THE RELEASE OF FREE AMINO ACIDS FROM GERMINATING POLLEN

H. F. LINSKENS and J. SCHRAUWEN

Botanisch Laboratorium, Universiteit, Nijmegen

SUMMARY

Synchronously germinating *Petunia* pollen in an aerated sucrose medium release free amino acids during the first minutes after contact with the aqueous medium. The release process between 1 minute and 6 hours follows an exponential equation. The content of free amino acids of the germinating pollen decreases sharply. Free amino acids set free from "dead" pollen reach their plateau after 40 minutes in the alcoholic extraction medium. Differences in the amino acid curves show that only a part of them is localized in the pollen wall, but the majority are peptide bound in the inner space of the microspore. Consideration of the data obtained by differential analysis of the amino acid curves suggests that amino acids and protein are synthesized in the early phase of pollen germination.

1. INTRODUCTION

Germination of pollen means the transition of resting microspores into active male gametophytes. This activation process is the first step of the progamic phase of fertilization (LINSKENS 1968). Germination normally occurs in nature when pollen grains come in contact with the surface of the stigma, where they take up water and swell. The activation of pollen can, to a certain degree, be imitated *in vitro*. *In vitro* germination experiments are, therefore, an important tool for molecular analysis of early activation processes.

It is well known that pollen activation starts with absorption of water from the environment. But immediately after this process starts, releasing processes begin in the other direction. The releasing of substances from the pollen can already be concluded from the population effect in germination (LINSKENS 1967b). Protein diffusion from germinating pollen was found by STANLEY & LINSKENS (1965). The new mass culture method, which enables synchronous start of germination for large quantities of pollen (SCHRAUWEN & LINSKENS 1967) made possible investigations on the release of free amino acids (F.A.A.) from germinating pollen. At the same time, determinations on the time axis of germination give some concept of the protein metabolism during the early phases of activation.

2. MATERIAL AND METHODS

Pollen grains from *Petunia hybrida*, clone W 166k (selfincompatible alleles S_1S_2) were collected on the day of anthesis from plants growing in greenhouses with artificial light (HPL Philips lamps). After 24 h drying in the dark at room

temperature, pollen was sifted out (sieve pores 0.4 × 0.4 mm) of the anther walls and stored at –20 °C for further use.

Germination took place in the mass culture vessel as described earlier (SCHRAUWEN & LINSKENS 1967) at 25 °C in a medium consisting of 10% sucrose and 0.01% boric acid. A concentration of 5 mg pollen in 1 ml of the germinating medium gave an optimal germination percentage. Contamination of the culture medium was controlled by microbiological tests.

Extraction of free amino acids (F.A.A.) was done with the following extraction medium (E.M.): 0.7 g citric acid and 10 ml thiodiglycol were mixed in 1000 ml 70% ethanol. Pollen was homogenized at 0 °C with quartzsand in a mortar and dispersed in 1 ml of the extraction medium for 2 minutes, and after centrifugation (7,000 g, 10 min) dispersed anew in 1 ml of extraction medium. The combined supernatants were shaken with 6 ml chloroform and after centrifugation (1000 g, 5 min) the water phase was used for analysis. As an internal standard 0.15 μmol norleucine was added to each sample before extraction[1]. The germination medium was treated in an analogous manner, but the extraction liquid was adjusted, so that the end concentration was 70% alcohol. "Dead" pollen means ungerminated pollen dispersed in E.M. The amides asparagine and glutamine were converted to their respective amino acids by a weak hydrolysis (2 hr, 100 °C, 1 N HCl). In the method used, the amides cannot be estimated.

Determination of the total amino acids (T.A.A.) in pollen was done after hydrolysis of 1 mg pollen with 0.4 ml of 6 N HCl and the addition of 0.15 μmol norleucine. The suspension was sucked into a pyrex tube and after freezing in liquid nitrogen the tube was evacuated and sealed. After hydrolysis of 24 h at 105 °C in the dark, the suspension was centrifuged and the supernatant concentrated twice in a rotating vacuum evaporator. The dry residue was used for analysis.

Amino acids determination was performed by the automatic amino acid analyser (Canalco) as described earlier (LINSKENS & TUPÝ 1966). All values are calculated for 1 mg pollen.

For *RNA and protein determination*, 50 mg of pollen was homogenized for 2 min in a cooled (0 °C) glass homogenizer with 0.2 ml buffer and 50 mg Alcoa powder and then mixed in 1 ml buffer for 5 min and centrifuged (10 min 12,000 × g, 0 °C). The pellet was washed with 1 ml buffer and centrifuged. The residue contains no estimable protein.

