Plant Physiol. (1985) 78, 652–654
0032-0889/85/78/0652/03/$0.1.00/0

Short Communication

Pollen Sporoplasts: Dissolution of Pollen Walls[1]

Received for publication January 16, 1985 and in revised form March 19, 1985

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ABSTRACT

4-Methylmorpholine *N*-oxide monohydrate (MMNO·H_2O), a potent solvent for polysaccharides, is an effective vehicle for release of membrane-enclosed male gametophytes (sporoplasts) from spore walls. This release occurs in minutes when pollen (*Lilium longiflorum* Thunb.) is suspended in a melt of MMNO·H_2O at 75°C. Continued heating at 75°C leads to distintegration of the exine 'shell' which coalesces into immiscible globules in the MMNO melt. These observations provide a general procedure for preparation of pollen sporoplasts and sporoplast outer membranes, and offer a new method for dissolving the sporopollenin component of the spore wall.

With few exceptions, pollen spores from flowering plants are encased by a tough, rigid outer coat, the exine, which is shaped and imprinted with a sculptured pattern that uniquely characterizes the species from which it arises (9, 15). Underlying the exine is a layer of polysaccharide-rich material, the intine, which envelops the membrane-enclosed male gametophyte. Access to organelles and internal structures of the microspore is limited by these layers and the usual experimental approach requires either mechanical rupture or spore germination (wherein metabolic events, triggered by external stimuli, initiate pollen tube development). Methods such as those used to release protoplasts from vegetative tissues (6) have been tried (1) but appear to have only limited application. A reliable procedure for release of the naked gametophyte from its encasement within the wall would stimulate fresh interest and new efforts to study the organelles and internal structure of pollen. Southworth (14) surveyed a number of reagents and solvents for their exine-solubilizing properties. Apart from strong oxidizing reagents and fused potassium hydroxide, only hot 2-aminoethanol dissolved exine and this action was limited to sculptured outer exine. She did not test the cyclic amine oxides. We have discovered that MMNO·H_2O[2] is an effective solvent of the intine layer and eventually dissolves the exine as well. MMNO·H_2O is a potent solvent for cellulose and other cell wall polysaccharides (2, 3, 8). Physical studies indicate that MMNO·H_2O acts as a real solvent and not as a derivatizing reagent (3, 10, 11). Experiments described in this report use lily pollen (*Lilium longiflorum* Thunb.) but other pollens (cattail, *Typus latifolia* L.; pear, *Pyrus communis* L.; ash, *Fraxinus americana* L.; and walnut, *Juglans regia* L.) also responded to MMNO·H_2O treatment.

We have chosen the term 'sporoplast' to describe a pollen grain which has been stripped of its exine encasement. Criticism may be leveled at use of this term in view of the high temperature used to effect sporoplast release and consequent nonliving state of sporoplasts. Since this report was submitted, conditions for rapid and complete release of sporoplasts at 20°C or lower have been found. Methodology and the biological properties of sporoplasts obtained therefrom will be the subject of a separate report.

MATERIALS AND METHODS

Lily pollen (*Lilium longiflorum* Thunb. cv Nellie White, Ace and Harbor) were harvested from a commercial bulb farm in Brookings, OR and stored at −20°C until used (12). Samples of pollen from 1981, 1982, and 1984 harvests were used in the present study. Pollenkitt (also refered to in the literature as tryphine or pollen coat, see Knox [9]) interfered with the optical studies so this material was removed by extraction with acetone, a treatment not considered damaging to mature lily pollen (7). Pollen (2–3 g) was suspended in acetone (50–100 ml, −70°C), shaken (2–3 min), and filtered. This process was repeated three to four times until the pollen grains were seen to be free of pollenkitt by microscopic examination. The final washed pollen was air dried and stored in sealed glass vials at 20°C until used.

MMNO·H_2O was purchased from Eastman Kodak or Aldrich Chemical Co. The monohydrate contains about 10% water and melts at 71 to 72°C.

