Virginia Journal of Science
Volume 52, Number 1
Spring 2001

Isolation and Fusion of Cotton Protoplasts

Michael H. Renfroe [1], Ronald C. Hartwig[2], and Roberta H. Smith[2], [1]Department of Biology
James Madison University, Harrisonburg, VA 22807 [2]
Department of Soil and Crop Sciences, Texas A&M University
College Station, TX 77843

ABSTRACT

Protoplasts were isolated from five species of *Gossypium*. Protoplast yield and viability were affected by incubation conditions, osmolarity, purification procedures, and cell source. Using an optimized procedure, highly viable protoplasts were isolated from cell suspensions, callus cultures, and leaf tissue of *G. hirsutum*, *G. arboreum*, *G. klotzschianum*, *G. harknessii*, and *G. herbaceum*. Protoplasts of *G. harknessii* were enucleated and successfully fused with protoplasts of *G. hirsutum*.

INTRODUCTION

Modification of plants through tissue culture methods, such as somatic cell fusion has the potential of providing immediate benefits to agriculture (Evans and Sharp, 1986), because genetic characteristics can be transferred by somatic cell fusion without the necessity of isolation and identification of the genes responsible for the trait. Many aspects of plant improvement through somatic cell hybridization have been reviewed previously (Evans and Flick, 1983; Schieder, 1982).

An important plant breeding tool is male sterility. Through sexual crosses, Meyer (1975) demonstrated that *Gossypium harknessii* Brandegee cytoplasm in the nuclear background of *G. hirsutum* L. resulted in plants with CMS. Production of these cotton plants required extensive backcrossing, and the seed set was limited.

Although the genetic basis for cytoplasmic male sterility (CMS) is not understood (Boeshore et al., 1985; Jigeng and Yi-mong, 1983; Levings and Pring, 1979), this trait has been transferred to a male fertile plant through protoplast fusion (Zelcer et al., 1978) and subsequent hybrid regeneration. The fusion of *G. harknessii* cytoplasts with *G. hirsutum* protoplasts should also produce *G. hirsutum* plants exhibiting CMS. As a first step in this process, a technique to rapidly obtain good yields of highly-viable protoplasts suitable for protoplast fusion was developed. Protoplast yields and viability exceeded other published accounts (Bhojwani et al., 1977; El-Shihy and Evans, 1983; Finer and Smith, 1982; Firoozabady and DeBoer, 1986; Khasanov and Butenko, 1979). Additionally, cytoplasts were prepared and fused with these protoplasts.

MATERIALS AND METHODS

Plant Material

Cotton plants *G. hirsutum* L. var. Coker 310, Stoneville 213, and Paymaster 145, were grown in potting soil in an environmentally-controlled growth chamber which

1 Address all correspondence to:Dr. Michael H. Renfroe, Department of Biology - MSC 7801, James Madison University, Harrisonburg, VA 22807

was programmed for 12 h of light with a temperature of 30 C and 12 h of darkness at 20 C. Plants were watered as necessary and fertilized with Osmocote slow-release fertilizer. Young, rapidly-expanding leaves were selected as source leaves for protoplast isolation.

Callus cultures of *G. hirsutum* L., *G. harknessii* Brandg., *G. arboreum* L., *G. herbaceum* L., and *G. klotzschianum* Anderss. were maintained on a medium consisting of: macronutrients and micronutrients of Murashige and Skoog (1962) (MS salts); 2 mg/L NAA; 1 mg/L 2-iP; 30 g/L glucose; a vitamin mixture consisting of 1 mg/L thiamine-HCl; 0.5 mg/L pyridoxine-HCl; 0.5 mg/L nicotinic acid; and 100 mg/L *myo*-inositol (Smith et al., 1977). Media were sterilized by autoclaving for 15 min at 121° C. Prior to autoclaving and addition of agar, the pH was adjusted to 5.7-5.8 and medium was solidified with 0.8% Difco Bacto agar. Cultures were subcultured at monthly intervals.

Suspension cultures of *G. hirsutum* and *G. harknessii* were maintained in a medium described above with the following modifications: NAA reduced to 1 mg/L; BAP, 0.1 mg/L substituted for 2-iP; agar was omitted. Suspension cultures were maintained at a 16:8 h photoperiod (60 rpm). Cultures were supplied with fresh medium weekly and serially subcultured every 3 weeks.

