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Influence of pH and sucrose concentration on nonenzymatic and enzymatic isolation of protoplasts from mature pollen of *Tulbaghia violacea*

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Abstract

Studies on protoplast isolation were carried out with mature pollen grains of *Tulbaghia violacea* Harv. (*Liliaceae*). Pollen grains drifted from surface sterilized crushed anthers were incubated either in a nonenzymatic solution composed of Nitsch medium and sucrose, or in the same solution supplemented with 1% cellulase Onozuka R-10 and 1% Macerozyme R-10. The process of protoplast release was studied as a function of pH and sucrose concentration of nonenzymatic and enzymatic solutions. For nonenzymatic isolation, the tested range of pH and sucrose concentration was from 3.3 to 13.1 and from 0.015 to 1.12 M (final solution osmolality from 200 to 1,300 mOs kg^{-1} H_2O), respectively. In the former case, the release of protoplasts occurred only at nonphysiological pH (12.2 to 13.1) and could be observed after several seconds to 120 min, depending on pH and sucrose concentration of medium. Under enzymatic incubation, viable protoplasts were released more rapidly (3 to 35 min) and in more physiological conditions, the optimum being pH 5.8 and final medium osmolality 652 mOs kg^{-1} H_2O. Speed, manner of protoplast release, number and quality of protoplasts were dependent on interactions of pH and sucrose concentration.

Introduction

Microspore and pollen protoplasts are an attractive research subject following the success of somatic protoplast culture. The production of haploids and doubled-haploids has been a useful tool in pollen biology, genetic studies and in plant breeding of many species. To realize certain experiments, however, the methods for the isolation of intact microspore and/or pollen protoplasts and their culture should developed.

Pollen protoplasts from mature pollen have been enzymatically isolated firstly from dicotyledons (Bajaj *et al.* 1974). Many recent papers focus on the isolation, culture and structure of pollen protoplasts from monocotyledons, especially *Lilium longiflorum* (Loewus *et al.* 1985; Baldi *et al.* 1987; Tanaka *et al.* 1987; Miki-Hirosige *et al.* 1988; Zhou & Wu 1990; Tanaka & Wakabayashi 1992). Isolation and culture of microspore protoplasts of *Asparagus officinalis*, from this same family *Liliaceae*, has recently been published (Kunitake *et al.* 1993). In such studies the protoplasts have been isolated either enzymatically or using a polysaccharide solvent (MMNO·H_2O). For the first time, nonenzymatic release of intact pollen protoplasts without the use of cell-degrading enzymes has been reported by Weaver *et al.* (1990) for dicotyledonous *Phaseolus vulgaris*.

This paper describes the nonenzymatic and enzymatic isolation of mature pollen protoplasts from the monocotyledon, *Tulbaghia violaceae* (*Liliaceae*).

Materials and methods

Plant material

Plants of *Tulbaghia violaceae* Harv. (*Liliaceae*), a garlic-like odour plant native of South and Tropical Africa, provided by the germplasm collection of the former Research Institute of Vegetable Growing and Breeding in Olomouc, were used as the source of mature pollen. The plants were grown in the greenhouse under natural light conditions at the temperature of 23 to 27 °C.

