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Glycosidases in pear pollen tube development

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During the in vitro germination of pear pollen, several hydrolases were released into the medium. They were apparently eluted from the pollen grain, since the activity was the same when germination was inhibited. These enzymes, once released, had no role in tube growth, since resuspension of pollen in fresh medium after 1.5 hr of incubation did not result in a change of the subsequent tube growth. Homogenates of the pollen suspension at different stages of development showed no significant changes in phosphatase, β-glucosidase, or β-galactosidase activity. However, patent β-glucosidase activity measured directly in suspensions of intact pollen did increase after germination in proportion to tube wall development. Nojirimycin, a specific inhibitor of glucosidases, reduced this β-glucosidase activity by 75% at 10^{-5} M and significantly reduced growth rate at 10^{-4} M.

Key words: Glycosidases — Nojirimycin — Patent ß-glucosidase — Pollen tube.

Pollen has been found to release proteins (*12*), including several hydrolases (*3*) soon after coming into contact with an aqueous solution. During the in vitro germination of pear pollen, β-1,3- and β-1,4-glucanase, and possibly also pectinase are released into the growth medium (*13*). Experimental evidence indicated that these enzymes could be involved in pear pollen tube growth (*10*).

In the present study, several types of hydrolase activities were found in the growth medium after the in vitro germination of pear pollen, and their involvement in tube growth was reexamined. Total hydrolase activity was measured at different stages of pollen tube development. β-Glucosidase was assayed to determine if an increase in activity associated with the cell wall could be correlated with the appearance of newly formed pollen tube wall.

Materials and methods

Pear pollen (*Pyrus communis* L., cv. Winter Nelis) was obtained from dry anthers purchased from Antles Pollen Supplies, Wenatchee, Wash. When stored frozen, the germination and growth capacities of the pollen remained unchanged for several months. The pollen (average grain diameter: 150 μm) was separated from the anthers by filtration through a fine plastic net (average pore diameter: 200 μm).

Abbreviations: CMC, carboxymethylcellulose; DMSO, dimethylsulfoxide; MES, morpholinoethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis 2-ethanolsulfonic acid.

Pollen tubes developed in a medium containing raffinose (0.42 M), boric acid (0.01%), and 2 mM $Ca(OH)_2$, adjusted to pH 5.9 with H_3PO_4. For most experiments, samples of 5 mg of freshly filtered pollen were incubated in 2 ml medium as described by Roggen and Stanley (*10*). For studies of the enzymes released into the germination medium, 50 mg of pollen was incubated in 20 ml of medium (50-ml Erlenmeyer flasks) for 3 hr. Germination could be completely inhibited by substituting mannitol for raffinose, or DMSO (*1*) for 10%[1] of the volume of the germination medium.

Pollen tube development was followed microscopically. Several 3-μl portions of the suspension were placed on glass slides, covered with cover slips, and sealed in with nail polish. This procedure halted tube growth immediately and preserved the tubes for several days. Percent germination was determined two or three times by examining at least 300 grains each time. A grain was considered germinated if the length of the tube was at least as long as one half the diameter of the grain. Average tube lengths were determined with three lots of 30 pollen tubes. The microscope was equipped with a drawing attachment which allowed tracing of the pollen tubes onto paper (165 × magnification); the traces were measured with a small graduated wheel. Pollen tube lengths were far from uniform: the maximum standard deviation of the population tended to be near 40%, and the standard error of the mean, between 5 and 10%.

Hydrolase activities released into the incubation medium were assayed after filtering off the pollen on a Sartorius SM 11302 membrane filter (pore size 3 μm) with gentle suction, and removing the low-molecular weight components of the medium by gel-filtration at 4°C on Sephadex G-25, equilibrated with 6.7 mM phosphate buffer pH 5.9.

Total activity of each hydrolase was measured in homogenates, which were prepared at 4°C by transferring the pollen suspension (with 5 mM MES buffer pH 5.9) into a 12-ml vibration flask of a Braun homogenizer and grinding it for 15 sec with glass beads 0.45–0.50 mm in diameter. The homogenate was recovered by flushing the beads three times with buffer.

Alcohol dehydrogenase was assayed spectrophotometrically at 30°C (pH 8.5).