Protoplast isolation

The general isolation procedure was developed using cotton cell suspension cultures of *G. hirsutum*. Basal isolation medium consisted of macronutrients of MS salt (Murashige and Skoog, 1962), 5 mM MES (2-(N-morpholino)ethanesulfonic acid), 0.7 M mannitol, 5% (w/v) Cellulysin cellulose and 1% (w/v) Macerase pectinase at a pH of 5.7. Effect of osmotic potential on protoplast isolation and viability was determined by reduction of mannitol to 0.5 M and 0.3 M. Effect of macronutrient composition on protoplast isolation and viability was determined by testing full and half strength MS macronutrients and full strength macronutrients from Gamborg's (1975) medium. Effects of enzyme concentration and length of incubation were tested by reduction of the enzyme concentration to 1/2 and by increasing the incubation period from 5 h to 24 h. Isolated protoplasts were purified either by filtration through a nylon mesh with 100 mm pores, by centrifugation at 125 RCF for 6 min layered over a 20% (w/v) sucrose solution, or by a combination of filtration and centrifugation treatments.

Prior to incubation in the isolation medium, cells from the suspension culture were plasmolyzed in a solution identical to the respective isolation medium without enzymes for approximately 30 min. Plasmolyzed tissue was incubated in protoplast isolation medium for 5 h at 28° C in a water bath reciprocal shaker at 40 CPM. Protoplasts used for enucleation experiments were also isolated using this procedure.

Cell counts were made using a haemocytometer. Cell viability was measured using the Evans' blue dye exclusion technique (Onyia et al., 1984). Protoplasts of the other species were obtained from callus cultures and young plant leaves using the procedure developed for cell suspension cultures.

Cytoplast Preparation and Protoplast Fusion

G. harknessii protoplasts were enucleated by centrifugation at 31,000 RCF for 1 h on an iso-osmotic step gradient (Lorz and Potrykus, 1980). Enucleation of protoplasts to form cytoplasts was verified using epi-fluorescence microscopy (Zeiss) following

TABLE 1. Yield and viability of protoplasts isolated in media of various osmotic strength after 5 hours incubation in protoplast isolation medium.

Mannitol (M)	Water Potential of Medium (MPa)	Yield (No/ml PCV[a])	Viability (%)
0.3	-0.97	3.2×10^5	96.7
0.5	-1.48	2.7×10^5	95.7
0.7	-2.07	1.9×10^5	91.9

[a] PCV =packed cell volume

incubation of protoplasts and cytoplasts for at least 1 h in DAPI (4,6-diamidino-2-phenylindole) at 1 mg/mL.

Protoplasts of *G. hirsutum* were mixed with an excess of *G. harknessii* cytoplasts (approximately 2:1 cytoplasts:protoplasts). A red pigmented cell line of *G. hirsutum* was used in some experiments to provide a visual marker for interspecific fusion. Protoplasts and cytoplasts were fused using the procedure by Evans (1983), modified by substitution of 0.5 M glucose for sorbitol in the enzyme wash solution. Fusion was promoted by a 50% (w/v) PEG (mw 6000) solution (Evans, 1983). The PEG fusing solution was eluted with either a glycine buffer (50 mM glycine, 50 mM $CaCl_2 \cdot 2H_20$, 0.3 M glucose, pH 10.5) followed by a wash with culture media, or by a Tris buffer (5 mM Tris , 50 mM $CaCl_2 \cdot 2H_20$, 0.3 M glucose, pH 7.0) followed by a wash with culture media, or eluted by washing with culture media alone (pH 5.7-5.8).

Protoplasts and fusion products were cultured in various media based on Murashige and Skoog's (1962), Gamborg's (1975) or Kao and Michayluk's (1975) formulations. Liquid culture, agar or agarose-solidified media and nurse cultures were all used in an attempt to promote growth of protoplasts and fusion products.

RESULTS

Protoplasts of high viability were obtained from all the *Gossypium* species examined and from leaf, as well as callus and suspension cultures, using this procedure. The highest yield and viability were obtained using the isolation medium with 0.3 M mannitol (Table 1). As the medium osmotic strength was increased, protoplast yield and viability decreased. Isolation medium containing 0.3 M mannitol was therefore selected for subsequent trials.