Protoplast isolation

Flower buds (shortly before opening) 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 Petri dishes, where they were crushed with a scalpel, and then flooded with 4 ml either of a nonenzymatic solution, or of an enzymatic solution. The process of protoplast release was studied as a function of pH and sucrose concentration of nonenzymatic and enzymatic solutions. The experimental solutions of individual pH and osmolality values were prepared in advance from mother stock solution composed of Nitsch medium (Nitsch 1969). For nonenzymatic isolation, the tested range of pH and osmolality was from 3.3 to 13.1 and from 200 to 1,300 mOs kg^{-1} H_2O, respectively. For the enzymatic isolation, the solution was, in addition, supplemented with 1% (w/v) cellulase Onozuka R-10 and 1% (w/v) Macerozyme R-10 (Fellner & Havránek 1992). In that case, the pH and osmolality of solution was from 3.6 to 9.6 and from 652 to 814 mOs kg^{-1} H_2O, respectively. The pH of both solutions was adjusted using 1 N HCl and 1 N or 6 N NaOH. The osmolality was measured with an osmometer based on freezing point. The incubation of pollen grains was carried out at 23 to 25 °C, with an illumination of 3,200 up to 3,600 lx, without shaking. The extent of protoplast release was recorded in the case of nonenzymatic isolation after 5 min from releasing of first protoplasts and after 30 and 60 min of enzymatic incubation, as percentage, related to the number of treated pollen grains. The percentage of released protoplasts (as well as percentage of protoplast viability) counted both partially as well as totally released protoplasts. The time when protoplasts first started to be released is also indicated. All experiments were repeated 3 times for nonenzymatic isolation and 7 times for enzymatic isolation.

The viability was assessed for fresh pollen grains flooded with the nonenzymatic solution of pH 5.8 and osmolality 650 mOs kg^{-1} H_2O and for protoplasts only enzymatically released after 1h-digestion for the experimental conditions in which the maximal release of protoplasts was observed. Viability was determined by FDA staining (Widholm 1972) and presence of the pollen cell wall was determined by the UV-excited optical brightener Rylux BSU staining (Synthesia, Pardubice - Semtín, Czech republic; optimum excitation from 340 to 380 nm, emission from 430 nm) (Hejtmánek *et al.* 1990) (sample of Rylux BSU was kindly provided by Professor M. Hejtmánek). Both were observed by fluorescent inverted microscopy (Leitz, Austria). Size of protoplasts was estimated only on the basis of microscope magnification.

Results

Nonenzymatic isolation

The results of nonenzymatic protoplast isolation from mature pollen grains of *Tulbaghia violaceae* are shown in the Table 1. At low pH, from 4.5 to 5.5 and between osmolality values from 200 to 840 mOs kg^{-1} H_2O, no released protoplasts were observed but some pollen germination. This process of pollen germination was not studied in detail but, 11 to 22% of pollen grains germinated at the earliest after 20 min and not later than after 73 min of the incubation in those experimental. solutions. At osmolalities higher than 840 mOs kg^{-1} H_2O the pollen germination was not observed in this pH range. The pollen tubes often burst at the lowest osmolality values (200–380 mOs kg^{-1} H_2O) after about 1 hour of incubation. For the pH 3.3 and 5.8 to 12.0, irrespective of osmolality, neither pollen germination nor protoplast release was observed.

The release of protoplasts occurred only at nonphysiological pH from 12.2 to 13.1. The pollen envelope was most likely degraded around the germination pore due to high alkalinity: pollen grains burst and released protoplasts. The period necessary for pollen grains to burst and to release protoplasts was strongly dependent on the pH and sucrose concentration of solutions. At lower osmolality (200 to 490 mOs kg^{-1} H_2O) the pollen grain burst very quickly, protoplasts did not keep their shape because they apparently lacked a plasmamembrane, and protoplasm was dispersed into the surrounding solution in the form of clots. With the increasing sucrose concentration, the pollen grains

Table 1. Pollen protoplast frequency as a function of sucrose concentration and pH of the experimental nonenzymatic solution. Numbers in the brackets indicate time (in minutes) when protoplasts first started to be released.

