

## The Effect of High pH and Calcium on Tobacco Leaf Protoplast Fusion

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High frequencies of fusion were induced between isolated tobacco leaf protoplasts when they were incubated at 37 °C in the presence of 0.05 M  $\text{CaCl}_2$  in 0.4 M mannitol at pH 10.5. Subsequent to the fusion treatment the protoplasts were washed and cultured in a suitable medium and within two weeks, actively dividing cell colonies were observed. The fusion treatment had no irreversible, deleterious effects on the protoplasts.

The induced fusion of isolated plant protoplasts with the aim of producing interspecific somatic hybrid plants has drawn the attention of many plant scientists<sup>1–5</sup>. Induced protoplast fusion refers to the achievement of fusion between protoplasts that are free in suspension and it differs from spontaneous fusion which may occur as a result of plasmodesmatal expansion during isolation and which cannot be utilized for somatic hybridization<sup>6,7</sup>. Carlson *et al.*<sup>8</sup> have recently reported the formation of a vegetative, somatic or, as they call it, parasexual hybrid (“burdo”, Winkler<sup>9,10</sup>) resulting from the induced fusion of protoplasts from two *Nicotiana* species.

Several methods have been reported to induce protoplast fusion. These include manipulation with a perfusion micropipette<sup>11</sup>, treatment with  $\text{NaNO}_3$ <sup>12,14,8</sup>, artificial seawater<sup>15,16</sup> lysozyme<sup>17</sup> and deplasmolyzing osmotic shock<sup>7</sup>. With existing methods it has been difficult to induce high frequencies of fusion and retain the viability of vacuolate protoplasts such as leaf protoplasts<sup>18,13,17,8</sup>. In the present paper we wish to report evidence for the stimulation of high frequencies of protoplast fusion by calcium ions at high pH conditions without irreparable damage to the protoplasts.

### Material and Methods

#### Source of protoplasts

The upper expanding leaves of three *Nicotiana tabacum* varieties were utilized for protoplast iso-

lation. The varieties were *N. tabacum* var. “Samsun” with 48 chromosomes growing in greenhouse conditions and two “haploid” chlorophyll deficient, light sensitive varieties of *N. tabacum* designated as  $sl_1 sl_2$  (from now on called “s”, sublethal) and  $vi-A_1$  (called “v”, virescent). The “haploid” forms with 24 chromosomes of the light sensitive varieties were produced by anther culture techniques<sup>19,20</sup> and were grown in climate chambers at 700–800 lx (continuous) at 28 °C and 75% relative humidity. Under these conditions the plants show light green leaves. They are multiplied by cuttings. The  $F_1$  hybrids produced by crossing  $v \times s$  with 48 chromosomes complement for a normal dark green chlorophyll character and they grow vigorously in high light intensities present in the greenhouse or produced artificially.

#### Protoplast isolation

The leaves were surface sterilized by a two-min immersion in a 0.3% commercial detergent solution followed by a fourmin immersion in 10 vol/% of a commercial disinfection solution,  $\text{NaClO}$ , containing 150 g/l active Cl. After a triple washing in distilled water the midribs were removed and the leaves were cut into narrow (2 mm or less) strips with a razor blade. The leaf strips were placed in 100 ml Erlenmeyer flasks containing 0.7 M mannitol and were then shaken at 120 strokes/min (5 cm stroke) at 25 °C for 30 min. The mannitol solution was replaced by a 0.7 M mannitol solution containing 0.5% (w/v) Macerozyme 4S (All Japan Biochemicals Co., Nishinomiya, Japan) and 0.5% (w/v) potassium dextran sulfate (Meioto Sangyo Co., Meitosangyo, Nagoya, Japan) adjusted to pH 5.7. The Macerozyme solution was drawn into the intercellular spaces by a vacuum infiltration period of one min.

The leaf pieces were then agitated at 120 strokes/min at 25 °C for 15 min. The supernatant of the first maceration treatment was discarded but the

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cells harvested from four successive 20-min macerations were collected for cellulase treatment. The cells were pelleted by centrifugation at  $50 \times g$  for 3 min and then suspended in a cellulase solution consisting of 2% (w/v) Onozuka cellulase 4S (All Japan Biochemicals Co.) and 0.1% (w/v) potassium dextran sulfate in 0.7 M mannitol at pH 5.5. The cells remained in the cellulase for 2.5–3 hours (without shaking 2 hours =  $24^\circ\text{C}$ ,  $1/2$  hour =  $37^\circ\text{C}$ , if necessary) during which they were converted to protoplasts.

