Nonenzymatic Release of Intact Protoplasts from Mature Pollen of Bean

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Abstract. A nonenzymatic technique using dilute salt solutions effected rapid release of viable protoplasts from mature bean (*Phaseolus vulgaris* L.) pollen. Protoplasm release started within 30 sec and was completed within 5 min in solutions of 0.02 to 0.06 M NaCl, or KCl, pH 6 to 9. The degree of release could be altered by changing the concentrations and ratios of CaCl₂ and H₃BO₃, and by adding sucrose to either solidified or aqueous salt media. The surface of nonenzymatically released protoplasts was partially digested by short-time exposure to a mixture of cell wall-degrading enzymes and then examined by scanning electron microscope.

Protoplasts were first released from leaf segments plasmolyzed in KNO₃ (Klerker, 1892). A few cells were cut without apparent damage to the protoplasts, and intact protoplasts were released by controlled deplasmolysis. Kuster (1909) used an aqueous solution of Ca(NO₃)₂ to produce subprotoplasts. In another study (Michel, 1937), leaf tissue was pretreated with KNO₃, then mechanically cut and protoplasts released using a viscous solution of sucrose. Protoplasts can be mechanically released only from cells in which a high degree of plasmolysis occurs followed by subsequent separation from the cell wall. Mechanical release, however, is slow, with recovery of relatively few intact protoplasts.

Large quantities of intact protoplasts are released using cell wall-degrading enzymes (Power and Cocking, 1969; Takebe et al., 1968). A major concern in the use of this method, however, is the potential damage to the physical structure and physiological activity of the protoplasts. Pilet (1972, 1973), using a few protoplasts mechanically released from root cells of *Allium cepa*, found that cell wall-degrading enzymes reduced transaminase activity. Research efforts to identify other potentially harmful effects have lagged, however, because of the inability to nonenzymatically release the large quantities of intact protoplasts needed for such studies.

Although intact protoplasts can be released enzymatically from somatic cells of most plant tissues, release from pollen grains presents special problems. At the immature tetrad stage, cell walls are only partially susceptible to enzymatic degradation (Bhojwani and Cocking, 1972; Deka et al., 1977), and release requires as long as 30 hr (Redenbaugh et al., 1980). The cell wall of mature pollen, however, contains sporopollenin, one of the most chemically inert natural plant constituents known (Gheradini and Healey, 1969) and one that is totally resistant to all of the commonly used cell wall-degrading enzymes. Baldi et al. (1985) released pollen protoplasts using a polysaccharide solvent, but the procedure required a very high temperature (75C) that reduced viability.

This study describes a unique, rapid process for the nonenzymatic release of intact viable protoplasts from mature bean pollen, and shows microscopic observations on physical alter-

ations to the surface of nonenzymatically released protoplasts by a mixture of enzymes.

Materials and Methods

Plants of 'Monroe' bean were grown in commercial planting medium (Fisons Sunshine mixture, Fisons Western, Vancouver, Canada) in 4.5-liter clay pots in a greenhouse at 25/21C (day/night), with a coincident photoperiod of 14/10 hr (day/night). Freshly opened flower buds were collected between 0730 HR and 0930 HR. Within 5 min of collection, pollen from each flower was shaken into the well of a 0.4 × 1.2-cm microcavity slide, followed immediately by the addition of two drops of an experimental protoplasm-release solution that contained various concentrations and combinations of NaCl, KCl, CaCl₂, and H₃BO₃ in glass-distilled water. Slides were then placed in closed chambers at 25C and 98% RH. Osmotic stabilizers, sucrose (0.03 to 1.2 M), mannitol (0.03 to 1.2 M), and sorbitol (0.03 to 1.0 M), were included along with the protoplast-release solutions to evaluate their influence on both quantity and extent of release and on prolonged stability of the released protoplasts. The pH of the release solutions was adjusted between 5.0 and 9.0 using 1.0 N HCl or 1.0 N KOH. In other trials, the same solutions were solidified with 0.5% or 1.0% agar. Pollen was shaken onto the surface of agar medium in a 9.0 × 1.0-cm petri dish and covered with a drop of phloxine/methyl green dye (Owczarzak, 1952).