pH	*Sucrose concentration (M)*						
	0.015–0.2	0.31	0.35–0.42	0.52–0.57	0.62–0.72	0.74–0.77	0.82–1.12
	Final solution osmolality (mOs kg^{-1} H_2O)						
	200–380	490	530–600	700–750	800–900	920–950	1000–1300
12.2	0	0	<1.0 (32)	0	0	0	0
12.3	0	0	<1.0 (30)	<1.0 (35)	0	0	0
12.4	0	0	<1.0 (32)	<1.0 (30)	0	0	0
12.5	0	< 1.0 (120)	18.0 (5)	<1.0 (4)	<1.0 (5)	0	0
12.6	0	<1.0 (120)	28.0 (4)	<1.0 (4)	3.1 (4)	<1.0 (7)	<1.0 (4)
12.8	0	< 1.0 (120)	34.6 (4)	30.0 (3)	28.5 (3)	21.6 (4)	31.0 (4)
13.1	0	< 1.0 (120)	62.8 (sec)	60.5 (1)	64.0 (1)	29.7 (3)	31.0 (4)

burst but protoplasts were released more slowly. Thus, released protoplasts largely retained their shape, being coated by plasmamembrane and partly by intine (Fig. 1) which was, however, soon separated from protoplasts (Fig. 2). Increasing the pH led to faster degradation of pollen coat and to faster bursting of pollen grain. Formation of numerous protoplasts was observed only from the osmolality of 530 mOs kg^{-1} H_2O at pH 12.8. Only at the osmolality range 530 to 600 mOs kg^{-1} H_2O were some protoplasts released at pH 12.5. The highest number of protoplasts was observed at pH 13.1 and osmolality range from 530 to 900 mOs kg^{-1} H_2O, being released within several seconds or minutes. In the other cases, the protoplasts were released exceptionally (in Table 1 labeled < 1.0%) and usually later. However, these protoplasts were soon lysed by the high pH.

The viability of protoplasts was not measured in the experiments on nonenzymatic isolation firstly, due to high background fluorescence of the solution at high pH values and secondly, to escape some errors in relation to an unknown behaviour of fluorescein diacetate at high pH and thus to avoid wrong interpretation of viability. The viability of fresh pollen grains in these sets of experiments was 69%, on average.

As to cell wall staining with Rylux BSU, even if the intensity of fluorescence was slight and pH-dependent and, in addition, the background was also high, it was possibly to distinguish that pollen envelope exhibited light yellow fluorescence, the intine-coated protoplasts and pollen tubes of germinated grains showed light blue fluorescence and protoplasts with plasmamembrane only did not exhibit any fluorescence (photo not shown).

Table 2. Frequency of enzymatically isolated pollen (%) protoplasts as a function of sucrose concentration and pH of enzymatic solution

pH	*Sucrose concentration (M)*		
	0.47–0.48	0.54–0.56	0.61–0.63
	Final solution osmolality mOs kg^{-1} H_2O_2		
	652–665	721–747	793–814
3.6	0[a] / 4.2[b] (35[c])	2.5 / 7.8 (21)	8.0 / 14.4 (11)
4.6	0 / 3.4 (31)	10.5 / 13.8 (15)	9.7 / 14.9 (13)
5.6	0 / 1.4 (29)	2.4 / 4.5 (18)	4.7 / 17.9 (11)
5.8	45.4 / 74.2 (5)	40.5 / 48.7 (5)	19.8 / 23.1 (3)
6.0	0 / 1.2 (33)	0.3 / 0.3 (25)	1.6 / 1.4 (11)
6.6	0 / 0	0 / 0	0 / 0.1 (23)

[a] Protoplast frequency 30 min after release of first protoplasts
[b] Protoplast frequency 60 min after release of first protoplasts
[c] Time (minutes) when protoplasts first started to be released

Enzymatic isolation

The results of enzymatic isolation of protoplasts from mature pollen grains are shown in Table 2. Protoplasts were released from pollen grains in either of two ways. In the first case, intine of the germination pore was enzymatically degraded, and a protoplast was released from the pollen grain through a narrow aperture. In the second case, the protoplasts were released through a wide aperture, which could be caused probably by enzymatic degradation of the pollen coat around the

