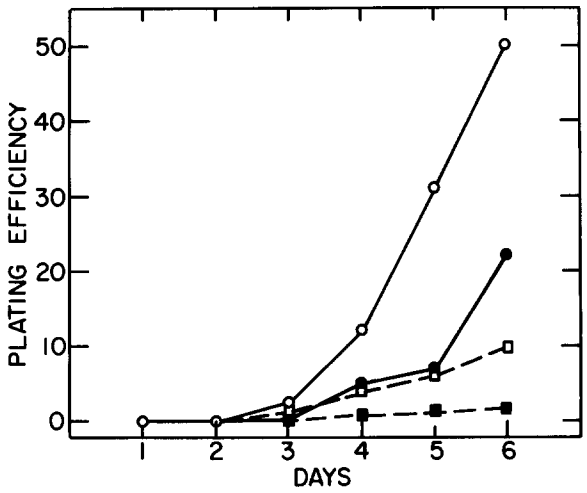

Fig. 2. Time course of initiation of cell divisions in mixed cultures of mesophyll and suspension culture protoplasts grown at high density. Protoplast cultures were initiated, as described for Fig. 1, at a cell density of 10^6 protoplasts/ml. The percentage of protoplasts which had divided (at least once) was determined daily. *Open symbols* suspension culture protoplasts; *filled symbols* mesophyll protoplasts: *Dashed lines* 1×K3S; *Solid lines* 1/2×K3S

due to the presence of large numbers of colonies in the cultures at this point. Those cells which have not divided by the eighth day show little further development.

Identification and development of heterokaryons

Following fusion heteroplasmic cells can be distinguished from parental protoplasts by the presence of green and non-green sectors within the same cell (Fig. 3 b). Because chloroplasts obscure the mesophyll cell nuclei it is impossible to tell, in vivo, if these fusion products are heterokaryons or cybrids. However, staining with carbol fuchsin (Kao 1975) indicates that most of these fusion bodies are true heterokaryons.

The coalescence of fusing cells has been followed microscopically. Fusions between mesophyll and suspension culture protoplasts take about 1 h to merge into a single spherical body (Fig. 4). This contrasts markedly with the situation for mesophyll-mesophyll fusions which merge in the span of 2–5 min (Bates et al. 1983). Probably the denser cytoskeletal network of the suspension culture derived protoplasts is a major factor in this difference. After fusion the mesophyll chloroplasts migrate slowly toward the suspension culture cell's nucleus around which they eventually form a dense layer (Figs. 3 b and 5 a). This packing of the chloroplasts around the nucleus persists for several days and is very useful in distinguishing heterokaryons from mesophyll protoplasts (where the chloroplasts are distributed more or less evenly throughout the cell).

Using the identifying characteristics described above, the percentage of heterokaryons formed in a given fusion can be determined by direct visual counts or by nuclear staining with carbol fuchsin. The production of heterokaryons in the experiments described in this work ranged from 3%–10% with an average of 6.2% (+/–1.2%, SE).

Cell wall regeneration in the heterokaryons begins after 12–24 h in culture as judged by the reappearance of nonspherical cells (Fig. 5 b). This occurs in parallel with wall regeneration in the suspension culture protoplasts but preceeds that for the mesophyll protoplasts by about one day. Development of the heterokaryons also resembles that of the suspension culture protoplasts in that both become lobed and highly asymmetric while the mesophyll protoplasts usually regenerate into oval or round cells.

