A rapid, efficient method for the mass production of pollen protoplasts from *Pinus bungeana* Zucc. ex Endl. and *Picea wilsonii* Mast.

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**Abstract**

An optimized protocol was established to isolate large numbers of mature living pollen protoplasts of *Pinus bungeana* Zucc. ex Endl. and *Picea wilsonii* Mast. Intact pollen grains of *P. bungeana* or pollen with short tubes were incubated with gentle agitation in a solution of 2% cellulase R-10, 1.5% macerozyme R-10, 15% sucrose, 0.01% H$_3$BO$_3$, and 0.01% CaCl$_2$. Intact pollen protoplasts with diameters of 40 µm were liberated, with an isolation rate of up to 70% after 6 h of enzymatic incubation. The optimal pH and temperature for the reaction were 5.8 and 24°C, respectively, and the optimal enzymatic digestion conditions were 6 h of incubation in the above solution. The method for isolating pollen protoplasts from *P. wilsonii* was similar to that for *P. bungeana*, except that the incubation medium contained 12% rather than 15% sucrose and the optimal enzyme concentrations were 3% cellulase and 2% macerozyme. The isolated pollen protoplasts were demonstrated to be living by microscopy in a fluorochromatic reaction with fluorescein diacetate (FDA).

**Keywords:** Isolation; Pollen protoplast; *Pinus bungeana; Picea wilsonii*

**Introduction**

Protoplasts are cells from which the cell wall has been removed, usually by digestion with enzymes. In general, cellulases are employed to digest the cellulose in the plant cell walls, and pectinase is used to break down the pectin. Protoplasts are an attractive model organism for studies of physiological and cytological processes related to cell wall formation, cell growth, and differentiation, and are used in basic research and plant improvement (Krautwig and Lörz 1995). Protoplasts are excellent tools for plant breeding through cell fusion and gene manipulation, and can be treated in a variety of ways to induce the uptake of DNA, including electroporation, incubation with bacteria, heat shock and high pH. Plants can then be regenerated with the cultured protoplasts, which constitutes a convenient method to produce genetically engineered plants (Puite 1992). Furthermore, protoplasts from unrelated species can be fused to generate plants that have desirable characteristics from both parents, such as disease resistance, flavor, and cold tolerance (Krautwig and Lörz 1995).

The pollen protoplast is enclosed in situ by two structures: the double-layered wall termed the exine, and the intine. The pollen protoplast became an attractive research system following the success of somatic
protoplast culture, since it is a useful haploid system for cell fusion, genetic manipulation, and mutation studies, as well as a new system for studying pollen biology. The isolation of pollen protoplasts is a useful approach toward the study of pollen ontogeny and physiology, in particular the significance of the exine. However, early efforts to isolate protoplasts from pollen grains were fraught with technical difficulties owing to the enzyme-resistant exine consisting of sporopollenin, which limited the quantities of pollen protoplasts obtained. Sufficient quantities of the isolated protoplasts must be living for use in further procedures such as culturing, fusion, or transformation. Isolation of living pollen protoplasts was first achieved with *Lilium longiflorum* pollen by treating the pollen grains with cell-wall-degrading enzymes (Tanaka et al. 1987). Since then, pollen protoplasts have been isolated from several other species of angiosperms, including *Iris tectorum*, *Zephyranthes grandiflora*, *Hemerocallis fulva* (Zhou, 1988), *H. minor*, *Gladiolus gandavensis* (Wu and Zhou 1990), *Hippeastrum vittatum*, *Brassica campestris* var. purpurea, *B. napus* (Li et al. 1992), *Allium* (Fellner and Havranek 1992), *Narcissus cyclamineus* (Zee et al. 1993), *Nicotiana tabacum* (Wang and Zhou 1995) and *Arabidopsis* (Fan et al. 2001).