Cellulase was assayed viscosimetrically. Enzyme (1.5 ml) was incubated for 2 hr at 30°C with 0.15 ml of 6.7 mM phosphate buffer (pH 5.9) and 1.35 ml of a filtered 1% solution of CMC (Hercules, 7 M-F) in 0.2 M NaF. The reaction was stopped by boiling for 10 min and insolubles were removed by centrifugation before viscosity determination (Ostwald viscosimeter). The activity was compared to that of Onozuka R-10 cellulase (Welding Co., Hamburg).

Glycosidases were assayed at 37°C by mixing 0.4 ml enzyme, 0.2 ml of the corresponding *p*-nitrophenyl-glycoside solution (2 mg/ml in H_2O) and 0.2 ml citric acid-phosphate buffer at the pH optimum of the enzyme (unless otherwise stated). The reaction was stopped with 0.2 ml of 1 M Na_2CO_3 and the amount of *p*-nitrophenol formed was determined from the optical density at 400 nm (A 400).

"Patent" β-glucosidase activity was assayed by adding 0.5 ml of fresh medium containing *p*-nitrophenyl-β-D-glucoside (final concentration 1 mg/ml) to suspensions

[1] This is twice as much as Dickinson and Cochran (*1*) used to inhibit lily pollen germination. However, germination of another batch of pear pollen, not used in these experiments, was inhibited by various DMSO concentrations to the same extent as these authors reported for lily pollen.

to intact pollen, incubating this for 15 min at 28°C, filtering it through SM 11302 membrane filter, mixing 1.0 ml of the filtrate with 3 ml of 0.25 M Na_2CO_3, and measuring the *p*-nitrophenol produced (A 400). The values were corrected for the A 400 of the substrate and of the suspension without substrate.

Laminarinase and pectinase were assayed by incubating the enzyme (1 ml) for 18 hr at 28°C with 0.5 ml of 0.3% laminarin (Fluka) in 50 mM dimethylglutarate buffer (pH 6.5) or with 0.5 ml of 0.6% polygalacturonic acid (Sigma) in 0.2 M Na acetate buffer (pH 5.3), in the presence of thymol to prevent bacterial contamination. The reducing groups formed upon incubation were measured according to Nelson (*5*).

Phosphatase was assayed at 37°C by incubating 0.1 ml of enzyme with 0.2 ml of 0.1 M citric acid-citrate buffer (pH 4.7), unless otherwise stated, and 0.2 ml *p*-nitrophenylphosphate (2 mg/ml) in a total volume of 0.8 ml. The reaction was stopped with 0.2 ml of 1 M Na_2CO_3 and the ammount of *p*-nitrophenol formed was determined from the A 400. The small amount of phosphate contained in the enzyme did not interfere with the assay.

For all enzymes, 1 μunit of activity corresponded to the utilization of 1 μmole of substrate per hour.

Protein was determined according to Lowry et al. (*2*) (10–200 μg) and to Schaffner and Weissmann (*11*) (1–20 μg).

Results

The germination medium was separated from the pollen by a rapid, but gentle filtration. Virtually no alcohol dehydrogenase activity was detected in the pollen-free medium by an assay that could have detected as little as 0.5% of the total activity present in the pollen. Therefore, protein found in the medium (average 2.5 μg/ml after 3 hr of pollen incubation) was not due to cytoplasmic contamination, but must have come from extracytoplasmic sites of the pollen.

The pollen-free medium was also tested for hydrolase activity. It was dialyzed overnight at 4°C against 2 × 1 liter of germination medium lacking raffinose, then concentrated to its original volume by dialysis against a 20% solution of polyethylene glycol 20,000. At pH 5.0, β-glucosidase, laminarinase, β-galactosidase, α-mannos-

Table 1 *Activities of hydrolases released by pear pollen into the incubation medium*

Activity tested [a]	No germination [b]	Normal germination
	μUnits/5 mg pollen	
Phosphatase	0.71	0.69
β-Glucosidase	0.18	0.15
β-Galactosidase	0.14	0.13
α-Mannosidase	0.05	0.03
β-Xylosidase	0.02	0.02
Laminarinase	0.03	0.02

[a] Hydrolases were assayed at 37°C at their pH optimum (see Fig. 1) after removing low-molecular weight materials by gel-filtration.

[b] Germination was inhibited by substituting raffinose with mannitol.

