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# Protoplast fusion of *Catharanthus roseus* cells by electrofusion of chemically-agglutinated protoplasts

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## Abstract

Mesophyll derived protoplasts of *Catharanthus roseus* cv. Little pinkie were fused with protoplasts derived from an habituated cell line of *C. roseus*. Polyethylene glycol was used as agglutinating agent while fusions were induced by square pulses. Best results were obtained by fusing protoplasts from primary leaves with those from three-day-old cell cultures. Adding calcium ions considerably enhanced heterofusion rate. Good cell viabilities indicated that this fusion process was not cytotoxic. The heterofusion frequency was up to 10 % or more. Most of the heterokaryons were able to regenerate their cell walls and underwent division.

## Introduction

Protoplast fusion is a powerful tool for the improvement of plant species (Evans 1983). It can be achieved with chemicals such as PEG (Kao and Michayluk 1974) or dextran (Kameya *et al.* 1981). Another method is electrofusion which needs tight adhesion of protoplast membranes before triggering fusions by electric pulses.

Dielectrophoresis phenomena have most often been used to attach protoplasts to each other (Zimmermann and Scheurich 1981). Nevertheless, this method has suffered from some limitations due to the small numbers of protoplasts that can be treated in one experiment or to the need to use media with low electric conductivity. Several improvements have therefore been proposed (Watts and King 1984, Tempelaar and Jones 1985), including an alternative electrofusion method using a chemical agglutination of protoplasts and a DC pulse as a fusion promoter (Kameya 1983, Chapel *et al.* 1984, 1986).

Fusion processes have seldom been used for obtaining of cell lines producing secondary metabolites (Yamada *et al.* 1987, 1989). This strategy might be of interest for at least two reasons: (1) deregulation of gene control or the mixed metabolic

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*Abbreviations:* AC - alternating current, DC - direct current, d.m. - dry mass, FDA - fluorescein diacetate, FITC - fluorescein isothiocyanate, f.m. - fresh mass, PEG - polyethylene glycol.

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pathways might result in the enhancement of some parental metabolites or in the production of new metabolites: this was found by Ninnemann and Jüttner (1981) in hybrid calli obtained after the fusion of *Solanum tuberosum* and *Lycopersicon esculentum* protoplasts. However, this result has to date remained unique; and (2) complementing useful traits of parental protoplasts might result in improved lines. For example, it might be of interest to fuse a cell line containing a large amount of alkaloids with a cell line that grows fast (Maeda *et al.* 1983).

As part of an investigation into the production of indole alkaloids in *Catharanthus roseus* cell suspension (Mérillon *et al.* 1989, 1991), we attempted to fuse *C. roseus* protoplasts derived from cells accumulating high level of alkaloids with those obtained from fast growing (but low accumulating) *C. roseus* cells. In the present paper, we report on optimal conditions for electrofusion of chemically agglutinated protoplasts. We obtained viable heterokaryons which resynthesized cell walls and underwent divisions.

## Materials and methods

**Chemicals:** Cellulase R10 Onozuka and Macerozyme R10 Onozuka were obtained from *Yakult Pharmaceutical Ltd.* (Tokyo, Japan). Pectolyase, FITC, Agar, Dextran 500 000 were purchased from *Sigma-chimic* (Saint Quentin Fallavier, France). PEG 6000 was obtained from *Merck* (Darmstadt, Germany) and FDA from *Prolabo* (Paris, France). In some experiments, PEG was purified as described by Kao and Saleem (1986).

**Plant materials:** Plantlets of *Catharanthus roseus* (L.) G. Don (cv. Little pinkie) (*Apocynaceae*) were grown in a greenhouse (24 ± 1°C, photoperiod 12 h). We used either cotyledons, the first (F1) or the third (F3) pairs of leaves developed above the cotyledons of a two-month-old plantlet. The leaves accumulated vindoline as the main alkaloid as well as catharanthine, ajmalicine and serpentine.

The habituated cell suspension culture (C20A) was maintained in an hormone-free Gamborg's *et al.* medium (1968) containing 58 mM sucrose (Mérillon *et al.* 1989). The cell suspension culture was albino and was growing fast. They accumulated 25 $\mu g\ g^{-1}$(d.m.) of ajmalicine and 20 $\mu g\ g^{-1}$(d.m.) of serpentine.