To the combined supernatants was added 1 ml of 20% TCA; after standing for 30 min at 0 °C, it was centrifuged (10 min 5,000 × g, 0 °C). The pellet was hydrolysed for 1 h at 37 °C in 0.5 ml 1 N NaOH, and the protein precipitated by adding 0.5 ml 2 N TCA (30 min, 0 °C). A pellet was obtained by centrifuging (10 min, 5,000 × g, 0 °C), which was used for protein estimation according to Lowrey. The supernatant was used to measure RNA content at E_{260} (1 mg RNA/ml corresponding with an absorbance value of 25).

Diffusion rate of free amino acids from germinating pollen was measured by

[1] This successful procedure was suggested by Mr. M. G. J. Buis.

dispersing the pollen in germination medium. At various time intervals, samples of pollen suspension were removed and the medium was sucked out through a metal sieve. The F.A.A. was estimated from these pollen samples and from their corresponding medium samples. All experiments were performed in duplicate, at least.

3. RESULTS

About 25% of the dry weight of ungerminated *Petunia* pollen is amino acids (calculated from *table 3*, column 6), partly free (6%) (calculated from *table 3*, column 4), partly protein bound (13%) (calculated from *fig. 5*), and peptide bound (about 6%).

Comparing living and "dead" pollen it can be seen in both cases that in the first minute after contact with the germination- or extraction medium high amounts of free amino acids (F.A.A.) are found in the environment (*fig. 1*). In the first 10 min, discharge from "dead" pollen in the alcoholic extraction medium is much faster than from germinating pollen in the germination medium. At the same time, there is a large decrease in the concentration of F.A.A. remaining in the germinating pollen, the decrease of the remaining F.A.A. goes uniformly after this time (*fig. 2*). The difference of release between "dead" and germinating pollen is shown also clearly (*fig. 2*). From "dead" pollen there is an output during the first 40 min, after this time no increase could be observed in the surrounding extraction medium. In contrast to this, *fig. 2* shows a continuous increase of the F.A.A. content in the germination medium. The different curves in *fig. 2* closely fit the equation $y = a + b \log t$ in which t is the time in seconds.

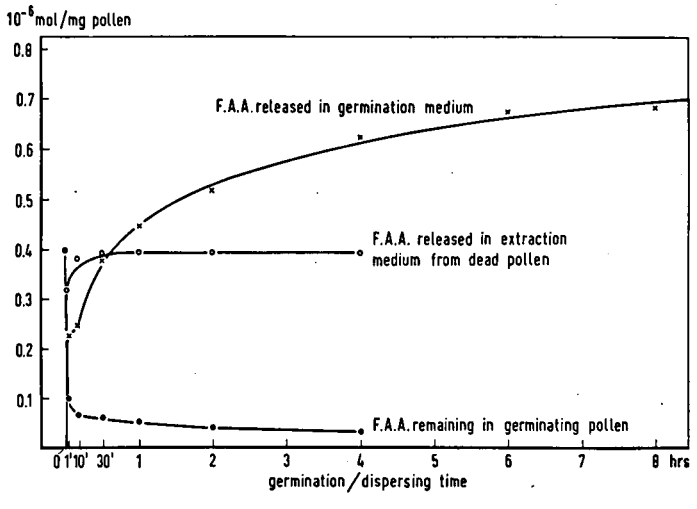

Fig. 1. Release of free amino acids (F.A.A.) from living and "dead" pollen with a linear time axis.

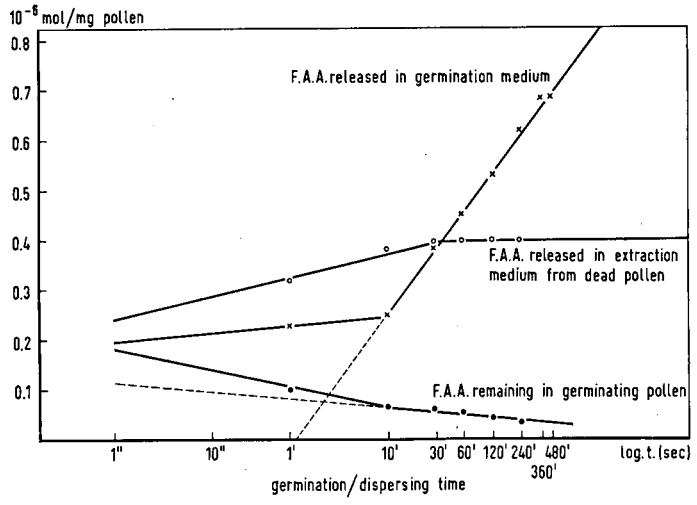

Fig. 2. Release of free amino acids (F.A.A.) from living and "dead" pollen with a logarithmical time axis.