Isolation medium macronutrient content had no effect on yield (Table 2). Similar results were obtained when macro-salts of MS were at full- or half-strength. Yield and viability were only slightly decreased by substitution of Gamborg's macronutrients. MS macronutrients at full-strength were chosen for routine use.

Reduction of enzyme concentration by half had no effect on viability over a 5 h period but did decrease protoplast yield (Table 3). An increase in incubation period from 5 h to 24 h resulted in decreased total yield and decreased viability. Loss of viability was slightly greater at the lower concentration of enzymes (Table 3). A 5 h incubation period using 5% Cellulysin and 1% Macerase was selected as the standard procedure.

Several purification procedures were compared for their effect on protoplast yield and viability (Table 4). Filtration of the protoplast suspension through a nylon mesh

TABLE 2. Effect on protoplast yield of various macronutrient formulations in the protoplast isolation medium. Protoplasts were isolated from suspension cultures of *G. hirsutum*.

Macronutrient Formulation	Strength	Yield (No/ml PCV)	Viability (%)
Murashige & Skoog	1X	4.4×10^5	96.2
Murashige & Skoog	0.5X	4.5×10^5	94.0
Gamborg	1X	4.3×10^5	91.7

TABLE 3. Effect of enzyme concentration and incubation period on yield and viability of protoplasts.

Cellulysin (% w/v)	Macerace (% w/v)	Incubation (hrs)	Yield (No/ml PCV)	Viability (%)
5.0	1.0	5	3.1×10^5	100
2.5	0.5	5	5.2×10^4	100
5.0	1.0	24	1.0×10^5	93.2
2.5	0.5	24	1.4×10^4	89.8

TABLE 4. Effect of protoplast yield and viability of several purification procedures by filtration, centrifugation, or their combination.

Purification Method	Recovery % of Protoplasts (%)	Number of Protoplasts/ mL PCV Yield	Initial Viability (%)	Viability After Purification (%)
Filtration, 100 mM Mesh	100	4.0×10^6	92.3	82.1
Floatation over Sucrose	68	1.7×10^6	88.4	77.8
Filtration and Floatation	26	9.0×10^5	86.9	85.7

with 100 mm pores, allowed protoplasts, cell fragments and cells with partially-digested walls to pass through resulting in an impure population of protoplasts. In contrast, purification by centrifugation was superior. Cell clumps and cell fragments sedimented into the sucrose layer while intact protoplasts floated above the sucrose in the suspension medium. Both purification by filtration and by centrifugation led to about a 10% decrease in viability. Purification by centrifugation gave approximately two-thirds the yield of filtration. When filtration and centrifugation procedures were combined, recovery was reduced more than would be expected by a simple additive effect of the two procedures.

The optimized procedure was used to isolate protoplasts from five different species of *Gossypium* (Table 5). Yields ranged from 10^5-10^7 protoplasts per g dw of tissue.

TABLE 5. Yield and viability of protoplasts isolated from various cotton species and tissue sources.

Species	Material	Yield (No/gdw)	Viability (%)
G. hirsutum	leaf	1.1×10^7	93.1
	callus	6.6×10^6	67.9
	suspension	1.4×10^8	89.5
G. harknessii	callus	4.8×10^7	85.1
	suspension	1.1×10^8	81.8
G. arboreum	callus	3.3×10^5	76.0
G. herbaceum	callus	8.6×10^7	85.3
G. klotzschianum	callus	5.4×10^7	84.8

Yields were higher for suspension cultured cells of *G. hirsutum* and *G. harknessii* than from callus of the respective species. Leaves of *G. hirsutum* yielded more protoplasts than did callus, but less protoplasts than did suspension cultures. All isolated protoplasts had good viability (Table 5). Protoplast yield from callus varied with the friability of the callus tissue. Callus of *G. arboreum* and *G. hirsutum* was very nodular and compact which resulted in lowered protoplast yields. However, callus of *G. herbaceum*, *G. klotzschianum* and *G. harknessii* was friable and soft and yields were accordingly greater.