**Protoplast isolation and purification:** Cotyledons and leaves were sterilized in 70 % ethanol (1 min), then in 0.5 % sodium hypochlorite (4 min) and rinsed four times with sterile water. After removing the lower epidermis, mesophyll protoplasts were isolated according to Constabel *et al.* (1980).

C20A protoplasts were isolated from à 3-5-d-old cell suspension culture by incubation in 2 % Cellulase, 1% Macerozyme and 0.05 % Pectolyase dissolved in osmoticum (0.55 M sorbitol, 0.5 mM $CaCl_2$, pH 5.5). Digestion was carried out for 16 to 18 h at 27 °C in the dark, with occasional shaking. Protoplasts were purified by filtration through a 100 µm mesh nylon screen, pelleted twice in osmoticum (100 *g*, 3 min), purified twice by overlayering 0.6 M sucrose, pelleted twice in

osmoticum (100 *g*, 3 min). The final pellet was resuspended in 0.5 M mannitol containing 0.5 mM $CaCl_2$ (pH 5.5). Protoplast viability was assessed by FDA staining (Power and Chapman 1985).

**Electrofusion:** An electropulsator (PS 10, *Jouan*, France) was used for delivering rectangular pulses which were electronically controlled on an oscilloscope. Parental protoplasts were mixed (1:1 ratio) at a final concentration of $5 \times 10^5$(protoplasts) $cm^{-3}$ in 0.5 M mannitol. A 75 µl drop was placed in a plastic Petri dish (3 × 55 mm, *Corning*). After 10 min settling, 75 µl of a filter-sterilized solution (0.45 µm, *Sartorius*), consisting of 10 % PEG, 0.05 mM $MgS0_4.7H_20$, 25 mM NaCl and 0.5 M mannitol (pH 5.9), was added to the drop. Thirty min later, two flat stainless-steel electrodes (10 mm long, 5 mm wide) were dipped into the agglutinated protoplasts and a single DC pulse (0.5 to 2 kV $cm^{-1}$) with a duration of 50 to 150 ms was applied. After the pulse, the electrodes were removed and the PEG was diluted 1 h later using a hypotonic solution (0.27 M mannitol, 0.13 M $CaCl_2$, pH 5.5).

**Estimation of fusion frequency:** The number of heterokaryons was estimated 24 h after application of the DC pulse. Heterofusion frequencies were expressed in percent of total protoplasts with at least three hundred to five hundred protoplasts being counted. Heterokaryons were recognized by the simultaneous presence of chloroplasts (mesophyll protoplasts) and dense cytoplasmic strands (C20A protoplasts). An alternative method was the staining of C20A protoplasts by FITC before fusion (Galbraith *et al.* 1984): heterokaryons showed both red (chloroplasts) and green (FITC) fluorescence.

**Culture of heterokaryons:** One hour after the PEG elution, 600 µl of culture medium were added to the protoplast mixture. The culture medium consisted of Murashige and Skoog's (1962) macronutriments, Heller's (1953) micronutriments, Gamborg's *et al.* (1968) vitamins, 4.5 µM 2,4-dichlorophenoxyacetic acid, 0.53 mM α-naphtalene acetic acid, 2.8 µM kinetin, 0.25 M glucose, 0.25 M sorbitol (pH 5.5). Protoplasts were grown at 26 ± 1 °C in the dark for one week, and then transferred to dim light. The osmolality of the medium was reduced at weekly intervals by adding aliquots of C20A culture medium.

Frequencies of heterokaryon divisions were determined at day 5 by counting heterokaryons that divided at least once.

## Results and discussion

**Culture of parental protoplasts:** Protoplasts were easily obtained from mesophyll and C20A cells (Fig. 3A,B). The yields were of $4.3 \times 10^6$ $g^{-1}$(f.m.) and $2.3 \times 10^5$ $g^{-1}$(f.m.), respectively. We first tried to culture parental protoplasts, and easily obtained microcalli from C20A protoplasts, but mesophyll protoplasts stopped growing after one or two divisions. This may have been due to the genotype used in our experiments, since calli were previously obtained from *C. roseus* mesophyll

protoplasts by Constabel *et al.* (1981). However, these authors reported that protoplasts needed a conditioned medium to grow, but we have enable to obtain better results by using different conditioned media.