The protoplasts suspension was filtered through a  $100\mu$  pore mesh, the protoplasts were pelleted (3 min at  $50 \times g$ ) and then resuspended in 0.8 M mannitol.

#### Protoplast fusion

The protoplasts in 0.8 M mannitol were pelleted and then resuspended in the fusion inducing solution which was buffered at pH 10.5 (0.05 M glycine-NaOH) and which contained 0.05 M  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  and 0.4 M mannitol. The protoplasts were again pelleted (max  $g = 50$ , 3 min) and the centrifuge tubes were placed into a  $37^\circ\text{C}$  water bath. Samples were removed at various time intervals and light microscopic observations were made to determine the degree of aggregation and the rate of fusion.

#### Protoplast culture

When the number of protoplasts undergoing fusion was considered to be maximal the tubes were removed from the water bath and the fusion-inducing solution was replaced by post fusion wash (0.6 M mannitol, 0.05 M  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ). After standing 30 min the protoplasts were washed twice with the culture medium of Nagata and Takebe<sup>21</sup> and were then plated in agar as described by Takebe *et al.*<sup>22</sup> or cultured as  $50\mu\text{l}$  droplets as described by Kao *et al.*<sup>23</sup> in plastic petri dishes sealed with parafilm. The dishes were maintained in the dark or in dim light (300 lx) for a period of 24–48 hours and were then moved to continuous light (3000 lx) at  $28^\circ\text{C}$  and 70% relative humidity<sup>24</sup>.

## Results

#### Protoplast fusion

The protoplasts isolated by the sequential method described above were generally quite uniform and underwent very low frequencies (1% or less) of spontaneous fusion (Fig. 1 \*). The initial isolation

of single cells by Macerozyme treatment caused the breakage of interconnecting plasmodesmatal strands which would have allowed spontaneous fusion to occur.

Observations subsequent to the fusion treatment revealed that protoplasts began to aggregate after 10–15 min of incubation. The degree of aggregation, as judged by the number of aggregates as well as the number of protoplasts within aggregates increased with time and after 25–30 min, fusion of protoplasts within the aggregates was observed. The fusion events involved from two or a few protoplasts (Fig. 2) up to large numbers undergoing multiple fusions (Fig. 3 \*). After 60 min of incubation many of the protoplasts, and especially the larger fusion bodies, became unstable and burst. In subsequent studies the fusion solution was removed after 40–45 min in order that the protoplasts could be cultured. The frequency of fusion was difficult to determine because of the multiple fusions and tightly aggregated clusters but in most instances 20–50% of the protoplasts were involved in a fusion event. A good preparation of viable protoplasts free of undigested or broken cells was necessary for fusion. Generally, large amounts of debris indicated a poor protoplast preparation in which the plasma membranes were apparently in a weakened condition unsuitable to undergo fusion. Washing the protoplasts with 0.8 M mannitol helped to remove debris and made conditions more conducive for fusion.

#### Factors affecting protoplast fusion

The calcium concentration was observed to be an important factor in fusion (Table I). If the calcium level was less than 0.03 M there was very little aggregation and fusion. At a 0.1 M  $\text{CaCl}_2$  concentration a very high degree of aggregation was observed but the fusion frequency was not greater than, and usually lower than at 0.05 M  $\text{CaCl}_2$ . If the osmotic stabilizer was replaced with 0.2 M  $\text{CaCl}_2$  a very extensive aggregation of protoplasts into large, easily visible clusters occurred but the fusion frequency remained quite low. If calcium was lacking from the system the protoplasts became loosely associated into long thread-like forms but the fusion frequency remained very low and it was not increased by subsequent addition of calcium.

\* Figs 1 and 2 see Table on page 740 a.

\* Fig. 3 see Table on page 740 b.