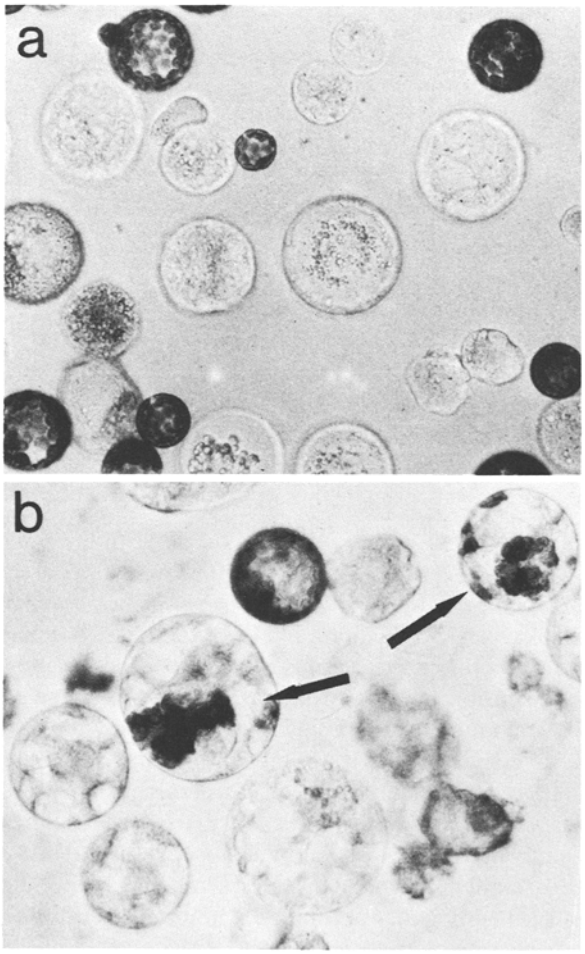

Fig.3 a,b. Protoplast suspensions before and after fusion. **a** mixed mesophyll (with chloroplasts) and suspension culture (no chloroplasts) protoplasts before fusion. **b** two heterokaryons (*arrows*) are in this field as well as several suspension culture protoplasts and a mesophyll protoplast

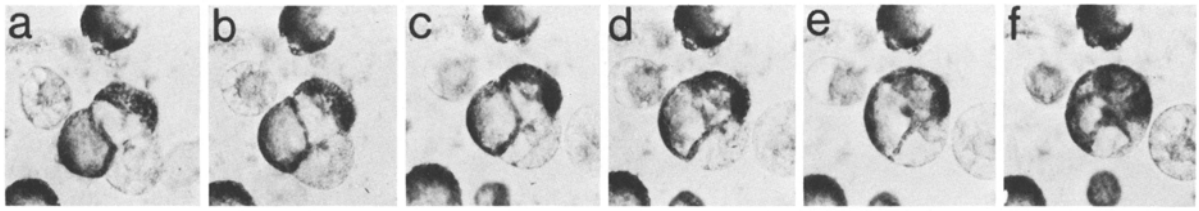

Fig. 4a–f. Time course of coalescence of a fusion body containing two mesophyll protoplasts and one suspension culture protoplast. **a** 5 min post fusion; **b** 15 min; **c** 30 min; **d** 40 min; 50 min; **f** 65 min

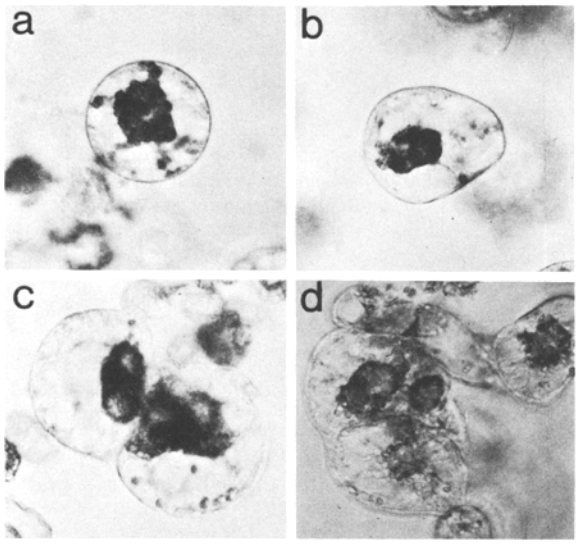

Fig. 5a–d. Development of hybrids in culture. **a** fusion product after 3 h in culture; **b** fusion product after 24 h in culture; **c** hybrid at the two cell stage and after 4 days in culture (1 day in K3S and 3 days in conditioned K3S); **d** 10-day old hybrid microcolony

After 2–3 days the suspension culture protoplasts begin to divide (see Fig. 2); the first divisions of heterokaryons (Fig. 5 c) are delayed to day 4–6.

By the third day of culture the chloroplasts in some mesophyll protoplasts begin to cluster around the nucleus in preparation for cell division. This makes it difficult from day 3 onward to distinguish the heterokaryons from the mesophyll cells. Therefore, 24–48 h after fusion the heterokaryons were manually picked out of the cultures. Using the procedure described in the "Materials and methods" a reasonably pure (> 75%) preparation of heterokaryons was achieved.