Protoplast to cytoplast fusion was initiated using a 50% (w/v) PEG fusion solution. The PEG solution caused protoplast adhesion to occur, but there was virtually no fusion until the PEG solution was eluted with high pH glycine buffer. Following elution, 50% of all visible objects were still single protoplasts that had not fused. Additionally, 38% of the viewed objects were multicellular fusion products. Only 20% of the objects were formed by adhesion of only two protoplasts. An additional 2% were bicellular fusion products in which the fusion event had clearly occurred at the time of evaluation.

However, when PEG solution was eluted with Tris buffer (pH 7.0) or with culture media (pH 5.8) there was virtually no fusion of adhering protoplasts. Clearly the higher pH of the glycine buffer was effective in stimulating fusion of protoplasts and cytoplasts. Furthermore, by increasing the ratio of cytoplasts to protoplasts from 1:1 to 2:1, the excess of cytoplasts increased the number of protoplast-cytoplast fusion products (4-24%).

DISCUSSION

Genetic modification of cotton through protoplast methods requires a procedure for isolating adequate numbers of viable protoplasts. We systematically evaluated isolation parameters and developed a system with widespread applicability to cotton species for the production of protoplasts suitable for fusion experiments.

In past studies of cotton protoplast isolation (El-Shihy and Evans, 1983; Finer and Smith, 1982; Firoozabady and DeBoer, 1986), enzyme concentration and incubation periods were evaluated, but concentrations were varied for a single, fixed time interval, or incubation period was varied, for one concentration of the enzymes. Results of these earlier studies indicate that low enzyme concentration for prolonged periods give best protoplast yield and viability (Potrykus and Shillito, 1986). Our results indicated that a higher concentration of enzyme used for a shorter period of time produced a greater yield of protoplasts with better viability. No benefits were derived from prolonging incubation periods, instead, viability and yield decreased.

Protoplast purification by filtration is the most commonly used technique (Evans, 1983). In our experiments, cotton protoplasts were isolated in greater numbers with greater viability with the flotation method. However, filtration did not remove ruptured cells, cell fragments, or cells with incompletely digested cell walls. The protoplast population that was recovered from flotation was extremely pure.

Changes in macronutrient composition in the isolation medium had almost no effect on yield, but had a slight effect on viability. This may have reflected differences in osmolality of the solutions. During purification, protoplasts should be maintained in solutions of similar osmotic pressure (Potrykus and Shillito, 1986). Therefore, it may be best to select macronutrients for isolation media with the final culture medium in mind. Macronutrient composition is probably of little overall consequence if the incubation and purification time is sufficiently short.

The largest component of osmotic pressure in our solution was mannitol. Mannitol is a commonly employed osmoticum and has been used in cotton protoplast isolation in the range of 0.4 M (El-Shihy and Evans, 1983; Firoozabady and DeBoer, 1986) to 0.7 M (Finer and Smith, 1982). Khasanov and Butenko (1979) tested mannitol concentrations over the range of 0.3-0.9 M and concluded that 0.5 M was optimum for yield. However, they did not evaluate protoplast viability. We observed a reduction in viability after only 5 h. It is likely that these differences would be more pronounced after an extended incubation period when the cells would have been exposed to the water stress of the high concentration mannitol solution for a longer period of time. The effects of water stress should be more widely considered, especially in procedures with extended incubation periods.

Our method of protoplast isolation and purification has wide applicability with cotton tissue sources and species. We isolated highly-viable protoplasts from five species of cotton and from leaf tissues as well as callus and suspension cultures. Suspension cultures and young, rapidly expending leaves from mature plants are good sources for the isolation of plant protoplasts. Khasanov and Butenko (1979) were unable to isolate protoplasts from cotton leaves, but could isolate protoplasts from cotyledons. Others have isolated protoplasts from cotton cotyledons (El-Shihy and Evans, 1983; Firoozabady and DeBoer, 1986), young leaves (Firoozabady and DeBoer, 1986) and callus cultures (Bhojwani, et al., 1977; Finer and Smith, 1982). In addition to evaluating leaves and callus, we extended the trials to include suspension cultures and found that cell suspensions invariably produced the highest yields of protoplasts.

Our overall procedure results in a high yield of protoplasts with good viability. Furthermore, the procedure is relatively quick compared to other published procedures and is advantageous for use in fusion experiments. Chemical fusion procedures are

harsh. Successful fusion and subsequent hybrid cell growth will be favored if the protoplasts are initially viable.

