NITROGEN METABOLISM ASSOCIATED WITH POLLEN GRAIN GERMINATION

YING-TZU LIN, TEH-YUAN CHOW[1] and CHIU-YUNG LIN[2]

Abstract: The germination of pollen grains collected from a long period blooming flower (Lily, *Lilium longiflorum*) and a short period blooming flower (Dish-Cloth Gourd, *Luffa cylindrica*) was studied. It was found that the germination pattern of the two plants was quite different.

The protein content in germinating pollen of lily increased steadily and reached a maximum in 90 minutes and then decreased. In contrast, gourd pollen had a larger amount of protein content before germination and decreased gradually during germination. It seems that for pollen germination, the lily required the synthesis of some new proteins but the gourd did not. The hypothesis was further proved by the cycloheximide inhibition test for protein synthesis. When cycloheximide was added to the germinating pollen at the beginning, lily pollen tube elongation was entirely inhibited, whereas gourd tube elongation continued. From the pattern of protein metabolism during germination, the mechanism for the initiation of the tube elongation of these two kinds of pollen may be different. RNA metabolism and free amino acids content were determined. Both the lily and gourd pollen synthesized new RNA for tube elongation. During germination, free amino acids tended to increase.

Based on the cycloheximide inhibition test, the germination patterns of eleven species of pollen grains could be separated into two groups, one was similar to the gourd type and the other belonged to the lily type.

INTRODUCTION

Early studies on pollen grain germination in vitro were based on the availability of tracer sugars as was reported by Ökelley[19]. He checked the respiration of pollen tubes by applying uniformly labelled sucrose-C^{14}, glucose-C^{14} and fructose-C^{14} as sole carbon sources and measured the specific activity of CO_2. He found that 36% of the CO_2 came from glucose, 66% came from fructose and 72% came from sucrose. For pollen grain germination and pollen tube elongation, calcium and boron[23,25] were found to be necessary. An optimal condition for lily pollen germination was developed by Dickinson[4] and he also indicated that intact pollen grains possessed a β-fructofuranosidase external to the cell membrane which hydrolyzed sucrose in the medium.

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Owing to its simple structure and active metabolism during germination, pollen is usually considered a good material for physiological study. For carbohydrate metabolism, Dickinson[6] found that several endogenous sugars could be reversed to starch, Larson and Lewis[15] examined pollen under the electron microscope and found that the enclosed substance was utilized for tube and pollen wall formation. Hellmer and Machis[9] noticed that pollen grains can metabolize a variety of mono-, di- and trisaccharides from the outside medium, they are then resynthesized and became polysaccharides. Utilization of exogenous sugars in biosynthesis of carbohydrates in germinating pollen has been indicated by Kessler[11], Kroch and Loewus[14] when labelled sugars were fed. Johri and Vasil[10] suggested that the presence of amylase in pollen can both digest and synthesize starch by taking sugar from the nutrient medium. Dickinson[6] presumed that the enzyme might be either starch phosphorylase or ADP-glucose pyrophosphorylase instead of amylase.

In nitrogen metabolism, Mascarenhas[17] using *Tradescantia* sp. and ^{3}H-uridine as materials concluded that RNA synthesis is necessary for tube growth, but he did not make a systematic study of the amino acids and protein in quantity.

The purpose of this experiment was primarily to find the optimal conditions for pollen tube growth, and to observe the effect of various protein and RNA synthesized inhibitors on pollen germination, protein and RNA synthesis and to get a further understanding of the relationship between the quantity of nitrogen metabolism and pollen germination.

MATERIALS AND METHODS

1. Collection and storage.

Anthers of lily were taken in the morning from freshly opened flowers of *Lilium longiflorum* Thunb. between the months of January and May. The anthers were placed in a desiccator at room temperature for 24 hrs., and the pollen was then collected and stored at 4°C for a maximum of 7 days. The pollen of the Dish-Cloth Gourd (*Luffa cylindrica* Raem.) was collected at night, between 6 and 7 pm from May to September, and was treated as above. Pollen of other plants was collected in June and used immediately.

2. Condition for germination.

For both lily and gourd pollen germination, a general culture medium was used as defined by Dickinson[4] and modified by others[1,23], which consists of 1.27 mM Ca $(NO_3)_2$, 0.162 mM H_3BO_3, 0.990 mM KNO_3, 10μg/ml tetracycline, 3.0 mM KH_2PO_4 (which should be omitted for gourd pollen) and 0.29 M of any one kind of carbon source which may be sucrose, glucose or pentaerythritol. The optimal pH and temp. for germination was pH 5.2 at 30°C for lily and pH 6.0 at 32° for the gourd. Culture medium for other pollen germination was that suggested by Brewbaker and Kwach[1], containing 10% sucrose, 100 ppm $MgSO_4$, 100 ppm H_3BO_3, 300 ppm $Ca(NO_3)_2 \cdot 4H_2O$,

200 ppm $MgSO_4 \cdot 7H_2O$. The pH was adjusted to 5.0 and maintained at 30°C.