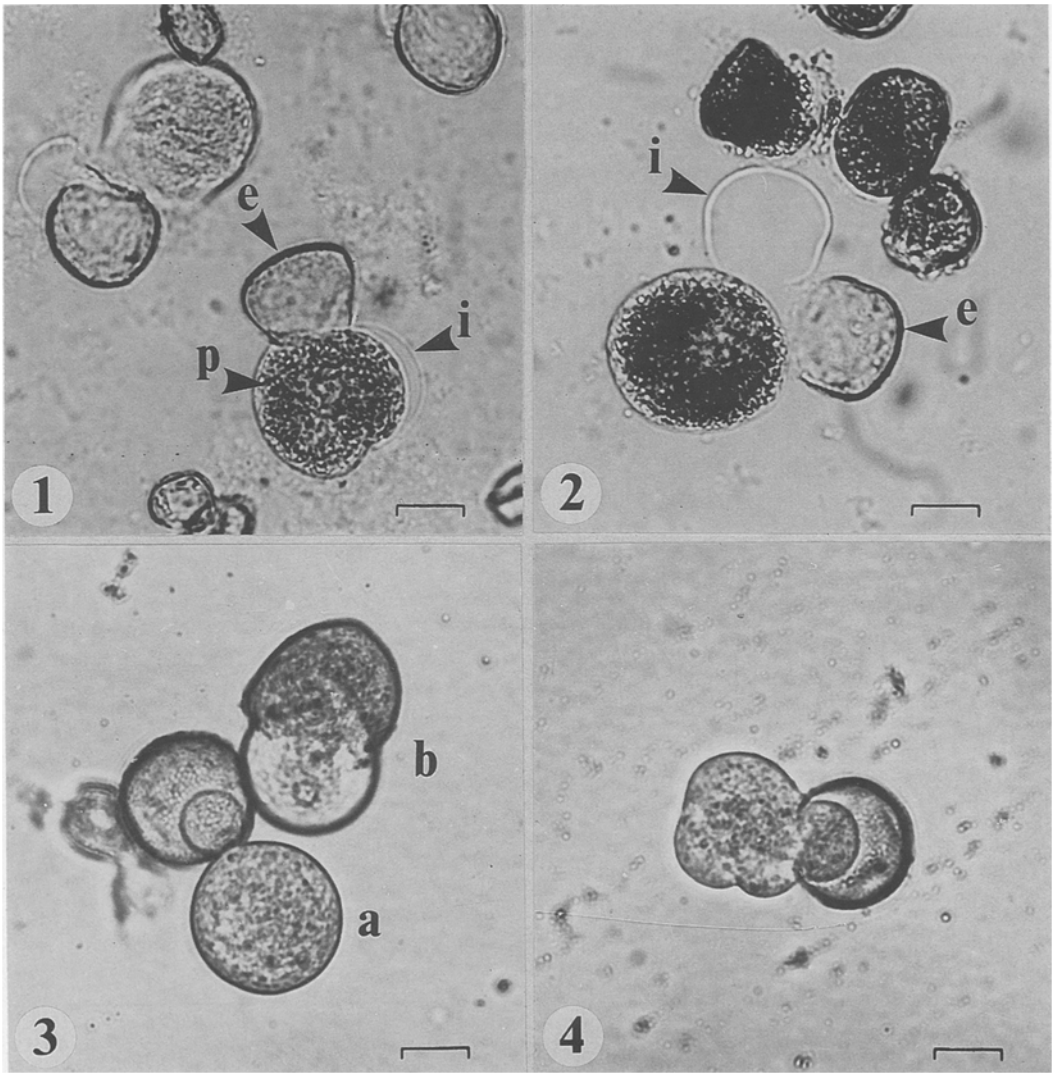

Fig. 1-4. **Fig. 1** Release of *T. violaceae* pollen protoplast (p) partially covered by intine (i) after 13 min in nonenzymatic solution (750 mOs kg^{-1} H_2O, pH 12.8); e = pollen coat (exine), (bar = 18 μm). **Fig. 2** *T. violaceae* pollen protoplast totally free from pollen coat (exine) (e) and intine (i) after 6 min in nonenzymatic solution (863 mOs kg^{-1} H_2O, pH 12.6), (bar = 18 μm). **Fig. 3** Release of pollen protoplasts of *T. violaceae* through both ways, narrow aperture (a) or wide aperture (b) in enzymatic solution after 30 min digestion (660 mOs kg^{-1} H_2O, pH 5.8), (bar = 12.6 μm). **Fig. 4** Nonspherical *T. violaceae* pollen protoplast covered by intine and releasing through a wide aperture in enzymatic solution after 30 min (660 mOs kg^{-1} H_2O, pH 5.8), bar = 12.6 μm.