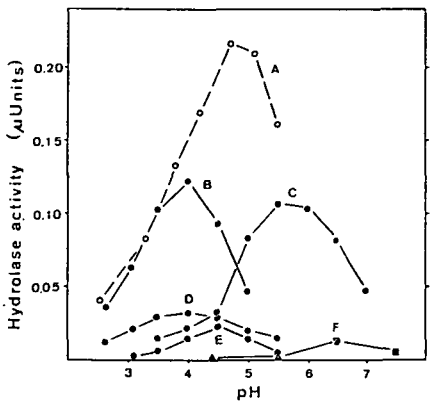

Fig. 1.

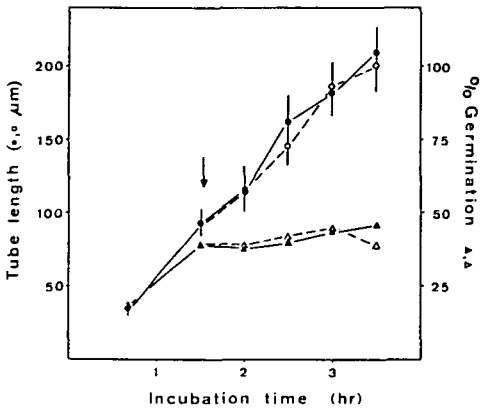

Fig. 2.

Fig. 1. *pH Dependence of hydrolase activity in the germination medium.* Activities refer to 5 mg of pollen. Enzyme activities: A, Phosphatase (activity×0.5); B, β-galactosidase; C, β-glucosidase; D, β-xylosidase; E, α-mannosidase; F, laminarinase. Buffers: citric acid-citrate (○), citric acid-phosphate (●), dimethylglutarate (△), NaPIPES (■).

Fig. 2. *Effect of medium renewal on pollen germination and tube growth.* After 1.5 hr of pollen incubation (↓), the medium was removed by centrifugation and either returned to the pollen (●, ▲) or replaced with fresh medium (○, △). Satndard errors of the average tube lengths are shown.

idase, β-xylosidase and phosphatase were found. The pH optima of these hydrolases are shown in Fig. 1. For weakly active enzymes, the optima were determined after removing interfering substances by gel-filtration on Sephadex G-25. Acid phosphatase, β-glucosidase, and β-galactosidase were the main activities found, but there was little or no β-galactosidase activity at the pH of the germination medium (pH 5.9). No detectable α-glucosidase, α-galactosidase or exopeptidase activity was found at pH 5.0.

Table 1 shows that hydrolase activity in normal growth medium after 3 hr of pollen tube development is the same as that found after suspending the pollen for 3 hr in mannitol-containing medium, which does not support germination. The same result was obtained for cellulase after 2 hr of incubation: DMSO-inhibited pollen (5 mg) released the equivalent of 1 μg Onozuka cellulase, whereas germinated pollen released two-thirds that amount. Hence, certain hydrolases appear to be eluted from the pollen whether or not germination and tube growth take place.

Hydrolases such as pectinase and cellulase released into the germination medium have been thought to play a role in pollen tube growth (*10*), but our results do not entirely support this view. The addition of pectinase (Mazerozyme, 0.07–0.7 units of polygalacturonase activity per ml of germination medium) resulted in a 14 to 27% increase in pollen tube elongation during 3 hr of incubation. Yet no endogenous polyglacturonase activity could be detected in the germination medium using an assay sensitive to 0.004 units of polyglacturonase/ml. To test the assumption that pectinase is released below the limit of the assay, we repeated an experiment of Roggen and Stanley (*10*), in which the medium was removed after 1.5 hr of incubation, and was either returned to the pollen or replaced with fresh medium.

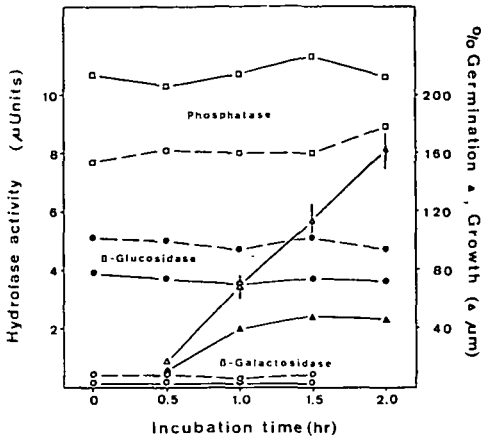

Fig. 3. *Total hydrolase activities during pollen tube development.* Samples of 5 mg of pear pollen were incubated in 2 ml of medium for 0 to 2 hr, then immediately frozen in liquid nitrogen. After thawing, they were homogenized, and assayed at 28°C and at pH 4.5 (---) and pH 5.9 (—) for phosphatase (□, activity × 0.5), β-glucosidase (●), and β-galactosidase (○) activities. Average tube lengths are plotted with their standard errors.