The total number of pollen grains from which protoplasts were released and the number of those totally released from the cell wall were determined microscopically 5 min after pollen application. The extent of protoplasm release was recorded as: 1) total, the number of pollen grains showing protoplasm release (partial plus free); and 2) free (Fig. 1B), protoplasts totally removed from the cell wall. Figure 2 shows several intact protoplasts from one treatment.

Each treatment mean included pollen from a minimum of 12 flowers picked at random, replicated three times, and repeated six times during a period of 30 days. At least 500 pollen grains were evaluated from each flower. All data were statistically evaluated by analysis of variance and treatment means were compared by LSD at *P* = 0.05 (Snedecor, 1956).

Viability of released protoplasts was determined using methylene blue (Vairo, 1961) and fluorescein diacetate (Heslop-Harrison and Heslop-Harrison, 1970).

To investigate the possible physical alteration of pollen pro-

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A

B

Fig. 1. (A) Bean pollen protoplast partially released after 55 sec in 0.03 M NaCl + 1.0 M sucrose, pH 6.5. Bar = 5 μ m. (B) Same bean pollen protoplast totally free from the cell wall after 60 sec.

toplasts by cell wall-degrading enzymes, pollen from each of 20 freshly opened bean flowers was shaken into the wells of two microcavity slides and covered with two drops of protoplast-release solution containing 0.03 M NaCl, 300 mg CaCl_2 /liter, 100 mg H_3BO_3 /liter, and 0.29 M sucrose at pH 6.5. After 5 min, a drop of protoplast-release solution containing 1% (w/v) Rhozyme HP-150 (Rohm and Haas, Philadelphia), 1% (w/v) cellulase R-10 and 1% (w/v) Macerozyme R-10 (both from Kinki Yokut Biochemical, Nishinomiya, Japan) was added to one slide and one drop of the protoplast-release solution without enzymes was added to another slide. After 15 min at 25C, pollen from each slide was washed with the same protoplast-release solution into separate 3-ml centrifuge tubes and centrifuged (4 min in a Sorvall GLC-1 with a relative centrifugal force of $45 \times g$). The wash solution was poured off, and the washing procedure was repeated. A drop of solution containing washed protoplasts from enzyme-free or enzyme-treated solutions was frozen in N_2 slush and transferred under vacuum using a Hexland CT 1000A Cryo unit (East Challow, Oxfordshire, England) for viewing in a Hitachi S-530 scanning electron microscope.

Results and Discussion

In nature, pollen hydrates within seconds of contacting the stigma. Water absorbed by the pollen from the stigma exerts a pressure on the interior of the cell wall. Because the outer layer covering the pore of the pollen is weaker than the cell wall, it

Fig. 2. Several bean pollen protoplasts released after 5 min in the same NaCl solution described in Fig. 1A above.

Table 1. Influence of salt concentration at pH 6.0 on release of bean pollen protoplasts.

Protoplast release	Salt concentration (M)						
	0	0.01	0.02	0.04	0.06	0.08	0.10
<i>Intact protoplasts released (no./100 pollen grains)</i>							
NaCl							
Total	20	91	90	91	92	75	50
Free ^z	20	68	85	85	84	60	0
LSD _{0.05}	NS	6	6	6	6	8	7
KCl							
Total	20	89	88	87	85	70	45
Free ^z	20	66	85	82	79	55	0
LSD _{0.05}	NS	6	6	6	6	6	6

Protoplasts totally free from the cell wall.

ruptures, and the protoplast protrudes through the pore initiating pollen tube growth. We believed that if this naturally occurring process could be greatly accelerated, the entire protoplast could be removed from within the cell wall in seconds.

Bean pollen hydrated rapidly in glass-distilled water, but most protoplasts (i.e., 80%) ruptured (Table 1). In 0.01 M NaCl solution, intact protoplasts were released from almost all pollen grains; however, of those released only $\approx 75\%$ were totally free from the cell wall. With 0.02 to 0.06 M NaCl, a little more than 90% of the protoplasts released were totally free of the cell wall. At a salt concentration of 0.08 M, the percentage of pollen grains that released totally free intact protoplasts had decreased to 80%, and at 0.10 M none of the protoplasts released were totally free from the cell wall. Potassium chloride solutions of the same molarity as NaCl gave similar results in protoplast release (Table 1). Even very low concentrations (<0.005 M) of supplemental

calcium chloride totally prevented the release of protoplasts (data not shown).