**Electrofusion conditions:** We achieved protoplast fusion with PEG (Kao and Michayluk 1974) or dextran (Kameya *et al.* 1981). However, as we always obtained less than 1 % heterofusion, we investigated an electrofusion process. We chose a method requiring firstly the agglutination of protoplasts by a chemical agent before triggering fusions by short DC electric pulses.

In the first set of experiments, we tried to find an efficient agglutinating agent. Unlike Kameya *et al.* (1981), we found that a 15 % (m/v) solution of dextran solution was toxic for protoplasts since all of them were lysed one day after the DC impulse. Therefore, we used a PEG agglutinating solution (Chapel *et al.* 1986, slightly modified). At a final concentration of 10 %, PEG had no obvious effect on protoplast viability and induced less than 0.1 % fusions by itself. On the contrary, DC pulses associated with PEG greatly increased the heterokaryon number. Ten-fold dilution of 10 times the PEG-treated protoplasts with a hypotonic solution facilitated microscopic observations, since viable protoplasts and heterokaryons floated on the surface whereas debris was still attached to the bottom of the Petri dish. We also found that the results were not improved by using deionized PEG in place of commercial PEG (data not shown).

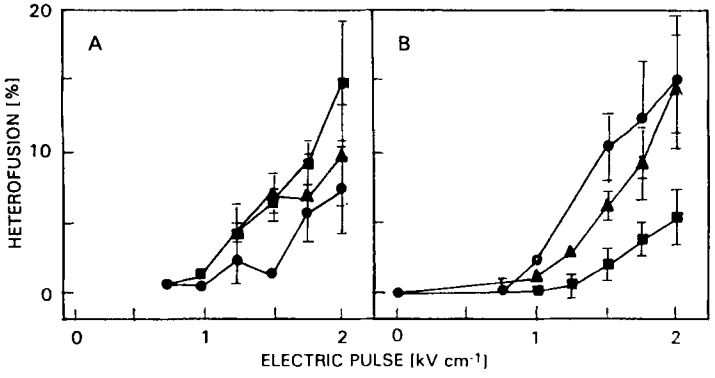


Fig. 1. Relation between heterofusion rate, electric pulse strength, and developmental stage of the cells. Under all conditions, one pulse with 100 ms duration was used. A - the mesophyll protoplasts were isolated either from the cotyledons (*circles*) or the first (F1 - *triangles*) or the third (F3 - *squares*) pair of leaves developed above the cotyledons on a two-month-old plantlet. Habituated protoplasts were isolated from a 4-d-old cell suspension culture. B - habituated protoplasts were isolated from the cell suspension culture on the 3rd (*circles*), 4th (*triangles*) or 5th (*squares*) day. Mesophyll protoplasts were isolated from F3. The heterofusion rate (estimated 24 h after electrofusion) is expressed in % of total protoplasts (mean ± standard error, n = 5).

In the second set of experiments, we varied the DC field strength. Levels of pulse voltage ranging from 0.75 kV $cm^{-1}$ to 2 kV $cm^{-1}$ were tested, while the duration and

number of pulses were maintained at preselected values (100 ms, 1 pulse). Fig. 1 shows that the heterofusion percentage increased with increasing voltage range. When 1.5 kV $cm^{-1}$ to 1.75 kV $cm^{-1}$ were applied, up to 10 % or more heterofusion could be obtained. By counting the protoplasts before and after DC pulses, we found that the bursting of protoplasts was kept low up to pulses of 1.75 kV $cm^{-1}$. On the other hand, protoplast bursting abruptly increased at 2 kV $cm^{-1}$ and therefore the heterofusion rate was over-estimated. So, we adopted pulses of 1.5 kV $cm^{-1}$ in routine experiments. Whatever experimental conditions, heterokaryons viability was about 95 %. In the same experiment, we investigated the effects of the physiological state of the protoplasts on heterofusion rate. We first investigated the effect of leaf position on the *C. roseus* plantlet by fusing C20A protoplasts (isolated from a 4-d-old culture) and mesophyll protoplasts isolated from different leaves (Fig. 1A). Heterofusion rates were higher when leaves were used as material, as compared to results obtained with cotyledons. Considering the problem of bursting at 2 kV $cm^{-1}$, Fig. 1 shows that similar results were obtained when using the first (F1) or the third (F3) leaves. Then, we fused mesophyll protoplasts isolated from F3 leaves and C20A protoplasts isolated at the 3, 4 or 5$^{th}$ day after subculture. Best results were obtained by using 3-d-old cells, whatever the field strength applied. Our results are similar to those reported by Kameya (1979) who found a higher percentage of heterofusion when cells from young fully expanded leaves and suspension cells in a logarithmic growth phase were used as fusion partners. This may be due to the favourable composition or organization of plasma membranes (Nea *et al.* 1987).

