FORMATION OF "INSTANT POLLEN TUBES"

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SUMMARY

After a pretreatment in water, aperturate pollen grains can be led to formation of "Instant Pollen Tubes" (IPoT) by treatment with strong inorganic acids, such as sulfuric acid. This IPoT formation is a pseudogermination dependent on the living state of pollen and a distinct period of swelling and enzyme activation. Conditions of pretreatment and acid treatment are investigated. IPoT formation seems to be a suitable object for further investigation of the molecular structure of the intine and the dependence of pollen tube formation on initiation of enzyme activation.

1. INTRODUCTION

The discovery of the pollen tube is attributed to AMICI (1824, 1830). Only a few years later, FRITZSCHE (1832) was able to distinguish natural from artificial pollen tubes. While normally pollen grains do not generate a tube until some minutes or hours after coming into contact with water or stigmatic fluid, artificial pollen tubes are formed in a few seconds after coming into contact with acid: "Kaum hatte die Säure den Pollen berührt, als dieser an drei symmetrischen Stellen aus seinem Innern darmähnliche Fortsätze hervortrieb, deren Länge den Durchmesser des Pollenkornes selbst überstieg, und deren Entwicklung ich mit größter Leichtigkeit verfolgen konnte" (FRITZSCHE 1832). Because of the danger of hydrochloric acid vapour to the optical system of the microscope, he used sulfuric acid (2 parts to 5 parts water). Checking the literature, FRITZSCHE found that this method of observing the structure of the pollen wall was not new. SPRENGEL (1817 p. 180) had already used nitric acid to clear up the pollen grain.

RASPAIL (1826, 1828) and BRONGNIART (1827) observed an explosion of the pollen grain after treatment with certain acids. Comparable results were obtained by ADRONESCU (1915) in 5% sucrose with corn pollen, who observed the formation of a long plasmatic thread, which was ejaculated with explosive power and immediately curled up. He called the process "pseudogermination". The formation of isolated tubes or tube parts after treatment of pollen grains with narcotics was reported by BOBILLIOFF-PREISSER (1917). This method was later introduced as a viability test of pollen by KEARNY (1923).

During recent research on the influence of antimetabolites on tube growth (TUPÝ c.s. 1965), 4% sulfuric acid was used to halt further growth. Attentive observation led R. STANLEY to the re-discovery of the old phenomenon, which he termed the formation of "*Instant Pollen Tubes*" (IPoT). These may offer a new approach to the study of the mechanism of tube wall formation. The present paper reports further details on the conditions of formation of IPoT.

2. METHODS

Anthers of ripe flowers were collected and dried for 24 hours at room temperature, so that the pollen could be sieved out and stored at -10 °C. The germination medium for *Petunia hybrida* clonal material was 10 % sucrose plus 0.01 % boric acid, pH 6.5 and for *Codiaeum variegatum*, 7.5 % sucrose as described by MÜLLER-STOLL (1956) was used.

Three different test methods were applied. (1) Slide method: An 0.04 ml drop of pollen was placed on a slide set in a Petri dish containing a saturated atmosphere. The concentration of the pollen suspension was always 5 mg/ml medium. (2) Petri dish method: On the bottom of a Petri dish, germination medium was added to a height of 0.5 mm to which pollen grains were added to a concentration of 5 mg/ml by sieving. After germination at 26 °C, the pollen suspension was centrifuged for 1 min at 500 × g and the sediment was taken for further experiments after decanting the supernatant. (3) Beaker method: 11 ml medium was placed into a 50 ml beaker and 55 mg pollen were added to obtain a concentration equal to those in (1) and (2). During the germination phase, the suspensions were stirred with a magnetic stirrer.

Germination percentage was determined after fixation with 0.1 % aniline blue in a mixture of lactic acid, glycerol, phenol and water (1:1:1:1, w/w). 200 pollen grains were counted in each experiment. All experiments were done at least in fivefold. Germination or IPoT formation was considered to have occurred when the protrusion was longer than half the diameter of the pollen grains.