We were able to demonstrate fusion not only of protoplasts, but also of protoplasts with cytoplasts using a standard fusion procedure (Evans 1983). Protoplast-cytoplast fusion products were obtained in 4-24% of all fusion events. Although numerous methods have been used for indirect selection of fusion products such as complementation (Carlson et al., 1972; Glimelius et al., 1978; Melchers and Labib, 1974) or inactivation (Medgyesy et al., 1980; Zelcer et al., 1978). We used a pigmented cell line to allow immediate visual scoring of fusion events.

Cytoplasmically-determined traits have been transferred when organelles were left in their native milieu inside an enucleated protoplast (Maliga et al., 1982) or in a nuclear-inactivated protoplast (Zelcer et al., 1978). To demonstrate the potential for such a system in cotton, we enucleated protoplasts using a published procedure (Lorz and Potrykus, 1980) to form cytoplasts.

Protoplast fusion was not promoted by PEG alone, as reported by Kao and Michayluk (1974), but required a high pH treatment as described by Keller and Melchers (1973). Elution of the PEG with a neutral buffer or with slightly acid culture medium did not promote fusion. For cotton protoplasts, it seems that a high pH elution step is essential for good fusion.

Regeneration of cotton plants from protoplasts has seemed intractable in the past (Bhojwani et al., 1977; El-Shihy and Evans, 1983; Finer and Smith, 1982; Firoozabady and DeBoer, 1986; Khasanov and Butenko, 1979) with protoplast cultures not growing well despite numerous approaches. However, for one cotton cultivar, plants have been regenerated from callus that developed from protoplasts (Peeters et al., 1994). We have taken the next step in demonstrating the potential for development of new cotton lines through protoplast-cytoplast fusions. Genetic modification by protoplast-protoplast or protoplast-cytoplast fusion may lead to agronomically-useful cotton hybrids.

ACKNOWLEDGEMENTS

RHS initiated and supervised the project and provided the cotton cell lines. MHR and RCH isolated protoplasts, MHR prepared cytoplasts and performed the fusions. This work was supported by the Texas Agricultural Experiment Station, College Station, TX.

LITERATURE CITED

Bhojwani, S.S., J.B. Power, and E.C. Cocking. 1977. Isolation, culture and division of cotton callus protoplasts. Plant Sci. Lett. 8:85-89.

Boeshore, M.L., M.R. Hanson, and S. Izhar. 1985. A variant mitochondrial DNA arrangement specific to Petunia stable sterile somatic hybrids. Plant Molec. Biol. 4:125-132.

Carlson, P.S., H.H. Smith,and R.D. Dearing. 1972. Parasexual interspecific plant hybridization. Proc. Nat. Acad. Sci. (USA) 69:2292-2294.

El-Shihy, 0.M., and P.K. Evans. 1983. Isolation and culture of cotyledon protoplasts of cotton (*Gossypium barbadense* cv. Giza 70). In: Potrykus, I., C.T. Harms, A. Hinnen, R. Hutter, P.J. King, and R.D. Shillito, eds. Protoplasts 1983. Lecture Proceedings. Sixth Int. Protoplast Symp., Basel, Aug. 12-16, 1983.

Evans, D. A. 1983. Protoplast fusion. In: Evans, D.A., W.R. Sharp, P.V. Ammirato, and Y. Yamada, eds. Handbook of plant cell culture. Vol. 1. Techniques for propagation and breeding. MacMillan Pub. New York. pp. 291-321.

Evans, D.A. 1983. Protoplast isolation and culture. In: Evans, D.A., W.R. Sharp, P.V. Ammirato, and Y. Yamada, eds. Handbook of plant cell culture. Vol. 1. Techniques for propagation and breeding. MacMillan Pub. New York. pp. 124-176.

Evans, D.A., and C.E. Flick. 1983. Protoplast fusion: Agricultural applications of somatic hybrid plants. In: Kosuge, T., C.P. Meredith, and A. Hollaender, eds. Genetic engineering of plants - an agricultural perspective. Plenum Press. New York. pp. 271-288.

Evans, D. A., and W.R. Sharp. 1986. Applications of somaclonal variation. Bio/Technology 4:528-532.