3. Germination percentage and average tube length.

Pollen was removed from a shaking culture at desired intervals, and fixed with a drop of 3% Lugole's solution (I_2KI) on a slide, they were then examined under a microscope. The germinating percentage was determined on the basis of 140–200 pollen grains. The average tube length was obtained from 45–100 pollen tubes with the aid of a micrometer. The hanging drop technique was applied for other varieties of pollen.

4. Chemical analysis.

10 mg (for amino acid analysis) or 30 mg (for protein or RNA analysis) of pollen was placed in a 25 ml flask containing 10 ml of culture medium. Samples were then taken for each analysis. The quantity of free amino acid was determined by the Moore and Stein modified Ninhydrin method[18]. The content of protein was measured by Lowry's method. RNA was extracted according to Smillie and Krotkov[22], and the amount was measured by the Key and Shannon[12] method.

RESULTS

1. Conditions for pollen germination.

The optimal condition for the germination of pollen is different for various plants, Table 1 shows that the optimal pH value for lily pollen germination is pH 5.2 but for gourd pollen is pH 6.0. The effects of temperature seems to be related to the blooming season, the blooming season of the gourd is in the summer while that of lily is in spring, therefore, the optimal temperature for gourd germation is 32°C, or slightly higher than that for the lily (30°C), Hellmer and Machilis[9] proved that the optimal temperature for the germination of pine pollen is about 25°C. Ca^{++} and BO_3^{---} are generally important for tube elongation.

Table 1. The effects of pH on germination percentage and tube elongation. The sample was incubated under optimal conditions for 90 minutes.

pH	% germination		tube length (mm)	
	Lilium longiflorum	*Luffa cylindrica*	*Lilium longiflorum*	*Luffa cylindrica*
3	0.0	0.0	0.0	0.0
4	74.0	88.0	0.3675	0.5722
5	75.1	89.2	0.5000	0.6758
6	75.0	88.5	0.1610	1.0792
7	0.0	89.1	0.0	0.8600
8	—	87.3	—	0.7384
9	—	72.5	—	0.3834
10	—	40.8	—	0.2130

It seems that sucrose is one of the best sugars for the germination of lily and gourd pollen (Fig. 1, 2). When the germinating pattern of these two plants are compared as seen in Fig. 3 and 10 they show a difference in the lag phase. For lily pollen the lag phase is 30 minutes while that of gourd pollen is less than 5 minutes.

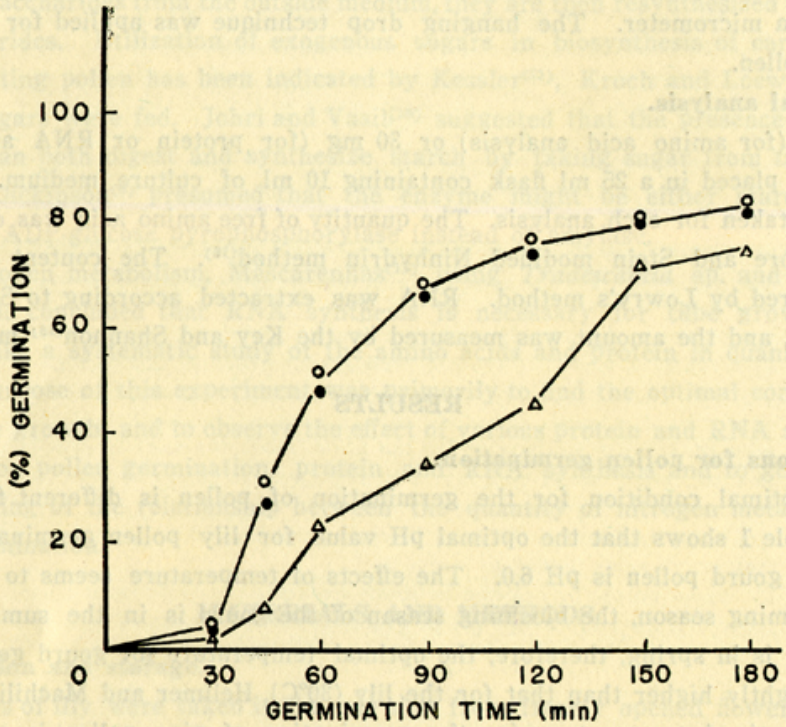

Fig. 1. The effect of sugars on germination percentage of lily pollen. Each value is the average of 3 experiments with 200 pollen grains as a basis for each experiment. -○- 0.29 M sucrose; -●- 0.29 M glucose; -△- 0.29 M pentaerythritol.