Pollen tube length was determined from micrographs made with the Leitz Orthomat automatic camera (magnif. 430 ×).

3. RESULTS

Under the standard conditions of germination for 20 min at 26 °C followed by the addition of sulfuric acid to a final concentration of 4 %, the percentage of IPoT (Fig. 1) formed was 70 % with the slide method (1) and about 50 % with methods (2) and (3). Several possible influencing factors were examined.

3.1 Effect of the pretreatment on IPoT formation

As seen in *fig. 2*, the duration of pretreatment, i.e., the period of germination *before* addition of acid, has a strong influence on the resulting percentage of IPoT. A higher percentage of IPoT can also be produced using the slide method. After about 20 minutes pretreatment, maximum IPoT formation is reached, whereas normal germination – less than 2 % – is practically nihil. If the pretreatment is prolonged beyond 20 minutes, the percentage of IPoT formation does

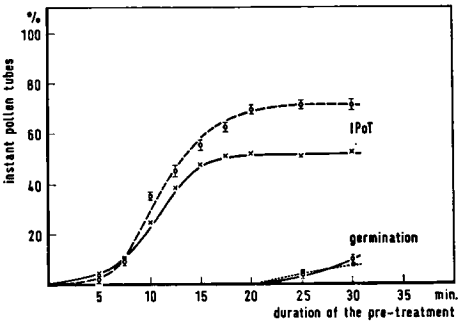


Fig. 2. Dependence of formation of IPoT on the duration of the pre-treatment (minutes in sucrose-boric acid solution), compared with normal germination. –x– Petri-dish method, –o– slide method.

Each point is an average of 5 experiments. Standard deviation is given by vertical lines.

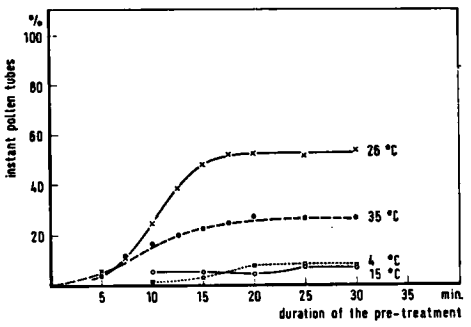


Fig. 3. Dependence of IPoT formation on temperature and duration of the pretreatment (sucrose-boric acid solution).

not increase. Adding acid then, only produces a certain increase in length of the normal tubes or induces rupture of their tips.

3.2 The influence of temperature on IPoT formation and IPoT length

The above experiments demonstrate that pollen give rise to IPoT only after a certain period of pretreatment. As seen in *fig. 3*, a distinct temperature optimum exists which yields the highest percentage of IPoT formation. There appears to be a parallel in temperature effect on normal germination and IPoT formation which reveals itself when normal germination and IPoT percentages are plotted against the temperature of the germination medium (*fig. 4*).

In addition, duration of pretreatment and temperature in the germination medium influence the length of the "Instant Pollen Tubes" (*fig. 5*). While there is no significant difference in lengths at 26° and 35°, IPoT lengths are about one-third shorter when the pretreatment is carried out at 4° or 15°C.

3.3 Concentration dependence of IPoT formation

According to FRITZSCHE (1832) an effusion from the pollen grain is formed after addition of a strong acid. But we observed that dry, resting pollen grains display only small swellings in the aperture region when treated with sulphuric acid (*fig. 6*) of concentrations between 4 and 20%. Also addition of acid to the germination medium at time zero does not lead to IPoT formation.

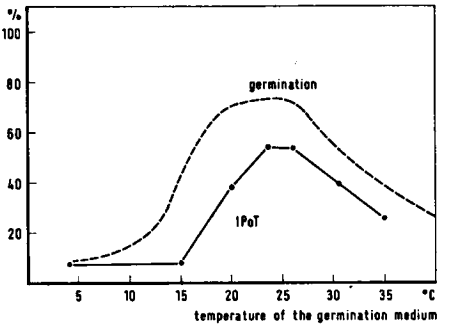


Fig. 4. Percentage of IPoT formation dependence on temperature of the germination medium. Pre-treatment: 30 minutes in sucrose-boric acid solution, compared with normal germination (after 4 hours).