**Effects of calcium ions:** Calcium ions are important for plasmalemma stabilization (Von Arnold and Eriksson 1977), membrane adhesion (Kanchanapoom and Boss 1986), protoplast fusion and cell wall regeneration (Nea *et al.* 1987). We therefore

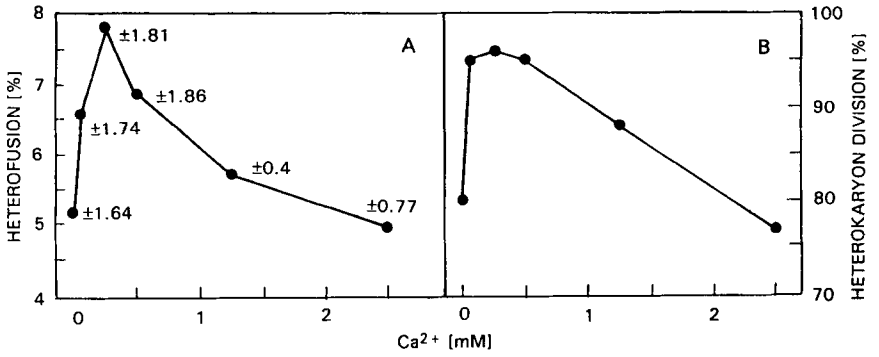


Fig. 2. Evolution of the heterokaryon rate and the percentage of heterokaryon division with $Ca^{2+}$ concentration. $CaCl_2$ was added before electrofusion. Conditions: 1 pulse of 1.5 kV $cm^{-1}$ with a duration of 100 ms. A - the heterofusion rate was estimated 24 h after electrofusion (mean ± standard error, n = 5). B - heterokaryon division was checked after 5 d of culture.

studied the influence of the addition of $CaCl_2$ on protoplast fusions. In these experiments we used 3-d-old cells and F3 leaves to prepare protoplasts, and

1.5 kV $cm^{-1}$ for electrofusion. Fig. 2 shows that heterokaryon production and cell division were increased by adding $CaCl_2$ concentrations ranging from 0.1 to 1.25 mM prior to fusion. No beneficial effect on heterofusion rate was noted when $CaCl_2$ was added just after the DC pulse.

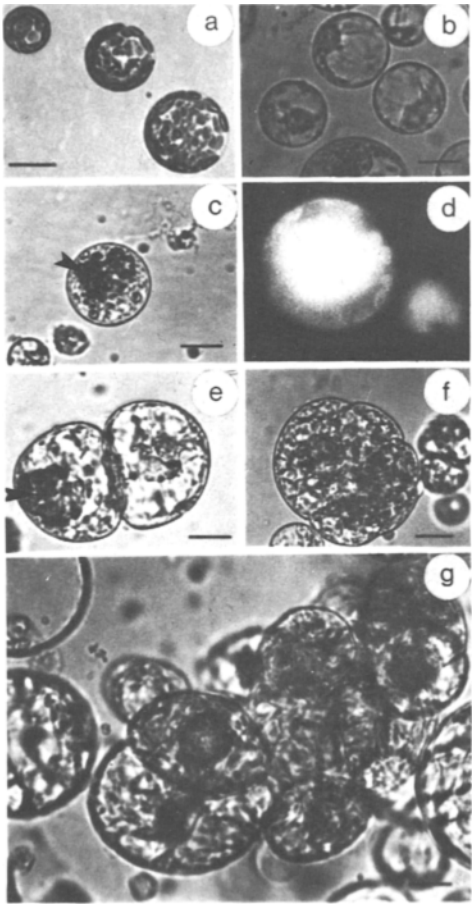


Fig. 3. Time course of heterokaryon culture.
A - freshly isolated mesophyll protoplasts of *C. roseus*,
B - freshly isolated protoplasts of habituated cell line C20A,
C - protoplasts and fusion products, 24 h after the electrofusion of mesophyll and habituated cell suspension protoplasts. (Arrows indicate chloroplast of the mesophyll protoplasts),
D - fluorescence microscopy (in UV light) showing dual (red/green) fluorescence of chlorophyll and FITC, 1 h after the electrofusion,
E - dividing heterokaryon at the onset of the fiirst division after 5 d of culture,
F - microcalli obtained after 10 d of culture,
G - cell colony derived from one heterokaryon after 15 d of culture.
Bars indicate 10 μm.