Table I. The effects of calcium concentration on aggregation and fusion of tobacco leaf protoplasts. The total osmolarity of the solutions (including the buffer and mannitol) was approximately 0.60 M. The pH level was 10.5. A represents aggregation and F represents fusion. Degree of aggregation or fusion is represented by +, ++, +++<sup>a</sup>, whereas absence of effect is represented by —.

Incubation time at 37°C [min]	Calcium (CaCl <sub>2</sub> ) concentration							
	0	0.01 M	0.02 M	0.03 M	0.04 M	0.05 M	0.1 M	0.2 M
0	A — F —	A — F —	A — F —	A — F —	A — F —	A — F —	A + F —	A + F —
15	A — F —	A — F —	A — F —	A + F —	A + F +	A + F +	A +++ F +	A +++ F —
30	A — F —	A + F —	A + F —	A + F —	A +++ F +	A +++ F +++	A +++ F +++	A +++ F —
45	A — F —	A + F —	A + F +	A + F +	A +++ F +++	A +++ F ++++	A +++ F +++	A +++ F —
60	A — F —	A + F —	A + F —	A + F +	A +++ F +++	A +++ F ++++	A +++ F +++	A +++ F —

<sup>a</sup> + Involving 5–10% of the protoplasts; ++ involving 10–25% of the protoplasts; +++ involving more than 25% of the protoplasts.

Chloride ions did not appear to be inhibitory to the fusion process since identical observations were made in the presence of Ca(NO<sub>3</sub>)<sub>2</sub>. Magnesium could not be substituted for calcium since it failed to induce either protoplast aggregation or fusion.

The pH of the fusion medium was not extremely critical (Table II). In the pH range of 8.5–9.0 there was a lower degree of aggregation and fusion than in the optimal range of pH 9.5–10.5. Levels of pH higher than 11.0 tended to cause protoplast damage after 30 min, although protoplasts from haploid v that had been treated at pH 11.0 for 30 min survived the treatment, divided in culture and produced colonies which later differentiated into shoots in the medium B3s of Sacristán and Melchers<sup>25</sup>. The type and concentration of the osmotic stabilizer also played a significant role in

the fusion system. If the mannitol concentration was raised from 0.4 M to 0.7 M both aggregation and fusion were retarded, but if the mannitol concentration was lowered to 0.1 M, aggregation was stimulated but the protoplasts were unstable and frequently burst. Mild hypotonic conditions were considered to be optimal for fusion. Substitution of sucrose for mannitol had no significant effect on fusion. Substitution of urea for mannitol stimulated the rate of fusion but it caused protoplast bursting within 30 min. Substitution of mannitol by KCl, NaNO<sub>3</sub>, or Mg(NO<sub>3</sub>)<sub>2</sub> resulted in very extensive aggregation but did not favor fusion.

Incubation temperature dramatically affected protoplast fusion rate. Aggregation and fusion were not observed if the protoplasts were incubated at 0 °C. At 23 °C the protoplasts aggregated slowly

Table II. The effect of pH level on aggregation and fusion of tobacco leaf protoplasts. The protoplasts were maintained in 0.05 M CaCl<sub>2</sub>, 0.4 M mannitol, 0.05 M buffer. The terms A, F, +, ++, +++, and — are the same as for Table I. D represents protoplast deterioration and death.

Incubation time at 37°C [min]	pH						
	8.5	9.5	10.0	10.5	11.0	11.5	
0	A — F —	A — F —	A — F —	A — F —	A — F —	A — F —	
15	A — F —	A + F —	A + F +	A + F +	A + F +	A +++ F +++	
30	A — F —	A +++ F +++	A +++ F +++	A +++ F +++	A +++ F +++	A +++ F ++++	
45	A + F —	A +++ F +++	A +++ F ++++	A +++ F ++++	A +++ F ++++	A +++ D	
60	A + F +	A +++ F +++	A +++ F ++++	A +++ F ++++	A +++ F ++++	A +++ D	

and underwent some fusion after two hours. Rapid aggregation occurred at 45 °C and protoplasts underwent fusion within 30 min but deterioration and bursting occurred after 30 min of incubation. A temperature of 37 °C was considered to be optimal for inducing high frequencies of fusion with a minimal effect on viability. Pelleting the protoplasts subsequent to the addition of the fusion solution had a marked effect on the degree of aggregation and fusion. Samsun protoplasts would aggregate and fuse quite readily without centrifugation but the "haploid" s and v protoplasts had to be pelleted in order to induce high frequencies of fusion.