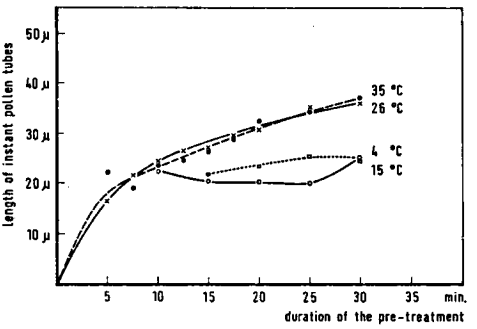


Fig. 5. Dependence of IPoT length on the temperature of the pre-treatment medium and the duration of the pre-treatment. Treatment: 4% sulfuric acid. Germination medium: 10% sucrose and 0,01% boric acid.

To examine the influence of concentration, pollen grains were treated after an optimal pretreatment of 20 minutes, with solutions of sulphuric acid ranging from 0.003 to 19.2% in concentration (*table 1*). Besides normal germination percentage and IPoT formed, incidence of instant tubes and formation of plasma threads were also calculated. Normal germination is completely halted by 0.01% sulphuric acid. Formation of IPoT begins at 0.05% concentration in medium and reaches its maximum at about 4%. In the range 0.003 to 0.13%, the acid affects some pollen grains by rupturing the intine and causing a spiral-like plasmatic thread to form (*fig. 7*). The diameter of the thread depends on the concentration, i.e. at higher acid concentrations, the threads are thicker. At

Table 1. Dependence of formation of Instant-Pollen-Tubes (IPoT) on the concentration of sulfuric acid.

Conc. H_2SO_4%	% ungerm. pollen	% germ. pollen	% IPoT	% ruptured IPoT	% pollen with plasm. threads
0.003	93.7 ± 2.3	4.1 ± 1.9			2.1 ± 1.2
0.005	78.0 ± 3.0	1.8 ± 0.8			20.2 ± 2.7
0.01	69.5 ± 4.2	0.0		7.2 ± 2.3	22.1 ± 4.0
0.03	66.8 ± 4.6			5.9 ± 3.2	26.9 ± 2.3
0.05	53.8 ± 5.1		4.1 ± 1.7	25.8 ± 1.8	36.3 ± 4.5
0.13	31.3 ± 4.6		9.1 ± 1.2	29.4 ± 3.9	30.3 ± 1.7
0.25	22.1 ± 6.1		15.8 ± 1.1	58.7 ± 3.9	3.2 ± 2.0
0.38	42.3 ± 4.9		28.3 ± 3.4	28.8 ± 3.8	0.0
0.50	47.2 ± 8.2		34.5 ± 2.9	18.3 ± 6.2	
0.75	45.6 ± 5.8		37.9 ± 3.5	16.4 ± 2.4	
1.0	41.0 ± 6.4		44.2 ± 6.0	14.8 ± 4.3	
1.5	43.6 ± 9.0		45.7 ± 5.8	10.6 ± 4.2	
2.0	41.1 ± 3.7		50.7 ± 3.1	8.1 ± 1.4	
3.0	38.0 ± 7.6		53.7 ± 5.3	8.3 ± 3.1	
4.0	30.2 ± 4.8		62.8 ± 4.9	0.0	
5.5	36.2 ± 4.3		63.8 ± 4.3		
7.2	35.2 ± 1.6		64.8 ± 1.6		
9.6	36.0 ± 4.8		64.0 ± 4.6		
14.4	32.6 ± 2.1		67.4 ± 1.5		
19.2	Total destruction of the pollengrains!				

concentrations higher than 6%, very often extrusions appear at all three colpi (*fig. 8*). At about 20% acid concentration, only total destruction of the pollen grains is observed.