**Culture of heterokaryons:** After electropulsation, unfused and fused protoplasts were cultured together. Heterokaryons were easily identified either as large protoplasts containing chloroplasts or after FITC staining (Fig. 3C, D). Mesophyll protoplasts failed to divide, while both C20A protoplasts and heterokaryons divided (Fig.3E). Electroporation was previously shown to induce physiological and biochemical changes (Joersbo and Brunstedt 1991) and to enhance protoplast division (Ochatt *et al.* 1988). In fact, we observed that the division rate of electropulsed C20A protoplasts (76.5 ± 12 %) was enhanced significantly, as compared to the division rate of non-electropulsed protoplasts (56.5 ± 10 %). Moreover, 76 ± 4 % of the heterokaryons divided at least twice. Heterokaryon-derived microcalli (Fig. 3F, G),

were recognizable until the 15th day of culture. Thereafter, they became difficult to identify because of chloroplast involution.

The method used in the present study gives a high percentage of heterokaryons and is applicable to a large number of protoplasts, without any restriction in electrolyte addition before electrofusion. Heterokaryons could be cultured directly in Petri dishes, avoiding the protoplast transfer which is necessary in the AC/DC method. After optimization of electrofusion, we routinely obtained 10 % hetero-fusion, *i.e.* a higher rate than those reported with other electrofusion methods (Al-Atabee *et al.* 1990, Gilmour *et al.* 1989). This method resulted in viable heterokaryons which resynthesized cell walls and underwent divisions. We also found that the low concentration of PEG (1 %) in the final protoplast culture medium had a slight beneficial effect on the survival and development of protoplasts.

## References


Al-Atabee, J.S., Mulligan, B.J., Power, J.B.: Interspecific somatic hybrids of *Rudbeckia hirta* and *R. laciniata* (Compositae). - Plant Cell Rep. **8**: 517-520, 1990.

Chapel, M., Montané, M.H., Ranty, B., Teissié, J., Alibert, G.: Viable somatic hybrids are obtained by direct current electrofusion of chemically aggregated plant protoplasts. - FEBS Lett. **196**: 79-86, 1986.

Chapel, M., Teissié, J., Alibert, G.: Electrofusion of spermine-treated plant protoplasts. - FEBS Lett. **173**: 331-336, 1984.

Constabel, F., Koblitz, H., Kirkpatrick, J.W., Rambold, S.: Fusion of cell sap vacuoles subsequent to protoplast fusion. - Can. J. Bot. **58**: 1032- 1034, 1980.

Constabel, F., Rambold, S., Chatson, K.B., Kurz, W.G.M., Kutney, J.P.: Alkaloid production in *Catharanthus roseus* (L.) G. Don. IV. Variation in alkaloid spectra of cell lines derived from one single leaf. - Plant Cell Rep. **1**: 3-5, 1981.

Evans, D.A.: Agricultural applications of plant protoplast fusion. - Bio/Technology **1**: 253-261, 1983.

Galbraith, D.W , Alfonso, C.L., Harkins, K.R.: Flow sorting and culture of protoplasts: Conditions for high-frequency recovery, growth and morphogenesis from sorted protoplasts of suspension cultures of *Nicotiana*. - Plant Cell Rep. **3**: 151-155, 1984.

Gamborg, O.L., Miller, R.A., Ojima, K.: Nutrient requirements of suspension cultures of soybean root cells. - Exp. Cell Res. **50**: 151- 158, 1968.

Gilmour, D.M., Davey, M.R., Cocking, E.C.: Production of somatic hybrid tissues following chemical and electrical fusion of protoplasts from albino cell suspensions of *Medicago sativa* and *M. borealis*. - Plant Cell Rep. **8**: 29-32, 1989.

Heller, R.: Recherches sur la nutrition minérale des tissus végétaux cultivés *in vitro*. - Ann. Sci. nat., Bot. Biol. vég. **14**: 1-22, 1953.