#### *Protoplast culture*

Protoplasts subjected to the fusion treatment and subsequently cultured in the medium of Nagata and Takebe<sup>21</sup> underwent division and developed into small colonies within two weeks. The large protoplast bodies formed by multiple fusions either failed to survive post-fusion washing or disintegrated within 48 hours in culture. We have not yet been able to determine the fate of fusion products involving two or a few protoplasts but it is certainly possible that such fusion products from two protoplasts could survive, regenerate a cell wall, undergo nuclear fusion, and ultimately undergo cell division.

#### **Discussion**

A calcium solution buffered at high pH induces leaf protoplast aggregation followed by high frequencies of fusion. The protoplasts have been able to tolerate the fusion treatment and could be successfully cultured. Although sodium nitrate has been reported to induce fusion between isolated protoplasts<sup>12</sup>, it has generally not been successfully used to induce high fusion frequencies between vacuolated protoplasts such as leaf protoplasts<sup>18, 3</sup>. Lysozyme has been reported to induce protoplast agglutination followed by fusion but it also appeared to be toxic<sup>17</sup>. Concanavalin A<sup>26</sup> and immunological methods<sup>27</sup> have been used to induce protoplast agglutination but apparently there was no reported increase in fusion.

A method of fusing erythrocytes by high pH and calcium has been reported<sup>28, 29</sup>, however, there are differences between this method and the method we have described for plant protoplast fusion. A pH

level of at least 10.0 was required to induce erythrocyte fusion<sup>29</sup>, but we observed fusion at lower pH levels. The fusion method described by Toister and Loyter<sup>29</sup> required the cells to be pretreated at high pH at 37 °C followed by treatment with calcium ions in the cold (0°–4 °C) to induce agglutination which in turn was followed by a high temperature (37 °C) treatment, to induce fusion. We have found optimal fusion conditions by treating the protoplasts with calcium and high pH at 37 °C.

Temperature level during the fusion treatment was an important factor determining rate and frequency of fusion. Similar observations were made by Ahkong *et al.*<sup>30</sup> when they reported a remarkable increase in the erythrocyte fusion at 37 °C.

It has been suggested that high pH conditions induce the formation of intramembraneous lysophospholipids such as lysolecithin and lysophosphatidylethanolamine<sup>29</sup>. Lysolecithin has been shown to induce animal cell fusion<sup>31</sup> and it is possible that intramembraneous lysolecithin would induce fusion between plant protoplasts. Isolated plant protoplasts are known to have a surface negative charge<sup>32</sup>. High pH conditions may change some characters of the membrane, thereby creating conditions favorable for aggregation. Calcium may act by stabilizing the protoplasts against lysis and by linking the membranes between different protoplasts.

The observation that protoplasts can be cultured subsequent to the fusion treatment would indicate that this method is promising for somatic hybridization studies. Experiments are in progress to determine the effectiveness of this method in fusing the complementing, haploid chlorophyll-deficient tobacco varieties s and c. If the selection pressure of the complemented diploid hybrid against the light sensitive haploid parents in high light intensities is good enough then this method could be generally used for fusion studies with many plants. Light sensitive chlorophyll deficient mutants are very common among higher plants.