3.4 The effect of other inorganic and organic acids on IPoT formation

Other inorganic acids are also able to induce IPoT formation (*table 2*), when added at proper concentrations. Size and pattern of IPoT are identical with those obtained after treatment with sulphuric acid. Alkaline treatment does not result in IPoT formation, but only in extensive destruction of the exine. Organic acids, so far as tested, did not cause significant IPoT formation, but these were comparatively weak acids.

Table 2. Effect of different trigger substances on the formation of IPoT. Optimal concentrations are given in the table. K_a: dissociation constant.

Trigger Substance	concentration	K_a	% IPoT
H_2SO_4	0,8 m	1	37,0 ± 3,1
HNO_3	0,81 m	1	74,3 ± 2,7
HCl	0,83 m	1	67,8 ± 1,6
$HClO_4$	0,8 m	1	56,5 ± 1,5
CCl_3COOH	0,8 m	$1{,}3.10^{-1}$	19,8 ± 3,4
HCOOH	0,8 m	$1{,}8.10^{-4}$	0,5 ± 0,3
CH_3COOH	0,8 m	$1{,}8.10^{-5}$	0,4 ± 0,2
Picric acid	0,8 m	$4.\ 10^{-1}$	1,6 ± 1,2
NaOH	0,78 m	1	0

3.5 Influence of acid treatment on non aperturate pollen

It was demonstrated that formation of IPoT takes place in many pollen species belonging to the aperturate type after a species-specific period of pretreatment prior to adding sulphuric acid. Thereafter we examined the influence of acid treatment on a non aperturate pollen, for which we selected *Codiaeum* (MÜLLER-STOLL 1956). Following application of sulphuric acid the exine ruptured and the grain content, still surrounded by the intine, was pressed out of the cracks (*fig. 9*). Formation of IPoT was not observed.

3.6 The presence of a wall around the IPoT

The regular form and size of the IPoT suggested the presence of a normal tube wall around them. Because the pollen tube wall normally consists of cellulose and pectin (MÜHLETHALER & LINSKENS 1953; MARTENS & WATERKEYN 1962; SASSEN 1964; VAN DER PLUIJM & LINSKENS 1966) specific colour reactions were employed. Colorations with chloride-zinc-iodine and iodine-iodine-potassium in 65% sulphuric acid, as well as with ruthenium red were positive. Checking the IPoT in a polarizing microscope showed a normal reaction between crossed nikols in the tube wall. Definitive evidence for the presence of a real wall around the IPoT could be given by electron microscope observation (*fig. 10*).

Fig. 1. Instant-Pollen-Tubes (IPoT) of *Petunia hybrida*. Petri-dish method. Pretreatment: 20 min in sucrose-boric acid solution. Treatment: 4% sulfuric acid. Magnification 1450×.

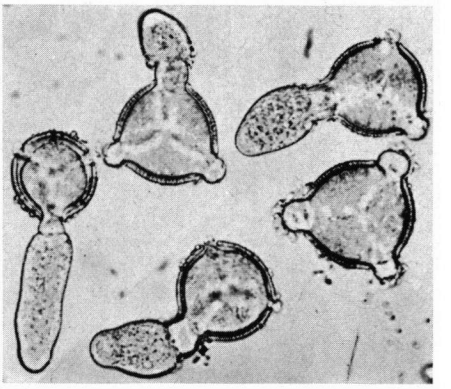
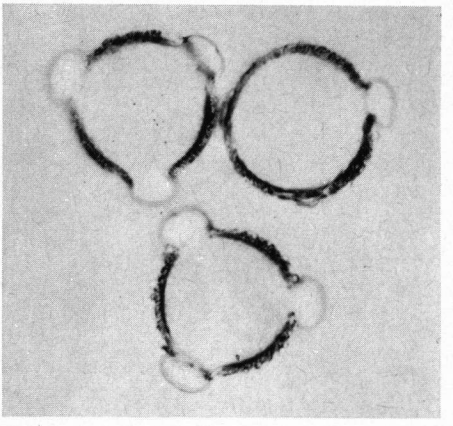
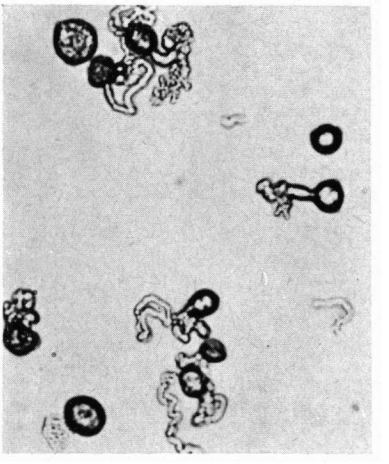