Joersbo, M., Brunstedt, J.: Electroporation: Mechanism and transient expression, stable transformation and biological effects in plant protoplasts. - Physiol. Plant. **81**: 256-264, 1991.

Kanchanapoom, K., Boss, W.F.: Osmoregulation of fusogenic protoplast fusion. - Biochim. biophys. Acta **861**: 429-439, 1986.

Kameya, T.: Studies on plant cell fusion: effects of dextran and pronase E on fusion. - Cytologia **44**: 449-456, 1979.

Kameya, T.: Studies on plant cell fusion by dextran: effects of pH, inorganic salts and electrical stimulus. - Cytologia **48**: 873-878, 1983.

Kameya, T., Horn, M.E., Wildholm, J.M.: Hybrid shoot formation from fused *Daucus carota* and *D. capillifolius* protoplasts. - Z. Pflanzenphysiol. **104**: 459-466, 1981.

Kao, K.N., Michayluk, M.R.: A method for high-frequency intergeneric fusion of plant protoplasts. - Planta **115**: 355-367, 1974.

Kao, K.N., Saleem, M.: Improved fusion of mesophyll and cotyledon protoplasts with PEG and high pH $Ca^{++}$ solution. - J. Plant Physiol. **122**: 217-225, 1986.

Maeda, Y., Fujita, Y., Yamada,Y.: Callus formation from protoplasts of cultured *Lithospermum erythrorhizon* cells. - Plant Cell Rep. **2**: 179-182, 1983.

Mérillon, J.M., Ouelhazi, L., Doireau, P., Chénieux, J.C., Rideau, M.: Metabolic changes and alkaloid production in habituated and non-habituated cells of *Catharanthus roseus* grown in hormone-free medium. Comparing hormone-deprived non-habituated cells with habituated cells. - J. Plant Physiol. **134**: 54-60, 1989.

Mérillon, J.M., Liu, D., Huguet, F., Chénieux, J.C., Rideau, M.: Effects of calcium entry blockers and calmodulin inhibitors on cytokinin-enhanced alkaloid accumulation in *Catharanthus roseus* cell cultures. - Plant Physiol. Biochem. **29**: 289-296, 1991.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 215-218, 1962.

Nea, L.J, Bates, G.W., Gilmer, PJ.: Facilitation of electrofusion of plant protoplasts by membrane-active agents. - Biochim. biophys. Acta **897**: 293-301, 1987.

Ninnemann, H., Jüttner, F.: Volatile substances from tissues cultures of potato, tomato, and their somatic fusion products. Comparison of gas chromatographic patterns for identification of hybrids. - Z. Pflanzenphysiol. **103**: 95-107, 1981.

Ochatt, S.J., Rech, E.L., Davey, M.R., Power, J.B.: Long-term effect of electroporation on enhancement of growth and plant regeneration of colt cherry (*Prunus avium* × *Pseudocerasus*) protoplasts. - Plant Cell Rep. **7**: 393-395, 1988.

Power, J.B., Chapman, J.V.: Isolation, culture and genetic manipulation of plant protoplasts. - In: Dixon, R.A. (ed.): Plant Cell Culture. Pp. 37-66. IRL Press, Oxford - Washington 1985.

Tempelaar, M.J., Jones, M.G.K.: Fusion characteristics of plant protoplasts in electric felds. - Planta **165**: 205-216, 1985.

Von Arnold, S., Eriksson, T.: A revised medium for growth of pea mesophyll protoplasts. - Physiol. Plant. **39**: 257-260, 1977.

Watts, J.W., King, J.M.: A simple method for large-scale electrofusion and culture of plant protoplasts. - BioSci. Rep. **4**: 335-342, 1984.

Yamada, Y., Morikawa, H., Sato, F., Yamamoto, Y.: Secondary plant products from cultured hybrid cells.- Proc. jap. Acad. **63**: 208-210, 1987.

Yamada, Y., Hashimoto, T., Endo, T.: Biosynthesis of tropane alkaloids. - In: Kurz, W.G.W. (ed.): Primary and Secondary Metabolism of Plant Cell Cultures. Pp. 133-137. Springer-Verlag, Berlin - Heidelberg 1989.

Zimmermann, U., Scheurich, P.: High frequency fusion of plant protoplasts by electric fields. - Planta **151**: 26-32, 1981.

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