Compared with those for angiosperm pollen and somatic cells, the isolation methods for protoplasts from gymnosperm pollen are much less established. Although Duhoux (1980) had some success with *Cupressus arizonica* pollen, the viability and the condition of the nuclei of the pollen protoplasts were inadequate with this method. To date, there has been no satisfactory method developed to isolate protoplasts from gymnosperm pollen, which has limited the study of gymnosperm pollen biology.

The purpose of this study was to develop a simple and reproducible method for the isolation of a large number of living protoplasts from *Pinus bungeana* Zucc. ex Endl., a widespread ornamental plant, as well as from *Picea wilsonii* Mast., pollen grains of which consist of a central body with sacci on both sides, between which there exists a distal sulcus. In the present study, an efficient protocol was developed for pollen protoplast isolation digested with cellulase R-10 and macerozyme R-10.

Materials and methods

Plant materials

Pollen grains were collected from *P. bungeana* and *P. wilsonii* trees growing in the Botanical Garden of the Institute of Botany of the Chinese Academy of Sciences on 18 April and 4 May 2003, respectively, when the majority of the flowers were ripe and some had begun to pollinate. The collected pollen was stored in Petri dishes for several days at room temperature, and then the dried pollen grains were stored at −20 °C before use.

Isolation of protoplasts

The stored pollen grains were equilibrated at room temperature for 30 min and then dispersed into culture medium containing 15% sucrose, 0.01% H3BO3 and 0.01% CaCl2, pH 5.8, to allow short pollen tubes to grow to length just greater than the diameter of the pollen grain. Whole *P. bungeana* pollen grains or pollen with short tubes were incubated in the above medium supplemented with varying concentrations of cellulase R-10 and macerozyme R-10 (Yakult Honsha Co. Ltd.), from 1% to 3%. The grains were incubated for times from 2 to 6 h in darkness with shaking at 24 °C. The enzymes acted on the germ aperture and the pollen tube, respectively, penetrating into the inner part of the pollen grain and subsequently degrading the intine and thus giving rise to pollen protoplasts. The protocol used to isolate *P. wilsonii* pollen protoplasts was similar to that for *P. bungeana* except that the sucrose concentration was 12% rather than 15% and the enzyme concentrations were higher. Details were described below.

Fluorescein diacetate (FDA, Sigma) was dissolved in acetone to produce a 1mg/ml stock solution. For viability tests, the FDA stock was added to the medium to final concentration of 1μg/ml. To determine the number of nuclei per protoplast, 4’6-diamidino-2-phenylindole (DAPI, Sigma) was used at a final concentration of 0.01 mg/l. Calcofluor White ST (CW, Sigma) was applied to determine whether the cell wall was present or absent on the surface of the isolated pollen protoplasts. After 15 min of staining, the protoplasts were collected and transferred into dye-free medium.

Observation and image analyses

Samples were observed under a Zeiss Q500 IW light or fluorescence microscope, the latter with UV illumination and an excitation filter 365 nm. Digital images were viewed and captured using a charge couple device (CCD) camera.

Results and discussion

Isolation and characterization of pollen protoplasts

We succeeded in establishing a rapid, efficient and reproducible system to isolate *P. bungeana* and *P. wilsonii* pollen protoplasts, with isolation rates of
up to 70%. The best conditions for *P. bungeana* were an enzyme mixture consisting of 2% cellulase R-10, 1.5% macerozyme R-10 and 15% sucrose, pH 5.8, with gentle shaking for 6 h at 24 °C. The protoplasts were gradually released through a small break in the exine located at an aperture devoid of sculptured exine. The isolation process is illustrated in Fig. 1. At the beginning, only a small part of cytoplasm was released from the exine (Fig. 1a) and nucleus remained inside the pollen grain. Afterwards more cytoplasm and one nucleus were released (Figs. 1b–d). Finally, the whole cytoplasm and two nuclei were completely released (Fig. 1e). In few cases, two or more subprotoplasts were released from the same pollen grain (Fig. 1f). In most cases, the entire protoplast was released from the pollen grain. If the two enzymes functioned non-uniformly on the pollen grain, subprotoplasts were released, with some of the cytoplasm remaining surrounded by the exine and intine. Subprotoplasts were also observed in the medium, identified by their smaller volumes, and contained zero, one, or two nuclei as indicated by DAPI fluorescence.