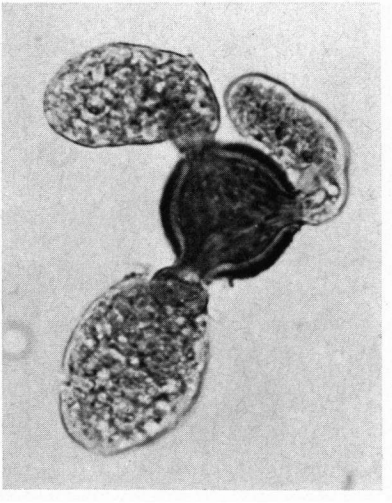
Fig. 6. Treatment of resting pollen (without pre-treatment) with 4% sulfuric acid: No IPoT formation. Magnification: 1450×.

Fig. 7. Formation of spiral-like plasmatic threads after treatment with 0,13% sulfuric acid. Pre-treatment 20 minutes, slide method.

Fig. 8. Formation of multiple extrusions in *Petunia* pollen. Pre-treatment 20 minutes sucrose-boric acid solution, treatment with 7,2% sulfuric acid. Magnification 1450×.







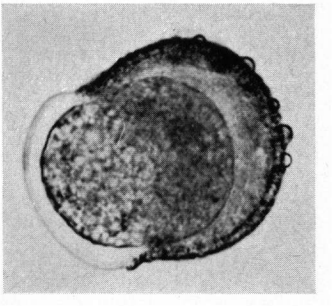


Fig. 9. Pollen grain content pressed out of the walls of a non-aperturate *Codiaeum* pollen. Pre-treatment: 30 minutes in 7,5% sucrose. Treatment: 4% sulfuric acid, slide method. Magnification 1450×.