A. For F.A.A. from germinating pollen in germination medium during the first 10 min a = 195; b = 17.
B. After 10 min for F.A.A. from germinating pollen in germination medium, a = –483; b = 264.
C. For F.A.A. from "dead" pollen in extraction medium, during the first 30 min a = 239; b = 48.

The results from these equations are, that:

1. Within 1 sec F.A.A. are present in the surrounding medium
   (In A. $y = 195 + 17 \log t$; $y = 0$ for $t = 10^{-11}$ sec)
   (In C. $y = 239 + 48 \log t$; $y = 0$ for $t = 10^{-5}$ sec)
2. A second release and different from the beginning starts at 1 min in germinating pollen. In B. $y = -483 + 264 \log t$; $y = 0$ for $t = 69$ sec.
3. All the F.A.A. have left the "dead" pollen within 40 min. In C. $y = 239 + 481 \log t$; $y = 400$ for $t = 2240$ sec.

The divergence between the released and the remaining amount of F.A.A. (*fig. 1*) both of which should be complementary, if there is no metabolic activity, can be explained by *table 1* and *fig. 3*. From these it can be seen that intact, germinating pollen shows a net synthesis of total amino acids (column 5). During this time of synthesis a diffusion of free amino acids is going on. After 10 minutes (during the first 2 hours) of germination, almost a linear increase of T.A.A. is observed (*fig.* 3). After 2 hours germination, the amount of T.A.A. decreases again.

Looking at the detailed patterns for the individual amino acids (*fig. 4*) we see the same tendency, except for glycine, which increases also after 2 hours germination.

Table 1. Amino acid concentration in n mol/mg pollen.

germination time	T.A.A. prot.(pept.+ A.A.) in pollen	F.A.A. in pollen	F.A.A. in germination medium	T.A.A. prot.(pept.+ A.A.) in pollen +germ. medium	A.A. from prot.+ pept. in pollen
1	2	3	4	5	6
0	2560	400	0	2560	2160
1 min.	2195	100	225	2420	2095
10 min.	2055	55	250	2305	1990
30 min.	2375	60	380	2755	2315
1 hr	2675	55	450	3100	2620
2 hrs	3510	40	520	4030	3470
4 hrs	2860	30	620	3480	2830
6 hrs			670		

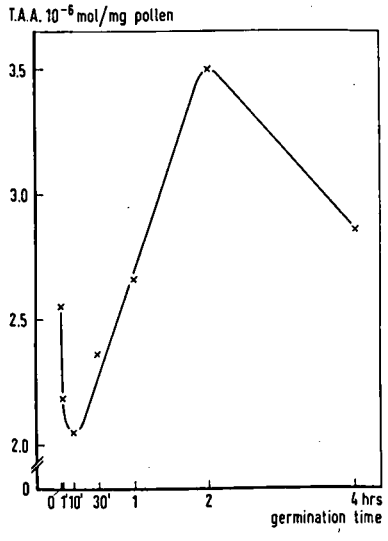

Fig. 3. Total amino acid (T.A.A.) content in the pollen grains during germination in liquid medium.

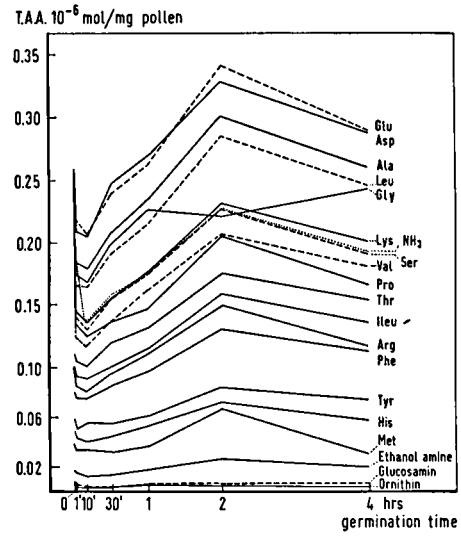

Fig. 4. Content of the individual amino acids in pollen grains during the germination phase.

The absolute amount of proline, in agreement with earlier information in literature, is very high: the total amount of free proline in non-germinated pollen is 230 nmol (= 2.6%) with only 26 nmol peptide bound; this is a small amount in comparison to the high content of the free proline (*table 3*). It is different in regard to the other amino acids present and the ratio F.A.A.: peptide bound amino acid shows this very nicely (*table 3*, column 4 and 5). This ratio is in non-germinated pollen for proline = 88, for threonine = 0.5 and for all other amino acids between 0,01 and 0,1.

