Plant Cell, Tissue and Organ Culture **29**: 275–279, 1992.

Isolation of *Allium* pollen protoplasts

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Received 14 June 1991; accepted in revised form 6 January 1992

Key words: Allium, pollen protoplast isolation

Abstract

Studies on protoplasts isolation were carried out with mature pollen grains of 29 samples of species of *Allium aflatunense*, *A. cepa*, *A. fistulosum*, *A. karataviense*, *A. longicuspis*, *A. nutans*, *A. odorum*, *A. sativum* and *A. schoenoprasum*. Surface sterilized pollen grains drifted from crushed anthers were incubated in an enzyme solution containing 1% (w/v) cellulase Onozuka R-10, 1% (w/v) Macerozyme R-10, 0,5 mol l^{-1} sucrose and the basal salts of Nitsch medium. Protoplasts were released within 3 to 120 min, either from the pollen grain, through a slightly disturbed germination pore (narrow aperture), or through a wider aperture, when the exine surrounding the germination pore was disturbed. For the first time, protoplasts were obtained from 13 genotypes of 6 *Allium* species, at a rate of 1 to 30% of the digested intact pollen grains, depending on the genotype.

Introduction

Experiments on the isolation and culture of pollen protoplasts could contribute to the clarification of some problems concerning pollen development and, especially in *Allium* species, to the understanding of pollen sterility problems.

The first experiments on the isolation and culture of pollen protoplasts started at the end of the sixties and beginning of the seventies (Bajaj 1977, and references therein). Some interesting reports on the isolation of protoplasts from binuclear pollen grains of *Lilium longiflorum* (Tanaka et al. 1989, and references therein) and from the mature pollen grains of *Hemerocallis fulva* and other plant species (Zhou & Wu 1990, and references therein) have been recently published. In addition, the culture of protoplasts isolated from the pollen tubes of germinating pollen grains (Pargney 1982; Kroh & Knuiman 1988; Rutten & Derksen 1990), and the non-enzymatic isolation of protoplasts (using osmotic shock only) from mature pollen grains of *Phaseolus vulgaris* (Weaver et al. 1990) have also been reported.

This paper describes the enzymatic isolation of mature pollen protoplasts from several species in the genus *Allium*.

Materials and methods

Plant material

Mature pollen grains of twenty-nine samples (varieties, forms etc.) from nine *Allium* species from the germplasm collection of the Research Institute of Vegetable Growing and Breeding in Olomouc were used (Table 1).

The plants, from which pollen grains were taken, were grown in a glasshouse, under natural light conditions at a temperature of 23 to 27°C, or in the field. The viability of protoplasts was

determined by FDA (fluorescein diacetate) staining (Widholm 1972).

Protoplast isolation

Flower buds (shortly before flowering) were surface sterilized (1 min) with 70% ethanol and then rinsed several times in sterile bidistilled water. Anthers were removed from the buds and placed in 60 mm plastic Petri dishes, where they were cut and crushed with a scalpel, and then flooded with 4 ml of enzymatic solution. For the isolation of pollen protoplasts from all genotypes a modified enzymatic solution (Tanaka et al. 1987) containing 1% (w/v) cellulase Onozuka R-10, 1% (w/v) Macerozyme R-10, dissolved in basal Nitsch medium (Nitsch 1969) with 0, 5 M sucrose (pH 5, 8) was used. The incubation of pollen grains was carried out at 23 to 25°C, with an illumination of 3,200 up to 3,600 lx. All experiments were repeated at least 3 times.

Table 1. Pollen protoplast production and digestion duration for *Allium* genotypes.

Number of sample	Species and genotype	Released protoplasts (%)	Isolation time (min)	Viability of protoplasts (%)
1	*A. fistulosum* cv. Tokyo Long White	25–30	3	100
2	*A. fistulosum* cv. Tsukuba Long White	10–12	20	100
3	*A. fistulosum* cv. Evergreen Hardy White	10–11	30	100
4	*A. fistulosum* cv. Multistalk	4– 5	30	100
5	*A. fistulosum* cv. Asagi Bunching Onion	4– 5	30	100
6+	*A. fistulosum* cv. Ishikura Long White	0– 1	5	100
7+	*A. fistulosum* cv. Ishikura Long White	0– 1	45	100
8	*A. aflatunense* wild species (Anzob)	0	–	–
9	*A.cepa* cv. Hanka	5– 7	120	100
10	*A. cepa* landrace (Peschpazak)	0	–	–
11	*A. schoenoprasum* cv. Jemna (purple mutant)	8–11	60	100
12	*A. schoenoprasum* cv. Jemma (white mutant)	5– 7	60	100
13	*A. karataviense* wild species (Aksu)	0– 1	45	100
14–17*	*A. longicuspis*	0	–	–
18–27**	*A. sativum*	0	–	–
28	*A. nutans* wild species	10–11	5	100
29	*A. odorum* wild species	7– 8	5	100