Release of Pollen Sporoplasts and Dissolution of Pollen Walls. Acetone-washed pollen was suspended in MMNO·H_2O and the progress of sporoplast release followed by compound microscope either continuously with the use of a heating stage or discontinuously by preheating glass microscope slides on a heating block which had been adjusted to the prescribed temperature. In the continuous procedure a small quantity of pollen (0.1–0.2 mg) was dispersed in a 200 µl drop of molten MMNO·H_2O on a 22-mm circular coverslip and covered with an 18-mm circular coverslip to facilitate manipulation without disturbing the sample. The sample was placed in the chamber of a United States Geological Survey gas-flow, heating/freezing stage apparatus at the Ore Deposits Research Laboratory, Washington State University (13). In this apparatus, the suspension was brought to a preselected temperature (70–120°C) within 30 s and held at that temperature for as long as the experiment lasted (up to 30 min).

[1] Supported by National Science Foundation grant PCM-8404157. Scientific Paper No. 6989, Project 0266, College of Agriculture Research Center, Washington State University, Pullman, WA 99164.

[2] Abbreviations: MMNO·H_2O, 4-methylmorpholine *N*-oxide monohydrate; PE, pentaerythritol.

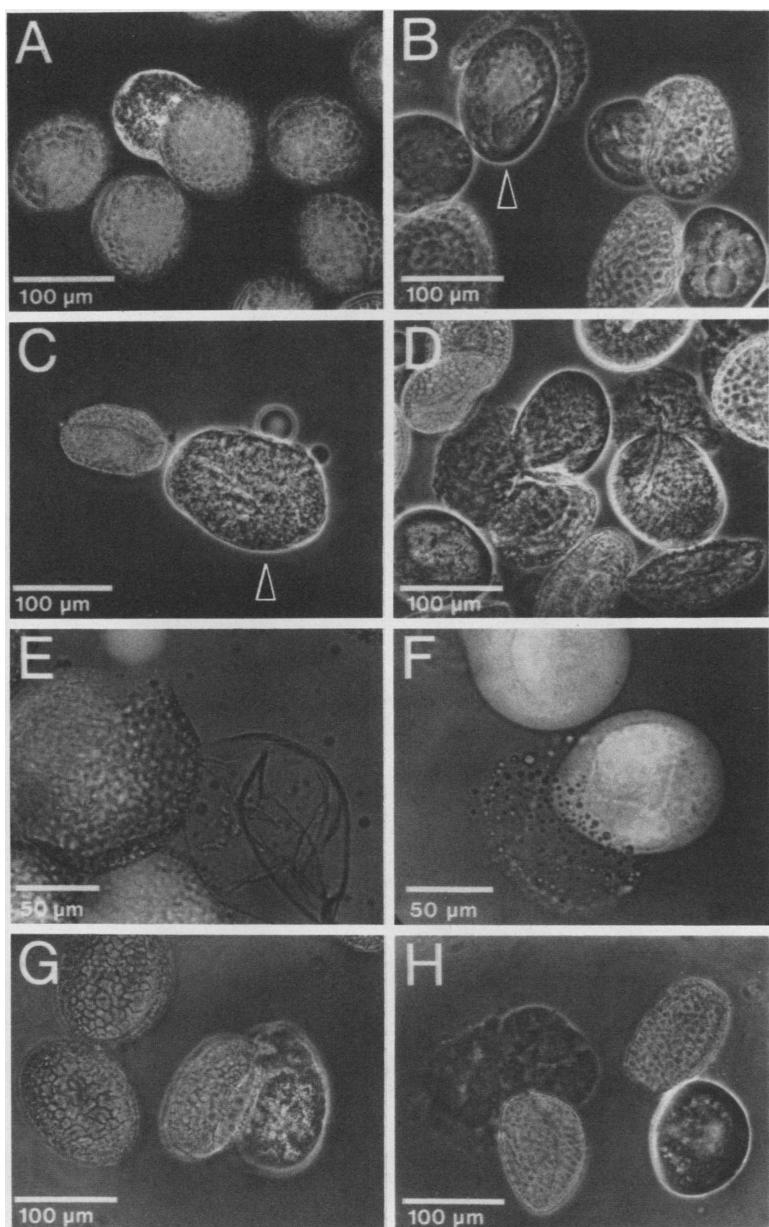

Fig. 1. Photomicrographs of the release of sporoplasts from lily pollen that was suspended in molten MMNO·H_2O. A through C, Phase contrast of pollen at 75°C for 2 min, (A), 4 min (B), and 10 min (C). D, Sporoplast rupture when sample used in (C) was heated to 125°C for 2 