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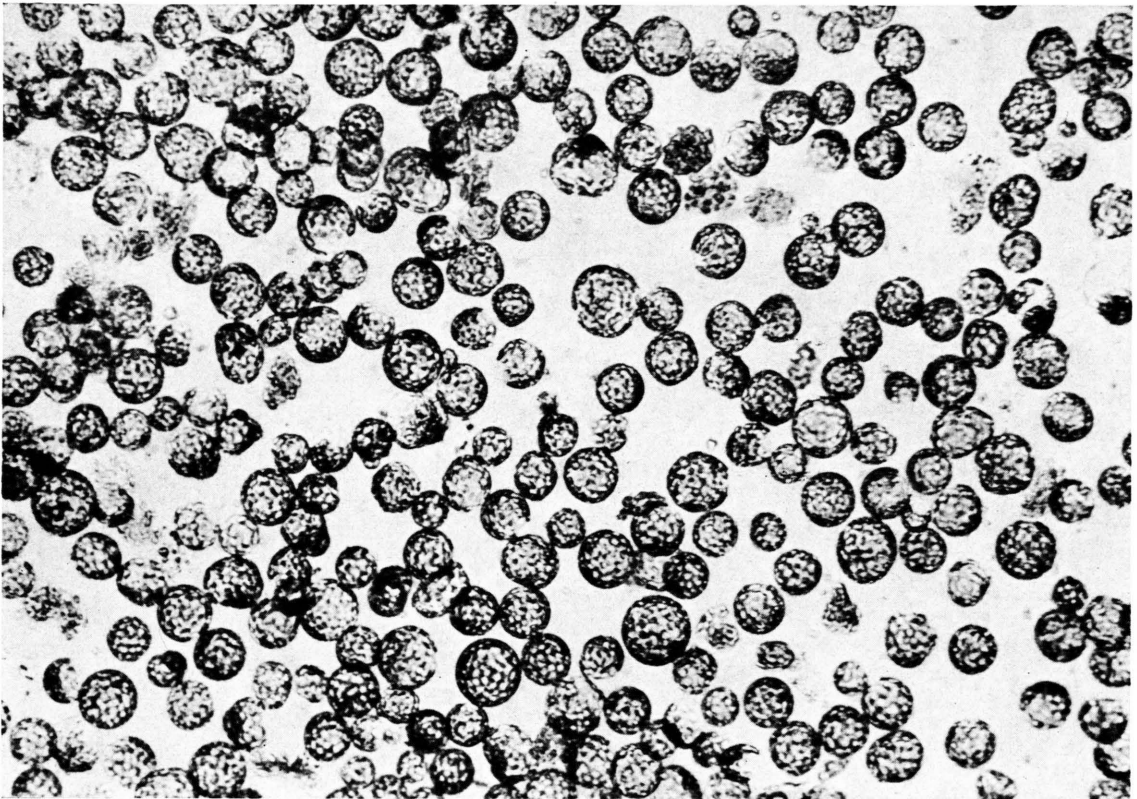


Fig. 1. Freshly isolated protoplasts.