+ Different clones provided by firms Takii (Japan) (sample 6) and Wagner (Germany) (sample 7).

* *A. longicuspis*: samples 14 to 17 were wild species Issyk-Kul, Urunga-sai, Univ Brusel, and cv. Jubilejnyj Gribovskij 55, respectively.

** *A. sativum*: samples 18 to 27, respectively, landrace (Osh, Bile Karpaty, Samarkand, Andizan, Dvurucka, Dzambulu, Osh and Samarkand), cv. Gribovskij and cv. D'Alsace Freres. Samples 18 and 26 are two different clones obtained at market in Osh, similarly, samples 20 and 27 are different clones obtained at market in Samarkand.

Results

The results for protoplast isolation from mature pollen grains of each sample are shown in Table 1. The number of isolated protoplasts is shown as a percentage, related to the number of digested pollen grains. The time when protoplasts first started to be released is also indicated.

Protoplasts were released from pollen grains in either of two ways. In the majority of cases (Fig. 1), the germination pore was enzymatically degraded, and an almost fully-expanded protoplast was released from the pollen grain, through a narrow aperture. Alternatively, protoplasts were released through a wide aperture, which could be caused by enzymatic degradation of the exine around the germination pore. Both methods of pollen protoplast release were observed simultaneously, in a same sample.

The osmolality of the enzyme solution was very important, with an optimum in the range of 670 to 710 mOs kg^{-1} H_2O, dependent probably on the overall physiological status of the donor plant. Preliminary experiments, with an enzymatic solution of a lower osmotic pressure showed that, since protoplasts lack a plasmatic membrane, they do not round off after passing through a germination pore, and are dispersed in the surrounding solution (deplasmolysis sets in). At a higher osmotic pressure, plasmolysis sets in even if the germination pore is degraded, and the protoplast is not released from the pollen grain.

The highest number of released protoplasts was observed for sample 1 (*A. fistulosum* var. Tokyo Long White) (Fig. 2), reaching 30% of the digested pollen grains. For *A. fistulosum* (samples 2–7), fewer protoplasts were released, i.e. between 1 to 12% of pollen grains, while an even longer number of protoplasts was released in samples 9, 11, 13, 28 and 29 (*A. cepa*, *A. schoenoprasum*, *A. karataviense*, *A. nutans* and *A. odorum*, respectively, Table 1), where the yield fluctuated between 1 and 11%. With *A. aflatunense*, *A. longicuspis* and *A. sativum* protoplast release was not observed, irrespective of the genotype tested.

The time necessary for release of pollen protoplasts varied, dependent on the plant species and genotype, ranging from several minutes (sample 1) up to two hours (sample 9). In *A. longicuspis*, *A. sativum*, *A. aflatunense* and *A. cepa* (sample 10) protoplasts were not released even after seven hours of enzymatic treatment.