Another remarkable result is shown in *table 3*. Column 8 shows the amount of

Table 2. Release of F.A.A. from germinating pollen in germination medium. Amino acid concentration in n mol/mg pollen.

A.A.	1 min.	10 min.	30 min.	1 hr	2 hrs	6 hrs	8 hrs
Asp	4.2	4.8	9.4	11.5	11.9	11.7	6.7
Thr	15.6	16.4	28.5	32.3	38.4	44.7	37.5
Ser	10.3	10.1	16.9	27.4	26.8	28.7	26.8
Glu	17.1	19.4	30.2	32.8	40.9	33.6	15.7
Pro	127	141	218	230	285	330	330
Gly	5.8	5.4	8.8	16.2	9.4	16.9	19.2
Ala	18.6	24.5	31.6	35.3	34.8	36.4	36.6
Val	2.8	2.8	4.4	6.2	6.0	12.8	15.9
Ileu	1.3	1.2	1.7	2.7	2.8	6.3	7.2
Leu	1.1	1.0	1.5	2.7	3.3	11.3	14.6
Tyr	0.8	0.7	1.4	2.4	2.2	7.4	8.7
Phe	0.6	0.5	0.8	1.4	2.3	8.0	9.3
Eth NH_2	0.7	0.8	1.7	2.3	9.7	20.5	23.5
γ NH_2 but.	1.1	1.7	2.8	2.6	6.2	38.7	60.6
NH_3	16.5	11.3	19.2	29.2	31	30	24
Orn	1.3	1.0	2.1	4.4			
Lys	1.0	1.0	1.6	2.6	4.4	17.7	22.5
His	1.4	1.4	2.5	3.5	3.8	9.7	10.5
Arg	0.7	0.6	1.1	1.7	3.3	11.2	12.4
Total	228	246	384	447	522	676	682

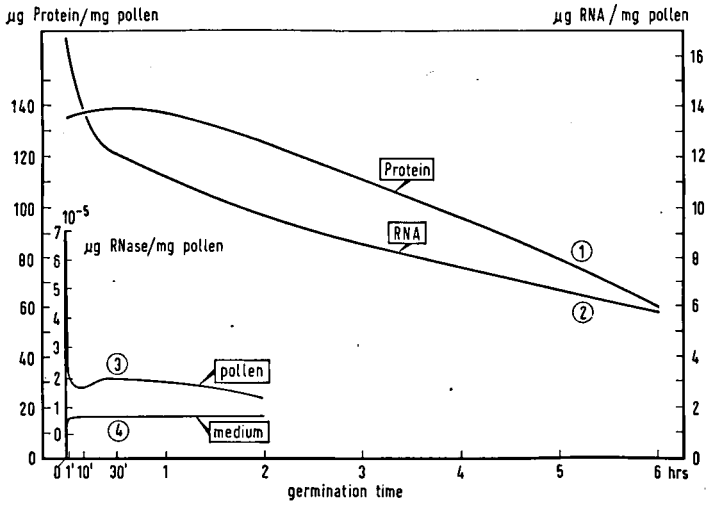

Fig. 5. Changes of the protein and RNA content in pollen during germination *in vitro*, as well as the RNase activity (measured, after 30 min incubation, the difference from $E_{260,\ 37°C} - E_{260,\ 0°C}$ and comparing with a standard).

Table 3. Amino acid concentration in nmol/mg pollen.

	4 hrs germinated			non germinated					F.A.A./T.A.A. from 4 hrs germ.: 1 min germ.
	F.A.A. in medium	T.A.A. in pollen	(1) + (2)	F.A.A. in pollen	Pept. A.A. in pollen	T.A.A. in pollen	(3) – (6)	(7) : (5)	
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
Asp.	11.8	285	297	7	250	257	40	0.16	1.4
Thr.	40	153	193	39	72	111	82	1.14	0.8
Ser.	27	190	217	12	133	145	72	0.54	1.7
Glu.	38	290	328	45	213	258	70	0.33	0.8
Pro.	309	165	474	230	26	256	218	8.40	2.1
Gly.	17	243	260	6	184	190	70	0.38	1.7
Ala.	35	259	294	29	179	208	86	0.48	1.0
Val.	10	180	190	5	130	135	55	0.42	1.5
Ileu.	3	137	140	2	101	103	37	0.37	1.6
Leu.	10	243	253	1	177	178	75	0.42	4.3
Tyr.	5	73	78	1	56	57	17	0.30	3.0
NH_3	30	194	224	10	204	214	10	0.50	3.4
Phe.	6	112	118	1	77	78	40	0.52	4.2
Lys.	15	200	215	2	147	149	66	0.45	4.3
His.	7	58	65	3	44	47	18	0.41	1.7
Arg.	8	117	125	7	91	98	27	0.30	1.0

amino acids formed during 4 hours of germination in relation to their peptide bound amino acids in non-germinated pollen. Whereas most ratios have the same magnitude, threonine and especially proline are notable exceptions.