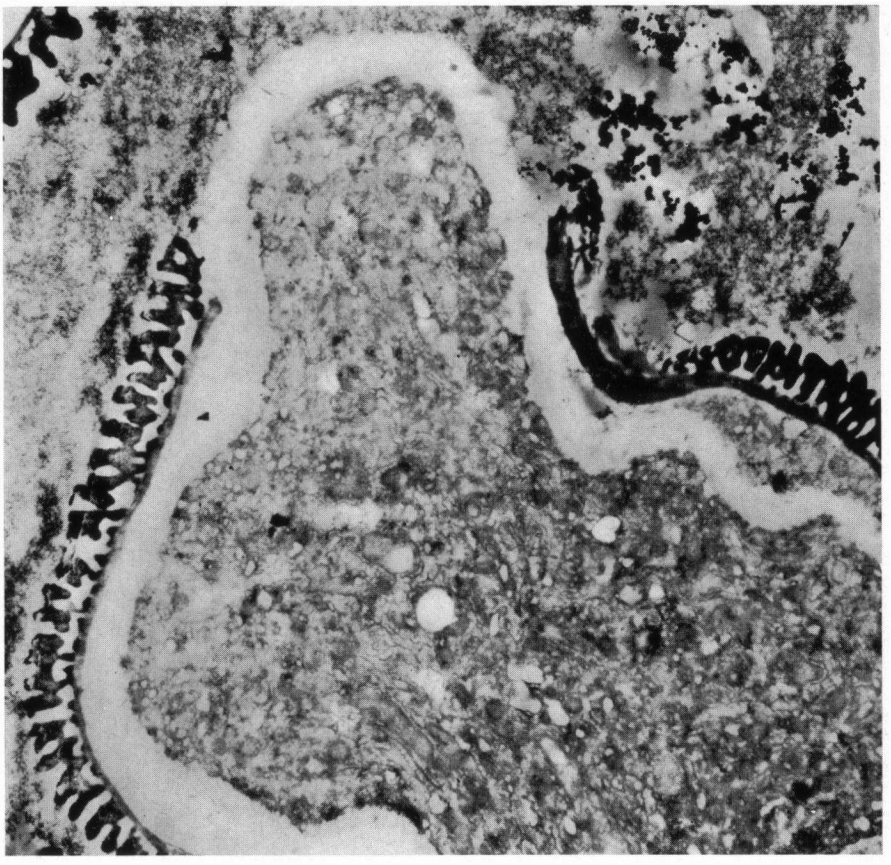


Fig. 10. Electron micrograph of a cross section through an IPoT of *Petunia*. Pre-treatment: 20 minutes sucrose-boric acid solution, beaker-method. Treatment: 4% sulfuric acid. Inbedding in methacrylate. Magnification 5300x. Foto taken by S. Reitsma.

Table 3. Influence of various cyanide concentrations on pollen germination and formation of IPoT.

KCN concentration	% "Instant pollen tubes"	Germination %
0.000 M. (control)	66.5 ± 1.7	69.3 ± 1.5
0.001 M.	18.6 ± 1.4	10.5 ± 1.6
0.002 M.	11.0 ± 1.4	1.8 ± 0.6
0.005 M.	2.6 ± 0.8	0.0

3.7 IPoT formation and oxygen consumption

It was shown by HELMERS & MACHLIS (1956) that CN^- ions inhibit oxygen uptake completely in germinating pollen. The dependence of IPoT formation on oxygen-requiring processes was investigated by adding cyanide to the pretreatment medium. As seen from *table 3*, increasing CN^--concentration inhibits normal germination; the formation of IPoT is also reduced while none are produced at a concentration of 0.006 M. Controlled oxygen uptake (Method: LINSKENS & SCHRAUWEN 1966) during pretreatment following by the acid treatment showed (*fig. 11*) that pollen, during activation in sucrose solution, consumes more oxygen than supplied by diffusion from the atmosphere. After application of sulphuric acid, oxygen tension increases to the level of the ungerminated pollen. That means that exhausted oxygen is quickly reversed after formation of IPoTs. It can be concluded that IPoTs are produced by the acid treatment and independent from the presence of oxygen. Control experiments with dead pollen show no change in oxygen tension in the medium, except for a short-lived peak after addition of acid, which is the consequence of the short heating of the medium during the mixing process.

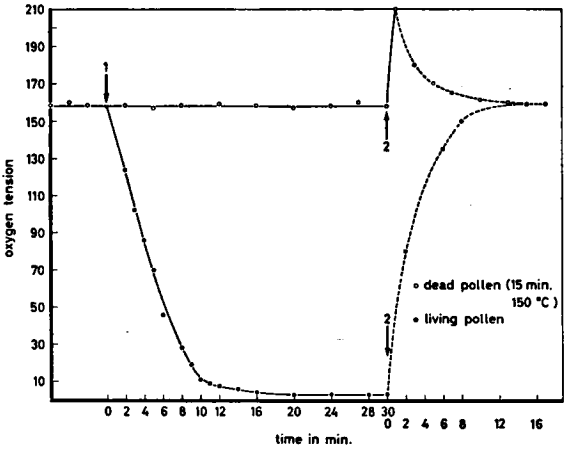


Fig. 11.
Changes in the oxygen tension in a sucrose-boric acid medium at 26°C, measured with a micro electrode (Method described by LINSKENS & SCHRAUWEN 1966) using the beaker method ↓1: addition of pollen grains to the pretreatment medium, ↓2: addition of sulphuric acid, above to the control medium, below to the germination medium with living pollen.