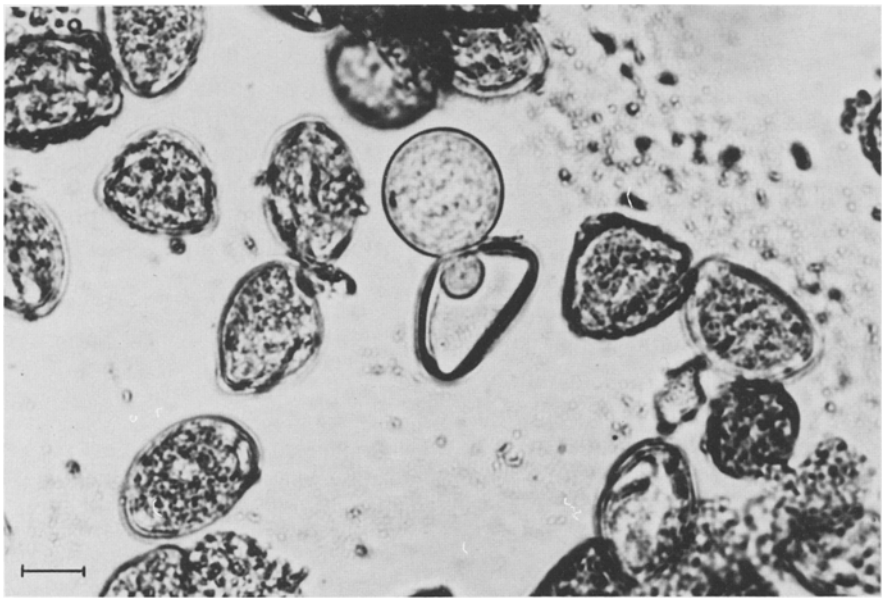

Fig. 1. Release of pollen protoplasts of *A. fistulosum* (sample 6) through a germination pore (narrow aperture) after 72 min digestion (bar = 13 μm).

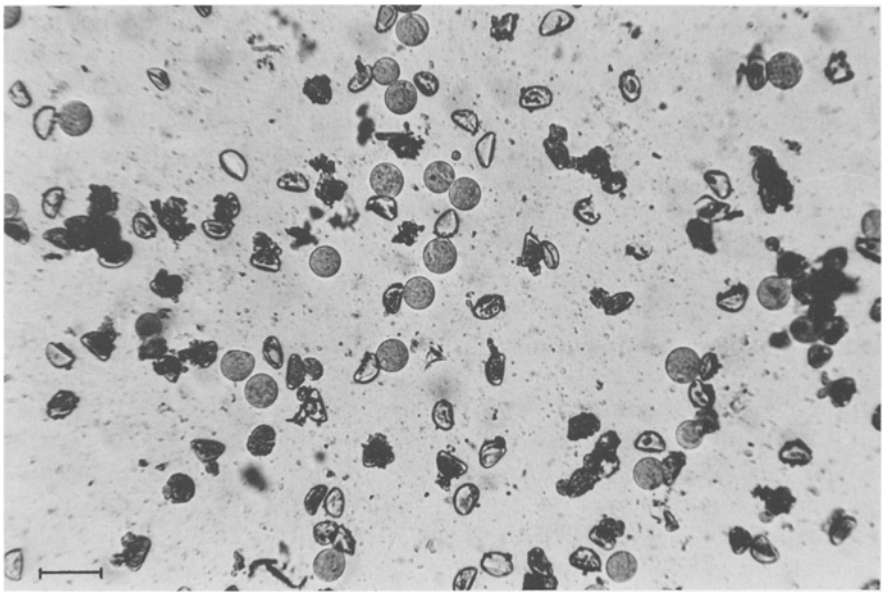

Fig. 2. Release of pollen protoplasts of *A. fistulosum* (sample 1) after 42 min digestion (bar = 56 μm).

All protoplasts released from pollen grains were viable, and, for all samples, their size ranged from 21, 8 to 33, 7 μm.

Discussion

In this paper pollen protoplast isolation from mature pollen grains of numerous *Allium* genotypes has been reported, by using a method described originally for the related species *Lilium longiflorum* (Tanaka et al. 1987). This method was modified by using Nitsch's medium instead of White's medium (White 1963) and omitting dextran sulphate from the enzymatic solution. With *Lilium longiflorum*, Tanaka and his collaborators (Tanaka et al. 1987, 1989) achieved a maximum protoplast yield of 80%, within 90 min. The protoplast yield in our experiments ranged from 0 up to 30% after 3 to 120 min of enzyme incubation. In former reports, several methods of isolation mature pollen protoplasts were described (Bajaj 1977):

- the protoplasts are released through a degraded germination pore which being formed only by intine, is disturbed by the enzymatic solution whereby the protoplast forces its way out;
- the exine partially degraded by the enzyme, is susceptible to mechanical disturbance, releasing protoplasts;
- although sporopolenin is not affected enzymatically, the enzymatic solution is able to partially dissolve exine which swells and disintegrates, enabling the protoplast to be released.

We observed that *Allium* pollen protoplasts are released probably by the first method or by a combination of the first and third method. In the first case, protoplasts are released from the pollen grains through a narrow opening only, while in the second case the protoplast is released through a wide one.

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