For the evaluation of the data presented it is important also to have some information on the changes of protein and RNA content during the investigated time interval. *Fig. 5* shows that the protein content (curve 1) in the germinating pollen remains more or less constant during the first 2 hours, decreasing then continuously. The RNA content (curve 2) diminishes rapidly during the initial phase of germination, followed by a continuous decrease comparable to that of the protein content.

4. DISCUSSION OF RESULTS

The results reported here confirm that germinating pollen freely releases amino acids which was observed earlier for enzymes and proteins (STANLEY & LINSKENS 1965).

Release of amino acids from the pollen takes place very quickly. During the first minute, about 50% of the free amino acids have left the living pollen grain (*fig. 2*); pollen dispersed in the extraction medium discharge during 60 seconds 80% (*fig. 2*) of its free amino acids. This means that the pollen wall has to be extremely pervious to these compounds. The different surrounding liquid is the reason that both plots are not the same at the beginning. The amino acids go more rapidly in solution in the acid extraction medium.

The first part of both curves gives information about the amount of amino acids which will be present in the wall of the pollen grain. From 1 mg of the germinating pollen within 1 sec about 200 nmol F.A.A. is present in the medium. It is almost impossible that these free amino acids were situated inside the wall, because the time necessary for water uptake by the pollen is more than 1 sec. The conclusion is that the amount of F.A.A. which is present inside the pollen grain and in the space of the exine structure is about the same (200 nmol). This information suggests that it is also the F.A.A. concentration after the microspore tetrad stage.

The large content of free proline in the pollen and the relatively high amount of proline synthesized during the germination process (*table 3*, column 8) suggests an important function of this amino acid (BRITIKOV *c.s.* 1964; ROSEN 1968).

It is possible to get information about the velocity with which the different amino acids diffuse out of the pollen. This can be obtained when the ratios F.A.A. (in the medium): T.A.A. (in the pollen) for 4 hours germination are divided by the values for 1 min germination (*table 3*, column 9). The results obtained in this way showed that the basic amino acids come out slower. The explanation for this phenomenon can be the absorption of the basic compounds on the acid exine layer.

The calculated difference between released and remaining F.A.A. (75 nmol) after 1 min germination may be a loss due to respiration.

During the first 2 hours of *in vitro* germination there must be strong amino acid and/or protein synthesis (*fig. 3*). Production between 10 minutes and 2 hours is almost 1.7 μmol. This means:

1. that the synthesized material has a low molecular weight. We do not see such increase in *fig. 5*,
2. that nearly 5 times more material is synthesized than can diffuse (300 nmol) out of pollen (*fig. 2*). Glutamic acid with its important position in the transamination cycle has apparently (*fig. 4*) a central function.

Fig. 5 shows a very small net increase of protein during the first 30 minutes only. Surprising is the increase of the T.A.A. in the germinating pollen grain (*fig. 3*) between 10 minutes and 2 hours, while the protein content drops after ½ hour. But there seems to be a correlation with the germination pattern: at 2 hours after beginning of activation, maximal pollen germination is reached: no more pollen grains are producing germination tubes, only pollen tube elongation continues to take place. After 2 hours, no fundamental changes in the kinetic processes are observed. That means no more enzymes are synthesized. This can be confirmed by the observation (LINSKENS 1967a), that the ribosomal pattern after 2 hours germination *in vitro* breaks down. Protein synthesis 2 hours after germination decreases, whereas there seems to be no net synthesis of RNA (*fig. 5*); RNA content decreases constantly. This can be explained, especially in the beginning, by the high activity of RNase in the pollen grains.

The complete lack of primary nitrogen supply in the germination medium, which only delivers energy, could be responsible for the depression of growth *in vitro*. The medium used must be considered the limiting factor to pollen tube growth.

As seen from *table 1*, column 5 the increase in T.A.A. is 4 — 2.3 = 1.7 μmol, that means 1.7 × 14 μg N_2 = 24 μg N_2. It can be concluded that in the pollen grain much more than 25 μg (that is only 2.5% of the dry weight) of non diffusible nitrogen compounds are present. The question arises, where this nitrogen is coming from.

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