Finer, J. J., R.H. Smith. 1982. Isolation and culture of protoplasts from cotton (*Gossypium klotzschianum* Anderss.) callus cultures. Plant Sci. Lett. 26:147-151.

Firoozabady, E., and D.L. DeBoer. 1986. Isolation, culture, and cell division in cotyledon protoplasts of cotton (*Gossypium hirsutum* and *G. barbadense*). Plant Cell Rep. 5:127-131.

Gamborg, 0. L. 1975. Callus and cell culture. In: Gamborg, 0. L., and L.R. Wetter, eds. Plant tissue culture methods. National Research Council of Canada. pp. 1-10.

Glimelius, K., T. Eriksson, R. Grafe, A.J. Muller. 1978. Somatic hybridization of nitrate-deficient mutants of *Nicotiana tabacum* by protoplast fusion. Physiol. Plant. 44:273-277.

Jigeng, L., and L. Yi-nong. 1983. Chloroplast DNA and cytoplasmic male sterility. Theor. Appl. Genet. 62:231-238.

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

Kao, K.N., and M.R. Michayluk. 1975. Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. Planta 126:105-110.

Keller, W. A., and G. Melchers. 1973. The effect of high pH and calcium on tobacco leaf protoplast fusion. Z. Naturforsch. 28:737-741.

Khasanov, M. M., and R.G. Butenko. 1979. Cultivation of isolated protoplasts from cotyledons of cotton (*Gossypium hirsutum*). Sov. Plant Physiol. 26:77-81.

Levings, C.S. III, and D.R. Pring. 1979. Molecular bases of cytoplasmic male sterility in maize. In: Scandalios, J.G., ed. Physiological genetics. Academic Press. New York. pp. 171-193.

Lorz, H., and I. Potrykus. 1980. Isolation of subprotoplasts for genetic manipulation students. In: Ferenczy, L., and G.L. Farkas, eds. Advances in protoplast research. Pergamon Press. New York. pp. 377-382.

Maliga, P., H. Lorz, G. Lazar, and F. Nagy. 1982. Cytoplasmic-protoplast fusion for interspecific chloroplast transfer in *Nicotiana*. Molec. Gen. Genet. 185:211-215.

Medgyesy, P., L. Menczel, and P. Maliga. 1980. The use of cytoplasmic streptomycin resistance: chloroplast transfer from *Nicotiana tabacum* into *Nicotiana sylvestris*, and isolation of their somatic hybrids. Mol. Gen. Genet. 179:693-698.

Melchers, G., and G. Labib. 1974. Somatic hybridization of plants by fusion of protoplasts. I. Selection of light resistant hybrids of "haploid" light sensitive varieties of tobacco. Mol. Gen. Genet. 135:277-294.

Meyer, V. G. 1975. Male sterility from *Gossypium harknessii*. J. Hered. 66:23-27.

Murashige, T., and F. Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Onyia, G.O.C., P.B. Gahan, and H. Norman. 1984. The use of new probes for protoplast integrity following isolation and purification of protoplasts from tubers of white yam (*Dioscorea rotunda*, Poir). Plant Sci. Lett. 33:231-238.

Peeters, M.C., K. Willems, and R. Swennen. 1994. Protoplast-to-plant regeneration in cotton (*Gossypium hirsutum* L. cv. Coker 312) using feeder layers. Plant Cell Rep. 13:208-211.

Potrykus, I., and R.D. Shillito. 1986. Protoplasts: isolation, culture, plant regeneration. Met. Enzymol. 118:549-578.

Schieder, D. 1982. Somatic hybridization: a new method for plant improvement. In: Vasil, I.K. W.R. Scowcroft, and K.J. Frey, eds. Plant improvement and somatic cell genetics. Academic Press. New York. pp. 239-253.

Smith, R. H., H. J. Price, and J. B. Thaxton. 1977. Defined conditions for the initiation and growth of cotton callus in vitro. I. *Gossypium arboreum*. In Vitro 13:329-334.

Zelcer, A., D. Aviv, and E. Galun, 1978. Interspecific transfer of cytoplasmic male sterility by fusion between protoplasts of normal *Nicotiana sylvestris* and X-ray irradiated protoplasts of male-sterile *N. tabacum*. Z. Pflanzenphysiol. 90:397-407.