germination pore. Both methods of pollen protoplast release were observed simultaneously (Fig. 3).

The pollen protoplast release was dependent at the same time on the pH and sucrose concentration of enzymatic solution. Protoplasts were released throughout the osmolality range tested and in the pH range from 3.6 to 6.6. The higher pH (from 7 to 9.6) was not conducive to protoplast release as in the case of nonenzymatic isolation. Protoplast release was highest for pH 5.8 in each osmolality tested. However, pH-dependent differences in the release of protoplasts diminished as the sucrose concentration of the enzymatic solution increased. The most rapid protoplast release was at pH 5.8. At a given pH value, the time for protoplast release shortened as the sucrose concentration increased. Unlike the protoplasts released through a narrow aperture, those released through a wide aperture were often covered with intine, evident

from their nonspherical shape (Fig. 4) and also from Rylux BSU staining. The intine was hydrolyzed by enzymes and protoplasts became spherical.

Viability of protoplasts released at pH 5.8, when their number was highest in each osmolality range tested, was measured. Mean protoplast viability after 1 h of enzymatic digestion reached 37, 38 and 19% for osmolalities 652–665, 721–747 and 793–814 mOs kg^{-1} H_2O, respectively. Average viability of fresh pollen grains was 73% in these experiments and it did not change effectively for 1 hour. The protoplasts released through a wide aperture were mostly dead. However, some protoplasts released through a narrow aperture without separating from the pollen grains showed lower viability than the free protoplasts which showed 100% viability.

Discussion

In this paper nonenzymatic and enzymatic protoplast isolation from mature pollen grains of *T. violacea* has been reported. The protoplast yield in the case of nonenzymatic isolation in these experiments ranged from 1 up to 64% after several seconds to 120 min of incubation. As reported by Weaver *et al.* (1990) for nonenzymatic isolation of protoplasts from mature pollen grains of bean, the protoplast release was strongly dependent on the pH of nonenzymatic solution and its $CaCl_2$, H_3BO_3 and sucrose concentration. In our experiments, protoplasts were not released from pH 3.3 up to 12.0, and at pH 4.5 to 5.5 only pollen germination was observed. Weaver and his collaborators did not mention pollen germination: on the contrary, they reported 65 and 95% release of intact protoplasts for pH 5.0 and 6.0, respectively, and similar percentages of released protoplasts for pH values up to 9.0. These different results might be attributed to different concentrations of H_3BO_3, $CaCl_2$ and sucrose in experimental solutions, factors shown to alter the degree of release, and its different final osmolality. Of course, the plant species, physiological status of pollen grains and the characteristics of the pollen wall itself could be very important factors for protoplast release. As Southworth (1974) mentions, the exine of monocotyledons is more resistant to chemical degradation than that of dicotyledons. Moreover, unlike Weaver *et al.*, we observed no effect on the exine, with the exception of pollen swelling, nor any protoplast release in bidistilled water at pH 5.8.