In a 0.03-M NaCl solution at pH 5.0, many protoplasts burst. Of those still intact, 38% were totally free from the cell wall (Table 2). Few protoplasts ruptured, and essentially all of the protoplasts released were totally free from the cell wall as pH was increased from 6.0 through 9.0. In a 0.03-M NaCl solution at pH 6.0, protoplasm release commenced between 30 and 45 sec after contact with the solution. Release was essentially completed within 5 min.

Total number of pollen that released intact protoplasts in agar-solidified salt media (Table 3) was similar to that obtained in liquid (Table 1) media at the same concentration. On 0.5% agar, the number of pollen grains releasing totally free intact protoplasts was significantly increased with each increment increase in salt concentration. On 1.0% agar, only a few pollen grains released totally free intact protoplasts when subjected to 0.005 and 0.01 M NaCl, but the number was significantly increased by 0.02 and 0.04 M NaCl. The number of pollen grains releasing totally free intact protoplasts was much less on 1.0% than on 0.5% agar at similar salt concentrations.

Boron and Ca are deemed essential to pollen germination (Brewbaker and Kwack, 1963; Vasil, 1964). When added to a 0.03-M NaCl solution at pH 6.0, neither element, at the concentrations used, greatly altered the total number of pollen grains that released intact protoplasts (Table 4). Almost all of the pollen grains releasing intact protoplasts were totally free from the cell wall when 50 mg H₃BO₃/liter was added to the medium in the absence of supplemental Ca. Addition of 100 and 200 mg H₃BO₃/liter in the absence of supplemental CaCl₂ resulted in a significant decrease in the number of pollen grains releasing protoplasts that were totally free from the cell wall. In the absence of supplemental H₃BO₃, addition of CaCl₂ at 50 mg-liter⁻¹ resulted in 82% of the protoplasts from pollen that released intact protoplasts being totally free from the cell wall. Addition of 100 and 300 mg CaCl₂/liter significantly reduced the percentage of totally free intact protoplasts to 30% and 4%, respectively. When CaCl₂ and H₃BO₃ were used together, the

Table 2. Influence of pH on the release of bean pollen protoplasts in a 0.03-M NaCl solution.

Protoplast release	pH				
	5.0	6.0	7.0	8.0	9.0
<i>Intact protoplasts released (no./100 pollen grains)</i>					
Total	65	95	88	89	83
Free ^z	25	90	84	85	80
LSD _{0.05}	7	NS	NS	NS	NS

^zProtoplasts totally free from the cell wall.

^{NS}Nonsignificant.

Table 3. Influence of salt concentration on release of bean pollen protoplasts in agar-solidified medium at pH 6.0.

Protoplast release	NaCl (M)							
	0.005		0.01		0.02		0.04	
	Agar (%)							
	0.5	1.0	0.5	1.0	0.5	1.0	0.5	1.0
	<i>Intact protoplasts released (no./100 pollen grains)</i>							
Total	85	80	81	87	82	86	79	81
Free ^z	25	5	45	7	60	18	68	16
LSD _{0.05}	6	7	7	6	7	7	7	6

^zProtoplasts totally free from the cell wall.

Table 4. Influence of supplemental CaCl₂ and H₃BO₃ on the release of bean pollen protoplasts in 0.03-M NaCl solution at pH 6.0.

Protoplast release	Salt and concn											
	CaCl ₂	0	0	0	0	50	100	300	50	100	100	300
	H ₃ BO ₃	0	50	100	200	0	0	0	50	50	100	100
<i>Intact protoplasts released (no./100 pollen grains)</i>												
Total		86	85	90	86	85	90	85	85	90	85	88
Free ^z		82	81	63	60	70	30	3	77	85	30	2
LSD _{0.05}		6	6	6	6	6	7	7	6	6	7	7

Protoplasts totally removed from the cell wall.

Table 5. Effect of sucrose concentration on the release of bean pollen protoplasts in 0.03-M NaCl solution at pH 6.0.