min. Sporoplasts, indicated by pointers in (B) and (C), are distinguished by their smooth outer membrane and absence of sculptured exine. In (C), the exine shell is the smaller illuminated object to the left of the sporoplast. E and F, Bright field photographs of pollen held at 90°C for 8 min (E) and 39 min (F). In (E), exine dissolution and a sporoplast outer membrane or ghost are evident. In (F), the dissolution of exine is almost complete and unruptured sporoplasts are starting to deform. Evans blue dye was added to the MMNO melt in (A) through (F) to establish the intact nature of sporoplasts, *i.e.* exclusion of the dye. G and H, Phase contrast of pollen grains suspended in MMNO (25% H_2O) at 60°C for 12 min (G) and 18 min (H). At this lower temperature, intact sporoplasts were extruded from small breaks in the exine rather than by a parting of the aperture. In (H) one sporoplast has ruptured and one remains intact.

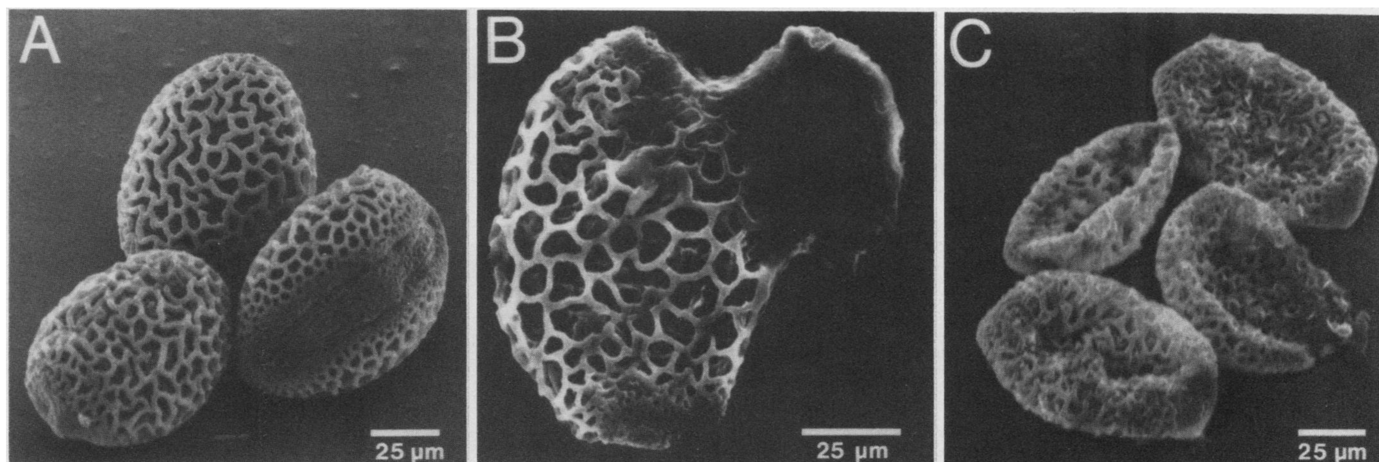

FIG. 2. Scanning electron micrographs of acetone-washed lily pollen (A), MMNO·H_2O-treated lily pollen during sporoplast release (B), and exine shells after sporoplast release (C).

Observations were made with an Olympus BS-2 microscope equipped with long-focus objectives.

In the discontinuous procedure, much smaller amounts of pollen were dispersed in 20 µl drops of molten MMNO·H_2O on preheated slides, as described above. Individual slides were removed and examined at intervals with a Leitz Orthoplan microscope that was equipped for interchangeable use under bright field, dark field, and phase contrast conditions.