In the following 2 hr or pollen tube growth, no significant difference was found between the two growth curves (Fig. 2). These results clearly show that if in fact hydrolases are involved in pollen tube growth, they do not influence growth after being released into the medium.

Germinating pollen is peculiar in that total enzyme activities seldom vary in the course of pollen tube development (*4*). After normal germination and tube growth (160 μm after 2 hr incubation), total phosphatase, α- and β-glucosidase, α- and β-galactosidase and α-mannosidase activities remained virtually the same as before germination (Fig. 3 and Table 2).[2]

β-Xylosidase activity apparently disappeared upon germination. Cellulase activity was found to be the same in homogenates obtained from germinated or ungerminated pollen. Five milligrams of pollen contained cellulase activity equivalent to that of 0.6 μg Onozuka R-10 cellulase.

Because of the futility of using either total enzyme activity or activity released in the germination medium to study the relationship between hydrolases and pollen tube development, an attempt was made to examine the "patent" β-glucosidase

Table 2 *Total hydrolase activities in pear pollen*

Activity tested[a]	Ungerminated	Germinated
	μUnits/5 mg pollen	
β-Glucosidase	2.90	2.84
α-Glucosidase	0.04	0.01
β-Galactosidase	0.10	0.08
α-Galactosidase	0.07	0.07
α-Mannosidase	0.06	0.04
β-Xylosidase	0.11	0
β-Glucuronidase	0	0.01

[a] Hydrolases were assayed at 28°C, pH 5.9.

[2] β-Glucosidase activity in the homogenate was higher at pH 4.5 than at pH 5.9, indicating that the pH optimum of internal β-glucosidases must differ considerably from that of the enzymes released into the medium alone (cf. Fig. 1).

activity of intact pollen suspensions. Fig. 4 compares the patent β-glucosidase activity of normally germinated and DMSO-inhibited pollen suspensions. Patent β-glucosidase activity was low when assayed just after suspending the pollen in the medium. In the DMSO-inhibited suspension, it reached a maximum after 0.5 hr when the pollen was completely wet, then decreased with time. On the other hand, the activity of germinated pollen increased steadily with time. The difference in activity between germinated and ungerminated pollen suspensions was closely correlated with the "growth product" (Fig. 4B, C), an estimate of the amount of tube wall present in the germinated suspension. This suggests a possible relationship between the amount of patent β-glucosidase activity and the quantity of the tube wall.