Protoplast release	Sucrose (M)									
	0	0.03	0.09	0.15	0.29	0.44	0.58	0.73	0.88	1.17
<i>Intact protoplasts released (no./100 pollen grains)</i>										
Total	80	90	92	90	88	80	75	76	75	71
Free ^z	78	87	88	86	85	75	50	41	25	11
LSD _{0.05}	7	7	7	7	7	7	6	6	6	6

^zProtoplasts totally released from cell wall.

reaction of pollen to release of totally free intact protoplasts was similar to that of pollen given only supplemental CaCl₂ at the same concentration.

Sucrose, in the absence of NaCl, did not effect a release of protoplasts (data not shown). However, addition of 0.03 to 0.29 M sucrose to a 0.03-M NaCl solution increased the total number of pollen-releasing protoplasts and the number of totally free protoplasts (Table 5). Addition of 0.44-M sucrose decreased both the total number of pollen releasing intact protoplasts and the number of totally free protoplasts. The number of pollen releasing intact protoplasts then remained the same with additions of 0.58 to 1.17 M sucrose. The percentage of pollen grains releasing totally free intact protoplasts, however, was significantly decreased with each successive increment increase of sucrose from 0.58 through 1.17 M.

Although sugars and sugar alcohols are preferred protoplasm stabilizers, salts can also be used (Meyer, 1974). However, protoplasts take up salt, and prolonged exposure to salt can be injurious. With the process described in this study, however, protoplasm release was so rapid (<5 min) that potential salt damage would be minimized.

Mannitol concentrations at 0.3 to 15.0 M and sorbitol at 0.3 to 1.0 M in dilute salt solutions did not alter the total number of pollen grains releasing intact protoplasts or the viability of protoplasts when compared to similar dilute salt solutions without sorbitol or mannitol (data not shown).

At least 80% of the protoplasts released in 0.02 to 0.06-M NaCl solutions retained their viability for at least 16 hr, as determined by methylene blue (Vairo, 1961) and by FDA (Heslop-Harrison and Heslop-Harrison, 1970).

Although only totally free protoplasts were evaluated in the study, partially free protoplasts (Fig. 1A) are of special interest, since they are less fragile and leave less cellular debris to interfere with microscopic examination. When sufficient protoplasm surface is exposed, they could prove useful for fusion and for transfer of genetic material by microinjection or electroporation. Although not shown in any of the tables, partials make up a large portion of the total protoplasts released. The number of partials produced and the amount of protoplasm release can be regulated by controlling the concentration of CaCl₂, H₃BO₃,

and sucrose used in the dilute salt solution and by controlling agar concentration.

All nonenzymatically released protoplasts, both totally free and partially free, remained intact. When nonenzymatically released protoplasts were incubated with cell wall-degrading enzymes, few (<5%) remained intact.

Exposure of nonenzymatically released protoplasts (Fig. 3A) to cell wall-degrading enzymes resulted in a severe disintegration of the protoplasm surface (Fig. 3B). The surface of a protoplast contains large quantities of carbohydrate constituents (Williamson et al., 1976), and their loss through enzymatic degradation could be detrimental to the function and longevity

of the protoplasm and could influence in vitro plant regeneration. Cell wall-degrading enzymes also release wall-bound enzymes such as peroxidase and catalase (Vairo, 1961; Strand et al., 1976), which could alter physiological processes associated with the protoplasm.

This study demonstrates for the first time that large quantities of viable intact protoplasts can be rapidly released from mature bean pollen without the use of cell wall-degrading enzymes. These results suggest the intriguing possibility that plants can be regenerated from mature pollen protoplasts. This procedure for nonenzymatically released protoplasts could be used for genetic manipulation through somatic hybridization and genetic transformation.

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Fig. 3. (A) Bean pollen protoplasm released as in Fig. 2 above. One drop of the solution containing protoplasts was frozen in N slush and transferred under vacuum using a Hexland CT 1000A Cryotrans unit for viewing in a Hitachi S-530 scanning electron microscope (SEM). Bar = 5 μ m. (B) Bean pollen protoplasm released as above and then subjected to an enzyme containing 1% each Rhozyme HP-150, cellulase R-10, and macerozyme R-10 at pH 6.5, for 15 min. One drop of the solution containing protoplasts was treated with N slush and examined by SEM as described in A above, except that the sample was left overnight in the SEM and viewed the next day.