At pH 10.0 to 12.0 it was only possible to observe, after a long time, formation of delicate accumulations and globules which originated most probably from the pollen coat partly degraded by high alkaline solution. These particles could originate from various molecules, including polysaccharides, proteins and lipids from the tapetum, which coat the pollen. But bursting of neither pollen coat nor intine covering the germination pore was observed in that pH range. The fluorescent line of pollen envelope (yellow fluorescence) and intine (blue fluorescence) was intact. However, at the pH over 12.0 the formation of these particles was more rapid. At these pH the pollen coat was apparently more degraded and pollen grain burst. Even though the sucrose concentration influenced the speed of bursting, it is also clear from our results that pH (adjusted by 6 N NaOH) played an important role in some pollen envelope degradation. Southworth (1974) reported that 4% (1 N) and 17.5% (4.4 N) sodium hydroxide at room temperature was incapable of dissolving sporopollenin of which mature pollen exine is composed. Apparently, pollen envelope components other than sporopollenin were degraded by high pH in our experiments and this was sufficient in combination with suitable sucrose concentration to burst the pollen grain. At the low osmolality (200 to 380 mOs kg^{-1} H_2O) for each pH value tested higher than 12.0, the released protoplasts were not rounded off after passing from the pollen grain, and were dispersed into the surrounding solution in the form of clots. Really spherical protoplasts were not observed. Spherical protoplasts were exceptionally found after 120 min at the osmolality 490 mOs kg^{-1} H_2O. We have no real explanation for this exception. Perhaps, after such a long time the osmotic value (and/or pH) of the experimental solution was changed so much that it allowed a release of protoplasts from remaining pollen grains with different physiological status. To underline this possible change of conditions, the time in the brackets (Table 1) is printed with *italic* letters in this case. On the contrary, increasing sucrose concentration at these pH values slowed the whole process, and the number of protoplasts decreased.

Enzymatic isolation of viable protoplasts from mature pollen *Tulbaghia* was realized by using a method described for the related plants of *Allium* genus (*Alliaceae*) (Fellner & Havránek 1992). *Tulbaghia* protoplasts were released from mature pollen grains in two ways, similarly to *Allium* protoplast release. However, in the case of protoplast release through a wide aperture, the protoplasts were very frequently intine-

coated at first. This phenomenon was not observed for *Allium* pollen protoplasts. Optimum solution osmolality for enzymatic isolation of *Tulbaghia* pollen protoplasts was similar to that observed for *Allium* pollen protoplasts at the same pH. Even if *Allium* and *Tulbaghia* are plants of related families, the differences in the protoplast forming through a wide aperture suggest some differences in the structure of pollen envelopes. Similar enzymatic release of intine-coated protoplasts was observed from mature pollen grains of *Zephyranthes grandiflora* (Zhou 1989) using a double concentration of enzymes. Tanaka *et al.* (1987) isolated intine-free protoplasts from binuclear pollen grains of *Lilium longiflorum* at pH 5.8 using an enzymatic solution very similar to ours. They achieved a maximum protoplast yield of 80% within 90 min. The protoplast yield in our experiments was optimal at the same pH value and ranged from 23 to 74% evaluated 60 min after first released protoplast. In contrast, a similar enzyme mixture was not effective when assayed for protoplast production from *Digitalis obscura* uninucleate or binucleate pollen (Arnalte *et al.* 1991). It is seen from our experiments that, as for nonenzymatic protoplast isolation, the process of enzymatic isolation is also very dependent on the sucrose concentration of solutions. Significant influence of mannitol concentration of a very similar enzymatic solution on yield of binucleate pollen protoplasts of *Asparagus officinalis* has also been reported (Kunitake *et al.* 1993). At pH 5.7 these workers obtained from 20 to 30% isolated protoplasts in the presence of 0.5 to 0.6 M mannitol in enzymatic solution, but, 0% and 4% isolated protoplasts in 0.4 M and 0.7 M mannitol, respectively. The pH seemed to be the major factor for protoplast isolation in our experiments because lower protoplast yields were observed for pH 5.6 as well as 6.0 compared with 5.8 at every sucrose concentration tested. This pH-dependence was attenuated at higher sucrose concentrations of enzymatic solution. The pH influence on enzymatic protoplast isolation from pollen has not previously been reported in such detail. The cited reports and our present results indicate that pH 5.7 to 5.8 is generally optimal for enzymatic protoplast isolation.

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