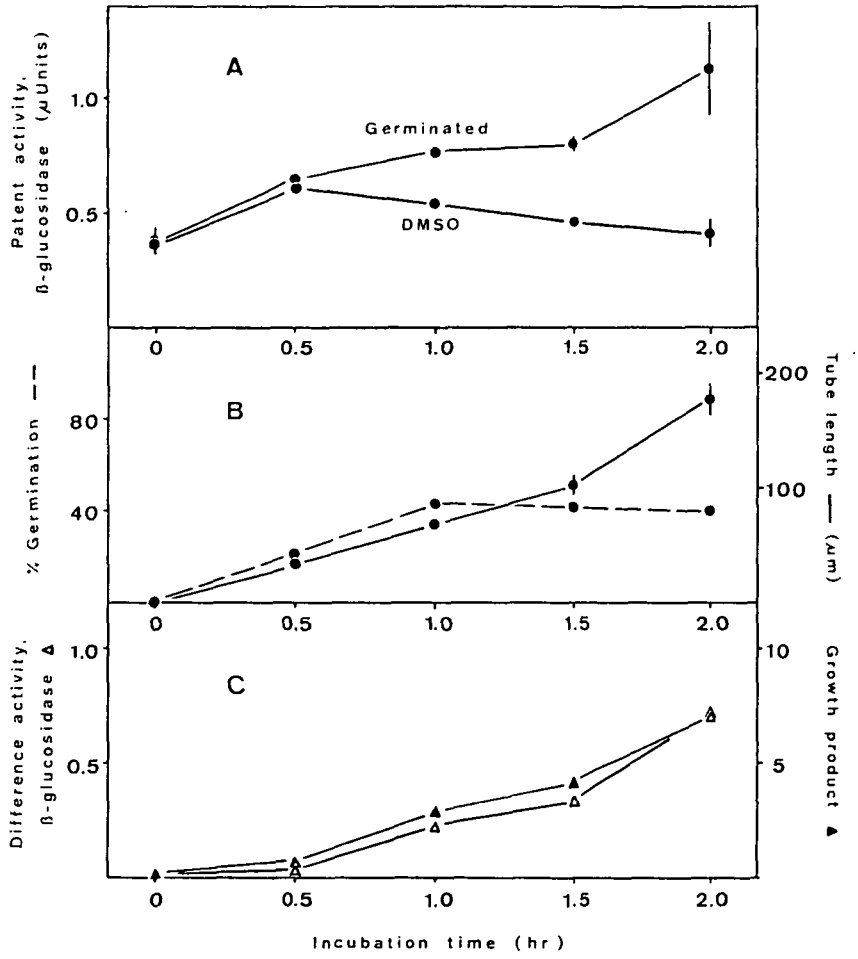

Fig. 4. *Correlation between pollen tube growth and tube wall β-glucosidase.* Samples of 5 mg of pear pollen were incubated in 2 ml of medium for 0 to 2 hr, then assayed for patent β-glucosidase activity (A). B, % germination and average tube length determined for the germinated pollen suspension. C, plots of the product of the curves in B and the difference between the curves in A. The "growth product" estimates the total amount of tube wall in the germinated suspension; the "difference activity" estimates the β-glucosidase activity associated with the tube wall.

Table 3 *Effect of nojirimycin on pollen tube development and patent activity of β-glucosidase after 1.5 hr of incubation*

Nojirimycin conc. (M)	Germinated suspension			DMSO-inhibited suspension
	Germination[a] (%)	Av. tube length[b] (μm)	Patent β-glucosidase activity[c] (μUnits)	Patent β-glucosidase activity[c] (μUnits)
0	40	155	1.02	0.43
10^{-5}	45	150	0.30	0.16
10^{-4}	43	151[d]	0.16	0.05
10^{-3}	9	103	0.27	0.25

[a] Average error: ±3%.
[b] Average error: ±12 μm.
[c] Average error: ±0.04 μUnits.
[d] See Fig. 5 for the effect of 10^{-4} M nojirimycin over 3 hr of incubation.

However, attempts to visualize β-glucosidase cytochemically according to Reiss (*9*) failed. Hyphae of *Neurospora crassa*, treated in parallel with germinated pollen, presented the expected blue-violet coloration. A likely reason for the apparent inactivity of pollen tubes is the comparatively low activity of the pollen enzyme.

Nojirimycin (5-amino-5-deoxy-D-glucopyranose) is a specific inhibitor of β-glucosidase which has been used to demonstrate the role of this enzyme in auxin-induced cell expansion (*6*). The effect of various concentrations of nojirimycin (bisulfite adduct) on pollen tube development and on the patent activity of β-glucosidase was assessed after 15 hr of pollen incubation (Table 3). Germination and tube growth were markedly reduced by 10^{-3} M nojirimycin, but were apparently not affected by concentrations of 10^{-5} M and 10^{-4} M. In separate experiments spanning 3 hr of incubation, 10^{-5} M nojirimycin still had no effect on pollen tube development. However, 10^{-4} M nojirimycin caused accelerated pollen tube initiation; after 23 min of incubation, 12% of the treated pollen grains had germinated compared with less than 2% in the control, resulting in an initial augmentation of