Scanning Electron Microscopy. Acetone-washed pollen (10 mg) was suspended in a melt of MMNO·H_2O (1 g) for 5 or 20 min at 75°C. Aliquots were removed, fixed in 3% glutaraldehyde uin 0.1 M sodium cacodylate buffer (pH 7.2) for 16 to 20 h, dried through an ethanol series (30 → 100% ethanol), air dried, and gold-coated (300 Å). Observations were made on an ETEC Autoscan (Electron Beam Technology/Perkin-Elmer, Hayward, CA).

Recover of Sporoplast Envelopes. Conditions as described by Joseleau *et al.* (8) were used with the exception that the sample size was reduced to 10 mg pollen per g of MMNO·H_2O to avoid the viscous conditions encountered with heavier suspensions.

RESULTS AND DISCUSSION

At 75°C, a suspension of pollen grains in molten MMNO·H_2O rapidly expanded (<5 min), notably in the region of the aperture, to assume a spherical form. Shortly thereafter the aperture parted and the sporoplast emerged. Photomicrographs taken at selected stages of expansion and sporoplast release are presented in Figure 1, A to C. Further heating at 125°C led to rapid rupture of released sporoplasts (Fig. 1D). Accumulation of sporoplast outer membranes (envelopes or ghosts) (Fig. 1E), and eventually (approximately 30–40 min after mixing the pollen in molten MMNO·H_2O), dissolution of the exine 'shell' occurred in melts maintained at 90°C. As exine dissolved, it formed immiscible droplets (Fig. 1F). Eventually, these droplets coalesced into larger immiscible globules. Figure 2 presents scanning electron micrographs of untreated pollen (Fig. 2A), a pollen spore in the process of releasing its sporoplast (Fig. 2B), and the empty exine shells (Fig. 2C).

When suspensions of pollen were heated rapidly (120°C, 2 min), spore walls ruptured expelling the contents of the grain before sporoplastic release could occur. Further heating at 120°C (20–30 min) eroded the outer wall (intine + exine) and eventually released empty sporoplast envelopes or ghosts which remained insoluble in MMNO·H_2O. Preparations of these ghosts were obtained in milligram amount by using the Joseleau *et al.* procedure for recovery of solubilized polysaccharides (8). After centrifugation (10,000*g*) of the dialyzed suspension, the ghosts were recovered as a pellet. An immiscible, viscid top layer of sporopollenin and lipid also accumulated.

Temperature, water content, and solute content affect the dissolution of a particular polysaccharide (3). When the water content of the MMNO·H_2O melt was increased from 13 to 25%, sporoplast release was obtained at 60°C without interference due to crystallization of the melt. The sporoplasts appeared to be extruded from small breaks in the exine and they did not assume the spherical shape of those released at higher temperatures (Fig. 1G). Prolonged heating caused sporoplasts to rupture (Fig. 1H). When PE (4) was added to the MMNO·H_2O melt in an amount sufficient to make a 0.3 M solution of the 13% water of hydration, pollen grains failed to release sporoplasts and rapidly expelled their contents as the temperature of the melt reached 80 to 100°C. When PE was added to MMNO melts containing 25% water (final concentration of aqueous component, 0.3 M PE), sporoplasts were released within 2 min at 125°C with virtually no membrane breakage. Under this latter set of conditions, exine rapidly disintegrated and coalesced into immiscible droplets within 10 to 15 min.

Production of wall-less gametophytes from angiosperms provides new opportunities to explore subcellular structures in mature (resting) pollen and, when living sporoplasts become available, the biological competence of such structures. As Williams (16) notes in her summary of methods applicable to the molecular biology of pollen, access to pollen protoplasts (sporoplasts) provides a convenient route for transfer of genetically engineered traits into higher plants. Recent reports on pollination (5, 17) herald the significance of such studies.

Acknowledgments—We are grateful to Dr. Darlene Southworth (Southern Oregon State College, Ashland) and Dr. M. S. B. Ku (Washington State University) for helpful discussions.

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