Groups of 20–50 heterokaryons were cultured in 50–100 µl of medium. K3S culture medium, regardless of its dilution, would not support growth at these cell densities; however, medium which had been conditioned by previous growth of suspension cell protoplasts gave sustained divisions by 30% of the isolated heterokaryons (Fig. 5 c, d). Fresh, conditioned medium was added to the cultures every 7–14 days. When the colonies were 0.5–1 mm in diameter they were separated from each other and transferred to solidified medium containing a 1 : 1 mixture of K3S (without mannitol) and conditioned K3S. After 4–6 weeks of further growth the individual calli were transferred to solid K3S (containing no mannitol). Regeneration of plants was begun by transferring calli, 1–2 cm in diameter, to RM medium. The resulting plantlets were rooted on P medium. Some of the calli gave rise to normal looking plants, some produced leafy shoots which fail to root, and some showed no morphogenetic potential.

Isozyme analysis

Leaf esterase profiles have been used previously to identify somatic hybrids between *N. tabacum* and *N. plumbaginifolia* (Menczel et al. 1982, 1983). Our results show clear cut differences in the esterase profiles of these species for callus as well as leaves (Fig. 6). Within a species the profile for callus and leaves are similar but not identical. *N. tabacum* callus yields 5 esterase bands. Only two of these are seen in the leaves, however the leaves express a unique band seen near the top of the gel. In the case of *N. plumbaginifolia,* callus and leaf esterase profiles are identical except that the pair of prominent bands near the bottom of the gel are expressed much more strongly in the undifferentiated tissue. These high mobility bands are only faintly visible in the *N. plumbaginifolia* leaf profile.

Comparison of *N. tabacum* and *N. plumbaginifolia* reveals no common bands. Based on these results it should be possible to identify somatic hybrids regardless of their state of differentiation. Lane A, Fig. 6, shows the esterase profile of a callus recovered after fusion which has not regenerated despite four passages on RM medium. This callus exhibits all of the *N. plumbaginifolia* bands and three of the *N. tabacum* bands. Consistent with the undifferentiated state of this sample the *N. tabacum* leaf band is missing. The callus also expresses one unique band (near the middle of the gel) which is not found in either parent. This is important evidence that this is a true hybrid and not a stable chimera. Lane B, Fig. 6, shows the profile of leaf material from regenerated shoots. This material was

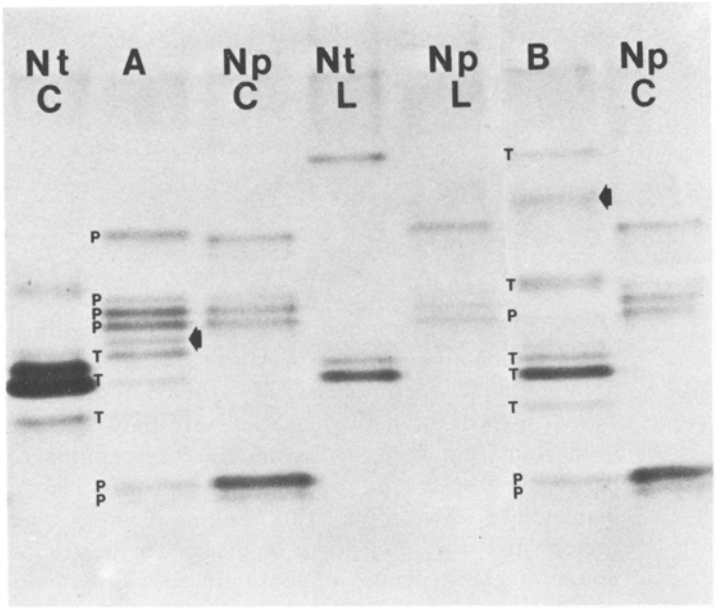

Fig. 6. Esterase isozyme profiles for callus and leaves of *N. plumbaginifolia* and *N. tabacum* and hybrids. *C* callus; *L* leaf; *Np N. plumbaginifolia; Nt N. tabacum; A* and *B* hybrids. Band assignations for samples *A* and *B* are indicated by the *small letters to the left* of these lanes (*P* for *plumbaginifolia* and *T* for *tabacum*). The *arrows* indicate unique bands

recovered from callus that had been grown from a single heterokaryon in a Cuprak dish. These clonal plantlets, which have yet to develop roots despite transfer to P medium, express all of the *N. tabacum* leaf and callus bands. Also expressed are one of the mid-mobility and the two rapidly migrating *N. plumbaginifolia* bands as well as a unique band (just below the *N. tabacum* leaf band near the top of the gel). Again, presence of a unique band indicates that this clonally derived material is a hybrid not a chimera.