The freshly isolated intact pollen protoplasts were spherical, with many starch particles and vacuoles occupying a large portion of the protoplast (Fig. 2a), and lacked cell wall remnants, as confirmed by CW staining (Fig. 2b). A large proportion of the pollen protoplasts had already separated from the empty pollen walls (Fig. 2c), although some isolated protoplasts were observed near their empty pollen walls (Fig. 2b). Strong FDA fluorescence and active cytoplasmic streaming observed by microscopy demonstrated the viability of the majority of the pollen grain.

**Fig. 1.** Isolation process of pollen protoplasts from *Pinus bungeana* Zucc. ex endl. indicated by DAPI labeling. Bar = 10 μm. (a) Small portion cytoplasm was released from the entire pollen. (b) Small portion cytoplasm but larger than that in (a) was released from the entire pollen, with a nucleus located at the aperture indicated by DAPI labeling. (c) Part of cytoplasm was liberated from the pollen grain with one entire nucleus. (d) Most of the cytoplasm was released from the pollen grain with one nucleus released and one located at the aperture. (e) Intact protoplast was released from pollen with two whole nuclei. (f) Two subprotoplasts were released from the same pollen grain.
Fig. 2. Isolation of pollen protoplasts from *Pinus bungeana* Zucc. ex endl. Bar = 10 μm. (a) An isolated pollen protoplast from *P. bungeana* with its exine. (b) An isolated pollen protoplast from *P. bungeana* with its exine. (c) A intact pollen protoplast of *P. bungeana*. (d) Fluorescein diacetate (FDA) fluorescence indicated viability of the pollen protoplast. (e) Pollen protoplast was released from pollen deprived of exine. (f) An isolated pollen protoplast labeled by 4',6-diamidinido-2-phenylindole (DAPI) showed two nuclei.
protoplasts (Fig. 2d). Pollen protoplasts could be obtained from de-exinated pollen by further enzymatic digestion (data not shown), and a greater proportion of these protoplasts were intact with this method (Fig. 2e). Each protoplast generally contained two nuclei that differed in volume and DAPI fluorescence intensity (Fig. 2f), the smaller nucleus being the generative cell nucleus and the larger nucleus the tube cell nucleus.

Factors affecting the isolation efficiency

The concentrations and ratio of the enzymes were crucial for optimal pollen protoplast isolation. High concentrations of the enzymes, up to 2% cellulase R-10 and 1.5% macerozyme R-10, were required for successful protoplast isolation. Furthermore, the ratio of cellulase to macerozyme was also important. Ratios of cellulase to macerozyme that were too high (over 2) or too low (below 0.8) were deleterious to the pollen protoplasts, resulting in ruptured plasma membranes. The combination of 2% cellulase R-10 and 1.5% macerozyme and enzymatic maceration for 6 h was found to provide the best conditions for pollen protoplast isolation (Table 1).

The intactness of the pollen protoplasts appeared to be associated with the ratio of the two enzymes. When the cellulase concentration was either more than twice that of the macerozyme concentration (ratios of cellulase to macerozyme greater than 2) or equal to or less than that of macerozyme (ratios of cellulase to macerozyme below 0.8), only a few intact protoplasts were released. Varying the duration of the treatment produced diverse results, with too short an incubation time leading to the release of subprotoplasts, and incubation times over 6 h resulting in decreased protoplast viability. There was a progressive increase in the isolation rate with increasing incubation times up to 6 h in the enzymatic solution at optimal concentrations, after which the rate remained relatively constant (Table 2). Therefore, an enzymatic incubation time of 6 h was adopted.

