Electrofusion of protoplasts from celery (*Apium graveolens* L.) with protoplasts from the filamentous fungus *Aspergillus nidulans*

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**Abstract.** A method was developed for electrofusion of higher-plant protoplasts from celery and protoplasts from the filamentous fungus *Aspergillus nidulans*. Initially, methods for the fusion of protoplasts from each species were determined individually and, subsequently, electrical parameters for fusion between the species were determined. Pronase-E treatment and the presence of calcium ions markedly increased celery protoplast stability under the electrical conditions required and increased fusion frequency with *A. nidulans* protoplasts. A reduction in protoplast viability was observed after electrofusion but the majority of the protoplasts remained viable over a 24-h incubation period. A small decline in protoplast respiration rate occurred during incubation but those celery protoplasts fused with *A. nidulans* protoplasts showed elevated respiration rates for 3 h after electrofusion.

**Key words:** *Apium* (protoplast fusion) – *Aspergillus* (protoplast fusion) – Electrofusion (protoplasts) – Protoplast fusion

**Introduction**

The use of high-strength electrical fields combined with the electrically induced formation of chains of cells (pearl chains) has been used to stimulate cell fusion (Zimmermann and Scheurich 1981). Such techniques offer more controllable and more efficient methods for protoplast fusion than chemical treatments. The use of electrofusion techniques avoids the need for toxic chemical stimulants and excessive washing of cells (Bates 1985). Chemical agents induce random aggregation and fusion events (Watts and King 1984). Electrofusion, however, combined with micromanipulation of electrodes, permits parentage of heterokaryons to be precisely defined (Koop and Schweiger 1985; Morikawa et al. 1988). Electrofusion can also be monitored microscopically, which aids determination of the electrical parameters required for heterokaryon formation and permits the observation of fusion events (Zachrisson and Bornman 1986). Additionally Arnold and Zimmermann (1984) have reported that zones of membrane disturbance can be restricted to zones of membrane contact, whereas chemical treatments affect the whole protoplast plasmalemma. Reduced membrane disturbance may maintain protoplast viability. Chemical treatments usually result in fusion frequencies of 1–5% (Zachrisson and Bornman 1986). Electrofusion can result in fusion frequencies, between plant protoplasts, in excess of 50% (Bates and Hasenkampf 1985; Watts and King 1984).

Electrofusion is a two-stage process. Initially, the subjecting of protoplasts to a non-uniform electrical field causes polarized protoplasts to move towards regions of higher field strength and the formation of pearl chains results (Arnold and Zimmermann 1981). This effect is termed dielectrophoresis (Pohl 1978). Subsequent application of short DC (direct current) pulses causes breakdown of aligned membranes and protoplast fusion results. After fusion, protoplasts round up into spheres (Zimmermann et al. 1985). The technique has been used successfully to fuse a range of protoplast types e.g. β-lymphocytes and myeloma cells, to produce hybridoma cells (Zimmermann and Vienken 1982); Friend cells with *Petunia* protoplasts.

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Abbreviations: AC = alternating current; DC = direct current
persed clones and subcultured at weekly intervals by inoculating, friable callus was selected 37 d after initiation and subcultured at 20 °C under a 12-h light/dark cycle. Fast growing cultures were continuously selected for fast-growing, finely dispersed clones and subcultured at weekly intervals by inoculating 0.25 mg 1-1 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg 1-1 N6_furfurylaminopurine (kinetin). Cultures were grown under a 0.25 mg 1-x 2,4-D, 0.5 mg 1-1 kinetin, with approximately 5 g 50 ml of MS medium supplemented with 30 g-1 1 sucrose, 0.6 M mannitol plus 0.5 mM CaCl2 2H2O.

Fungal culture. Aspergillus nidulans. BDUN 33 (University of Nottingham collection) was maintained on 1% (w/v) malt extract (Oxoid, Basingstoke, UK) solidified with 1.5% (w/v) agar (Difco). Cultures grown on agar slopes in 300-ml medical flat bottles at 37°C for 2 d were used as sources of conidia.

Liberation of fungal protoplasts. Protoplasts were liberated from A. nidulans mycelium grown in liquid culture (18 h) by the method of Isaacs and Gokhale (1982). A final concentration of 0.2 M phosphate buffer pH 5.8 plus 0.4 M mannitol was used as buffer/stabiliser with Novozym 234 (Novo Industri, Copenhagen, Denmark) at 1 mg ml-1. Lytic incubation was with gentle shaking at 30°C for 3 h. Protoplasts were harvested as described by Peberdy and Isaacs (1976) and resuspended in 0.6 M mannitol plus 0.5 mM CaCl2 2H2O.

Nuclear staining. Nuclei of protoplasts were observed microscopically using the method of Kunkel et al. (1987). A solution of DAPI (4,6-diamidino-2-phenylindole; Sigma, Poole, Dorset, UK) was prepared in 0.1 M (Tris) 2-amino-2-(hydroxymethyl)-1,3-propanediol-HCl buffer pH 7.0, plus 0.1 M NaCl and 0.01 M ethylenediaminetetraacetic acid (EDTA). Protoplast samples were fixed in osmotically stabilised 2% gluteraldehyde solution buffered with 0.1 M Tris-HCl buffer pH 7.0 for 30 min at room temperature. Samples were resuspended in buffer/stabiliser solution. A drop of protoplast suspension was mixed with DAPI (diluted to give 300 ng ml-1 on the slide) and allowed to stand for 5 min to allow uptake of the stain. Observations were made under ultraviolet illumination.

Electrofusion apparatus. Electric fields were generated by a Zim- mermann Cell Fusion™ System (GCA Corporation, Chicago, Ill., USA), described by Zimmermann and Scheurich (1981). Two types of fusion chamber (supplied by GCA Corporation) were used. A flat fusion chamber (7 μl capacity), similar to that described by Pilwat et al. (1981) and a helical chamber (200 μl capacity) of similar construction to that described by Zimmermann and Vienken (1984).

Electrofusion of celery protoplasts. Alignment and fusion parameters for celery protoplasts (1·105 protoplasts ml-1) were determined in a 100-μm flat fusion chamber, and used to fuse celery protoplasts unless otherwise stated. Protoplasts were aligned in a 1.5 MHz, 200 V cm-1 electric field and the number of aligned protoplasts assessed. After approx. 45 s, protoplasts were fused using two 150 V cm-1 fusion pulses of 99 μs duration, with a 1-s period between pulses. After the second pulse the AC (alternating current) field was smoothly damped to zero V over a 30-s period. The number of adhering protoplast pairs (membrane contact areas) which fused, was determined (fusion events) by microscopic observation. For larger volumes a helical fusion chamber was used.

Electrofusion of fungal protoplasts. Fusion parameters were determined in a 100-μl fusion chamber, loaded with 1·105 proto-