4. DISCUSSION

The observations presented here demonstrate that the formation of "Instant Pollen Tubes" (IPoT) requires a living pollen grain. Dead pollen do not give rise to IPoT and each treatment which inhibits normal metabolism also prevents IPoT formation. As a further prerequisite to IPoT formation, the living pollen grain must move from the resting stage into the active stage through swelling and enzyme activation. This transition needs only a few minutes (STANLEY & LINSKENS 1965) and reaches its optimum in *Petunia* after about 20 minutes. This demonstrates the swiftness of pollen grain activation under optimal conditions. The transition needed for IPoT formation seems to be the same as for induction of germination. All factors influencing germination which were examined, also influenced the transition to IPoT formation.

Furthermore, it was found that the concentration of the acid added after pretreatment determined in part the percentage of IPoT formed. The effect can be explained by a bipartite hypothesis. Due to low pH effects on the structure of the pollen grain, the ions can invade the grain immediately, resulting in an increase in the interior osmotic pressure. The tension on the pollen wall causes the grain to rupture at the sites of least resistance which are the germination pores in operculate pollen. As the intine is still rigid, the plasmatic contents are splashed out. Only when the intine is weakened by suitable pretreatment does a change occur which leads to IPoT formation. This instant tube is not identical with the normal pollen tube, but its wall has all the properties of the extending wall of the normal pollen tube.

A second effect of the added sulphuric acid may be to increase the extensibility of the intine. The intine consists of a network of cellulose microfibrils inbedded in a matrix of pectin (SITTE 1953). After hydrolysis of the matrix in strong acid, the microfibrils can be more easily moved. Thus, the intine can be stretched at the expense of the thickness of the existing wall.

The observation of plasmatic thread formation in the concentration range 0.003%–0.05% H_2SO_4 can be due to the low concentration of the acid which may be too weak to hydrolyse the matrix. The cytoplasm is then squashed out and coagulates immediately in the surrounding acid medium. It becomes rigid and quite visible under the microscope (HOFMEISTER 1956). When higher concentrations of sulphuric acid are employed, the instant tubes are the result, which consist of coagulated cytoplasm surrounded by the expanded intine, as seen from colour reactions and in electron micrographs. The wall present cannot be newly synthesized, as occurs, for example, in isolated plasmatic portions of living pollen tubes according to BOBILLIOFF-PREISSER (1917). As a consequence of the acid treatment, the grains are killed immediately, which is demonstrated by respiration measurements.

The shorter IPoT lengths found after shorter pretreatment times can be explained as a result of quick inactivation of synthetic processes. LARSEN (1965) has already demonstrated that synthesis of wall material is initiated with activation of the pollen grain. A longer period of pretreatment means, therefore, more intine material available for the formation of IPoT.

IPoT cannot be reactivated to normal growth. They are dead. Plasmatic streaming, as well as respiration, is stopped immediately. IPoT formation can therefore be defined as artificial germination in the sense of FRITZSCHE (1832). Formation of IPoT can only be demonstrated in aperturate pollen grains. In non-aperturate pollen, the exine tears off and the entire grain content is released into the medium, so that a tube-like structure is not formed.

The rediscovery of the "Instant Pollen Tubes" has brought to our attention a potential means of acquiring additional information on the intine, the arrangement of microfibrils in the pollen wall and the molecular transformations which are linked to the processes preceeding the formation of pollen tubes.

ZUSAMMENFASSUNG

Nach einer Vorbehandlung von einigen Minuten in Wasser oder Zuckerlösung können aperturate Pollen-Körner durch Behandlung mit starken organischen Säuren in Sekundenschnelle zur Bildung von "Schnell-Pollen-Schläuchen" gebracht werden. Die so produzierten Schläuche müssen als Ergebnis einer Pseudo-Keimung betrachtet werden. Voraussetzung dafür ist jedoch, dass der Pollen lebend war, und der Säurebehandlung eine gewisse Periode der Quellung und Enzym-Aktivierung vorausgeht. Die Bedingungen der Vorbehandlung und der Säure-Behandlung werden näher definiert.

Die Bildung von "Schnell-Pollen-Schläuchen" scheint eine geeignete Methode zu sein, um die molekulare Struktur der Intine und die Abhängigkeit der Pollenschlauch-Wand-Synthese von der Einleitung der Enzym-Aktivierung zu untersuchen.

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