The presence of an osmotic stabilizer was essential for the isolation and viability of the pollen protoplasts. Two different carbohydrates were employed as osmotic stabilizers: mannitol or sucrose in the present study. The use of solutions containing either mannitol or sucrose led to pollen protoplast isolation, but sucrose was more suitable, with an optimal concentration of 15% sucrose for *P. bungeana*. Therefore, sucrose was chosen as the osmotic stabilizer. Although various concentrations of mannitol were tested, the results were inferior to those obtained when sucrose was used.

Both intact pollen grains and pollen with short tubes were tested as the starting material for protoplast isolation. The isolation rate from pollen with very short pollen tubes was low. In contrast, from whole pollen grains, protoplasts were obtained with high levels of intactness and viability. Therefore, whole pollen grains were used as the starting material for data collection and analysis. Owing to the sacci, *Pinus* pollen grains always float on the surface of medium without aspiration, whereas protoplasts sink to the bottom of the medium, by taking advantage of which the isolated pollen protoplasts were purified.

Different isolation protocols for different taxa

Pollen protoplasts were also successfully isolated from *P. wilsonii* (Fig. 3a). For this species, different results were observed, entire protoplasts in most of the cases (Fig. 3b), occasionally one subprotoplast, or two or more subprotoplasts released from the same pollen grain (Fig. 3c). FDA fluorescence and active cytoplasmic streaming indicated the viability of the isolated pollen protoplasts under a fluorescence microscope (Fig. 3d). The protocol for pollen protoplast isolation was similar for both species, except for the enzyme concentrations and time of incubation. Higher enzyme concentrations were required to isolate *P. wilsonii* pollen protoplasts.
than for *P. bungeana*. A combination of 3% cellulase R-10 and 2% macerozyme R-10 was found to be optimal for *P. wilsonii* pollen protoplast isolation, as well as a lower concentration of sucrose, 12% rather than 15%. The *P. wilsonii* pollen protoplasts were much larger than those of *P. bungeana*, with diameters of 80 and 40 μm, respectively.

There are some differences between the optimal methods of isolation of pollen protoplast from angiosperms and gymnosperms. For angiosperm pollen protoplast isolation, the enzymes used are generally cellulase and pectinase at the same concentration. In the present study, with gymnosperm pollen, macerozyme rather than pectinase was used in combination with cellulase. Varying the concentrations of cellulase and macerozyme while holding other parameters constant revealed that 2% cellulase and 1.5% macerozyme were the most suitable concentrations for pollen protoplast isolation in terms of the isolation rate and the viability (Table 1), with isolation rates of up to 70%.

![Fig. 3. Isolation of pollen protoplasts from *Picea wilsonii* Mast. Bar = 10 μm. (a) An isolated pollen protoplast from *Picea wilsonii*. (b) An intact pollen protoplast with its exine left. (c) Three subprotoplasts were released from the same pollen grain. (d) Strong fluorescence labeled by FDA indicated the viability of the pollen protoplast.](image)
The enzyme concentrations used in this investigation differed from those used for angiosperm pollen protoplast isolation, possibly owing to the stronger action of macerozyme as compared to that of pectinase. Furthermore, the optimal concentrations and ratios of the enzymes differed for the isolation of *P. bungeana* and *P. wilsonii* pollen protoplasts, suggesting that these factors are species specific. The most appropriate osmotic stabilizer also proved to be specific to the species. In the present study, the soluble carbohydrates sucrose and mannitol were examined as osmotic stabilizers, revealing that sucrose was the superior stabilizer. Mannitol led to poor protoplast yields, in contrast to protocols designed for angiosperms (Wang and Zhou 1995).

In summary, a rapid, efficient, and reproducible enzymatic maceration protocol was established to isolate living pollen protoplasts at high rates from the gymnosperms *P. bungeana* and *P. wilsonii*. Protoplasts could be used as an experimental material for studies aimed at the characterization of membrane properties and ion transport processes such as nutrient uptake, ion homeostasis, and the uptake of inorganic carbon by pollen (Fan et al., 2001). This approach provides a baseline for further studies of the properties of pollen protoplasts, with respect to gene transformation, fusion with somatic protoplasts, and other techniques.

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**References**