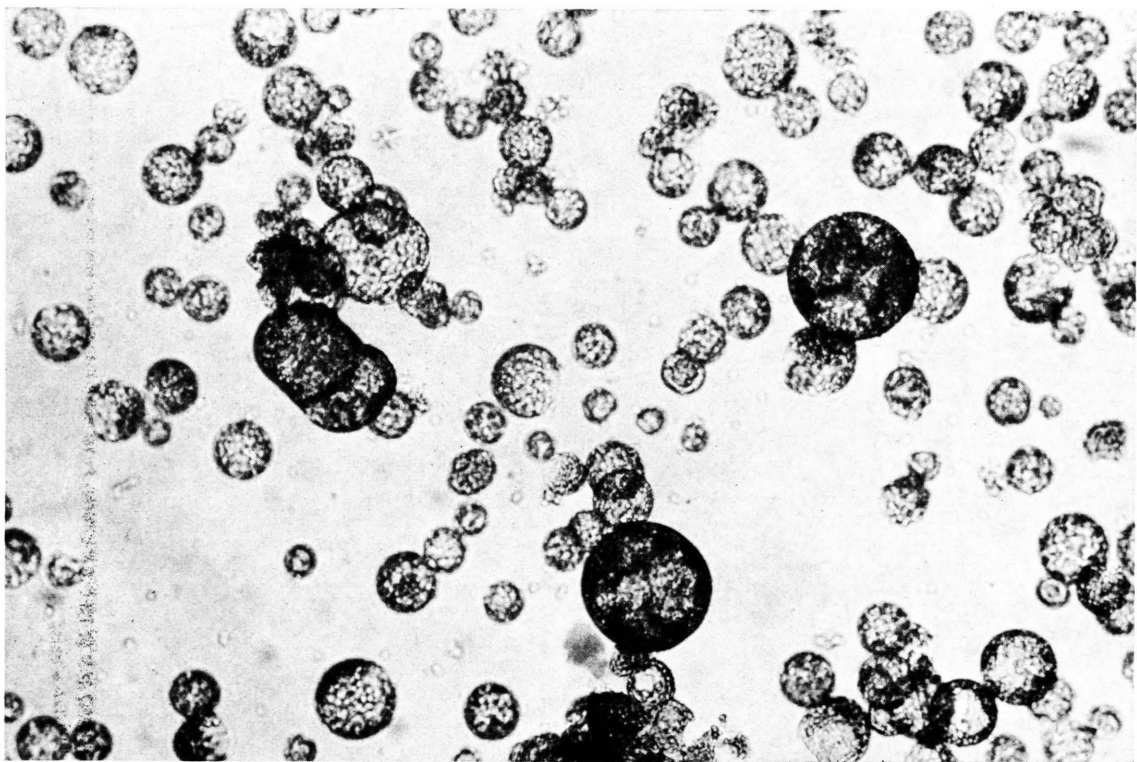


Fig. 2. Fusion events involving a few protoplasts.

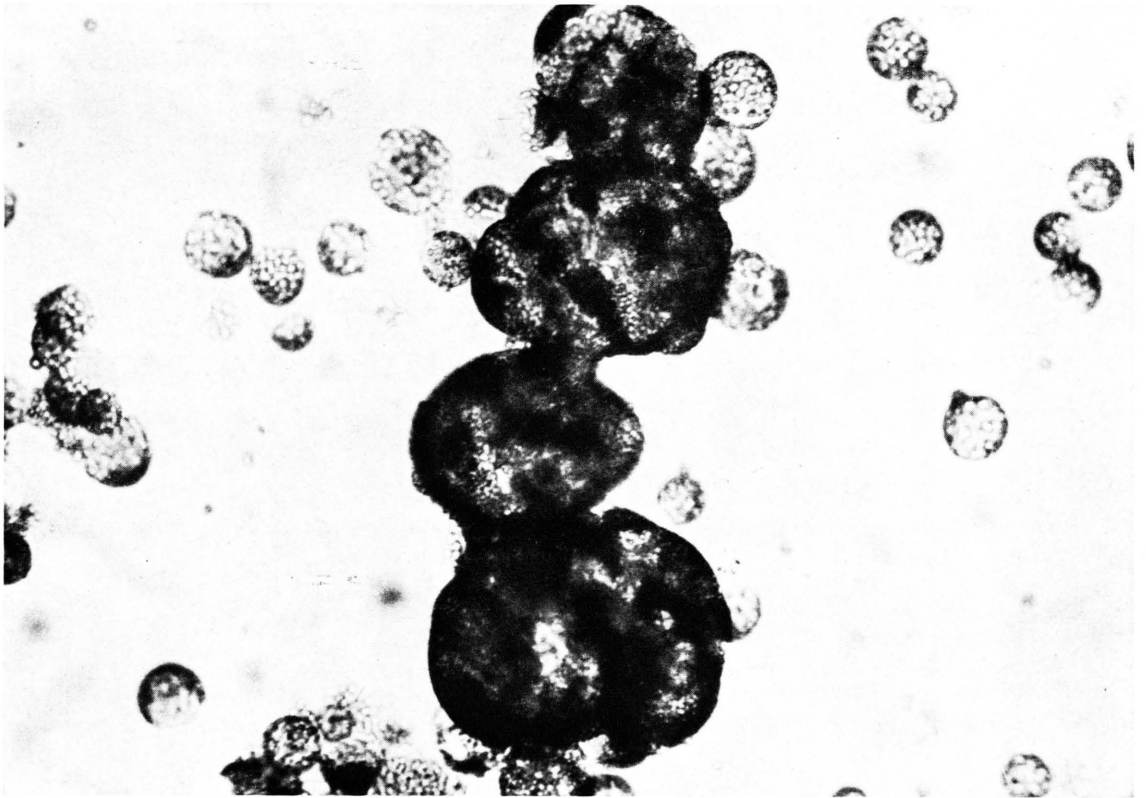


Fig. 3. A fusion event involving many protoplasts.

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