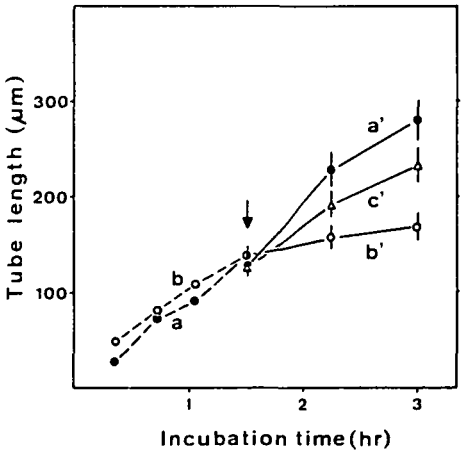

Fig. 5. *Effect of nojirimycin (10^{-4} M) on pear pollen tube growth.* In two separate experiments (dotted and solid lines), 5 mg samples of pollen were incubated in the presence of 10^{-4} M nojirimycin (b and b') and compared with an untreated control (a and a'). After 1.5 hr of incubation (↓), 0.2 ml of medium was added. In c' (previously untreated sample), this added medium contained nojirimycin to bring its final concentration to 10^{-4} M.

the average tube length (Fig. 5). But nojirimycin also slowed down the rate of subsequent tube growth so that the average tube length was virtually the same as that in the control after 1.5 hr of incubation (see also Table 3), and was more than a third smaller after 3 hr. Nojirimycin had an immediate effect on pollen tube growth if applied after all the viable pollen grains had germinated (1.5 hr). At 10^{-4} M nojirimycin, the tube growth rate was reduced but not as much as when the inhibitor was present throughout the incubation (Fig. 5, curve c'). At 10^{-3} M, tube growth was completely stopped.

Patent β-glucosidase activity was strongly reduced by as little as 10^{-5} M nojirimycin, and reduced still more by 10^{-4} M (Table 3). At 10^{-3} M nojirimycin, however, β-glucosidase inhibition was less pronounced both in the germinated and ungerminated suspensions. The patent activity attributable to the presence of pollen tubes, calculated by subtracting the activity of an ungerminated suspension from that of a germinated one, was reduced 75% by 10^{-5} M, 82% by 10^{-4} M, and nearly 100% by 10^{-3} M nojirimycin.

Discussion

Pear pollen releases a number of hydrolases into its incubation medium. The activities of phosphatase, β-glucosidase, β-galactosidase, α-mannosidase, β-xylosidase, laminarinase and cellulase released into the medium are the same whether or not the pollen has germinated. No signifieant change in pollen tube growth is found when these enzymes are removed from the medium after 1.5 hr of pollen incubation. These results clearly demonstrate that the enzymes released by pear pollen into the incubation medium are eluted from the pollen grain, and are not required for normal tube growth. This latter finding is contrary to that reported by Roggen and Stanley (*10*), who noticed a growth-stimulating effect of exogenous cellulase and pectinase. In contrast, we found that the endogenous cellulase activity is not correlated with pollen tube growth. Moreover, endogeneous pectinase can not be detected in the incubation medium.

The total activities of pear pollen hydrolases do not vary during pollen tube development. Yet, with regard to possible involvement of β-glucosidase in a wall-loosening process, only the activity associated with the cell wall is relevant. The investigation was therefore focussed on the "patent" activity of β-glucosidase which appears upon the incubation of intact pollen with substrate. Since no significant leakage of cytoplasmic enzymes has been found during pollen incubation, and since the plasmalemma of pollen tubes is unlikely to be permeable to *p*-nitrophenyl substrates, the activity measured must be due to extracytoplasmic enzymes. It follows that a difference in patent β-glucosidase activity between a germinated and an ungerminated pollen suspension can be attributed to enzymes present in the tube walls. We found this difference in activity increased in close correspondence with the amount of tube wall present in the suspension, suggesting that the enzyme is probably distributed uniformly over the entire length of the tubes.

The fact that nojirimycin reduces pollen tube growth may imply that a β-glucosidase (or an exo-β-glucanase) is important in tube wall formation, since this glucose analogue is considered a specific inhibitor of glucosidases (*7*, *8*). Yet, the difference between the concentration dependency of patent β-glucosidase inhibition

and that of tube growth suggests that the enzyme involved in wall formation is less sensitive (or less exposed) to nojirimycin than the one distributed over the non-growing part of the tube wall.

The inhibitory effect of nojirimycin on pollen tube growth is apparently different from that observed in the extension growth of coleoptiles (*6*). In pollen, normal tube growth is inhibited without delay if nojirimycin is applied after all the viable pollen grains have germinated. In coleoptiles, only auxin-stimulated growth is inhibited by low concentrations of nojirimycin and this effect is only noticeable after a considerable delay.

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