Discussion

The procedure described here for fusing plant protoplasts is extremely simple and rapid. Once isolated, drops of protoplasts can be fused and placed into culture literally in minutes. We have no evidence that electrical fusion has any lasting negative effect on protoplast viability. Indeed, the fact that the protoplasts can be successfully cultured after fusion without first being washed or re-isolated is clear evidence that this fusion technique is nontoxic. Our maximum plating efficiencies for unfused protoplasts are in the range of 50–60%. In one experiment 30% of the isolated heterokaryons were found to be capable of cell divisions. Considering that half of the heterokaryons contain more that two nuclei (Bates 1985), and therefore might not be expected to grow, a heterokaryon plating efficiency of 30% is quite respectable.

Our isozyme studies also indicate that viable heterokaryons are being recovered after fusion. Esterases have rarely been used for analyzing hybrid calli (as opposed to regenerated plants) before (but see Menczel et al. 1983). This appears to be a valid approach however, at least for hybrids between *N. tabacum* and *N. plumbaginifolia.* Both of the hybrids shown in Fig. 6 express esterase bands not found in either parent. Appearance of new isozymes often occurs in somatic hybrids (Davey and Kumar 1983).

The primary difficulties encountered in culturing electrically fused protoplasts were those often associated with microdrop culture: controlling cell density and medium composition. As a practical procedure we find that electrical fusion is most efficient when carried out at cell densities approaching 5×10^5 protoplasts/ml. Because it is not possible to recover all of the protoplasts from the fusion chamber it is difficult to control the final protoplast density in culture. The data presented in Figs. 1 and 2 show that protoplast plating efficiency can be made much less cell-density dependent by simply diluting the culture medium.

The actual percentages of protoplasts fused in these experiments are not any greater than those often reported for fusion by polyethylene glycol and is much lower than the fusion rates sometimes reported for electrofusion (Zimmermann and Scheurich 1981). We observed an average heterokaryon production of 6.2%. The total fusion percentage (homokaryons + heterokaryons) can be determined by staining the cells with carbol fuchsin and counting the number of nuclei per cell. This leads to an overall average fusion rate of 15.4% which is very close to the value reported by Zachrisson and Bornman (1984) but is still well below maximum achievable values predicted by our own previous work (Bates et al. 1983) as well as that of Zimmermann's group (Zimmermann and Vienken 1982). A primary reason for the fusion rate being less

than expected is that in using the fusion chamber described in this paper it is impossible to get all of the protoplasts into the effective portion of the electrical fields. The gap between the electrodes in the D.E.P. chamber holds 5–10 µl of protoplast suspension and we routinely place a 25–50 µl drop onto the chamber. Although the AC electric field draws protoplasts into the electrode gap many protoplasts in the chamber are beyond the field's reach and are never collected into chains. Undoubtedly the fusion rate for protoplasts in the gap between the electrodes is considerably higher than 15%. As a solution to this problem we have designed a fusion chamber in which 300–500 µl of protoplast suspension can be exposed to the electric fields. The results obtained with this chamber as well as an in-depth discussion of the numbers and types of fusions obtained in electric fields, will be presented in a separate publication (Bates 1985).

With fusion rates of the magnitude obtained here it is imperative that some method for selection of the hybrids be used. By fusing suspension culture and mesophyll protoplasts it was possible to identify the hybrids visually and isolate them manually (Kao 1977; Gleba and Hoffmann 1978; Menczel et al. 1978). Although this approach is more tedious than a genetic or biochemical selection it has the advantage of not restricting fusion experiments to particular auxotrophic or toxin-resistant cell lines. If one is able to find techniques for cultivating protoplasts at low cell densities then visual identification and manual isolation of hybrids can be very straight forward and successful. With tobacco, conditioned culture medium was sufficient for low density culture; however, species more difficult to culture will undoubtedly require the use of feeder layers such as described by Shneyour et al. (1984).

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