2. Amino acid content in germinating pollen.

When 10 mg of pollen is extracted with 80% of hot alcohol and the amount of amino acid determined by the Moore and Stein method[18]. Fig. 4 shows that the the free amino acid of the gourd is three times as much as that of the lily before germination, and the amount of amino acid increases gradually for both the lily and gourd after germination.

3. Protein content in germinating pollen.

When the protein content of ungerminated pollen of the lily and gourd were compared, lily contains 11%, and increases steadily until 90 minutes after germination, it then decreases again. On the contrary, gourd pollen contains a larger amount of protein which is about 34% and decreases to 24.5% in a duration of three hours (Fig. 6).

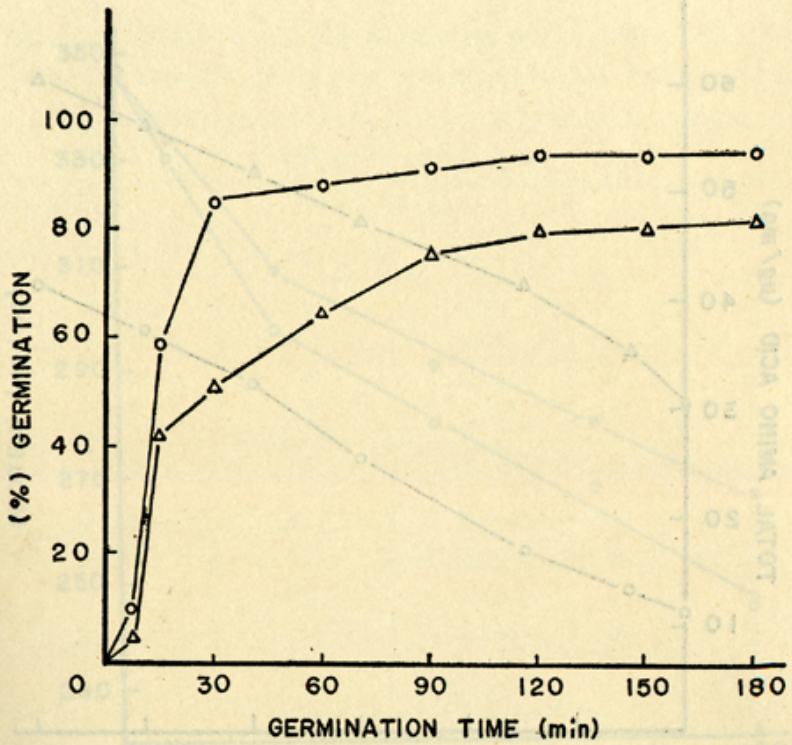

Fig. 2. The effect of sugars on germination percentage of gourd pollen. The method as Fig. 1. -○- 0.29 M sucrose; -△- 0.29 M pentaerythritol.

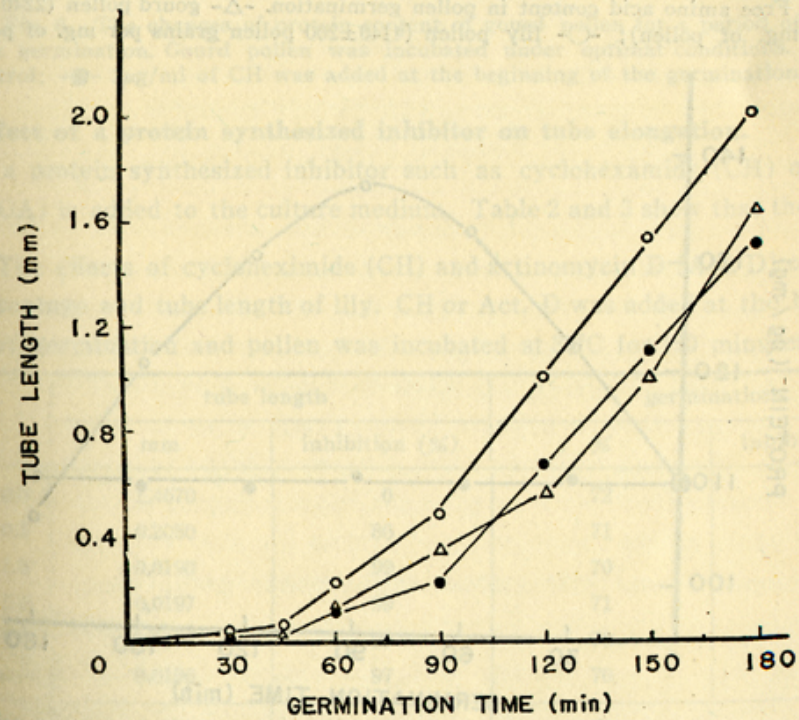

Fig. 3. The relationship between sugars and lily tube length. Each value was the average of 100 pollen tubes. -○- 0.29 M sucrose; -●- 0.29 M glucose; -△- 0.29 M pentaerythritol.

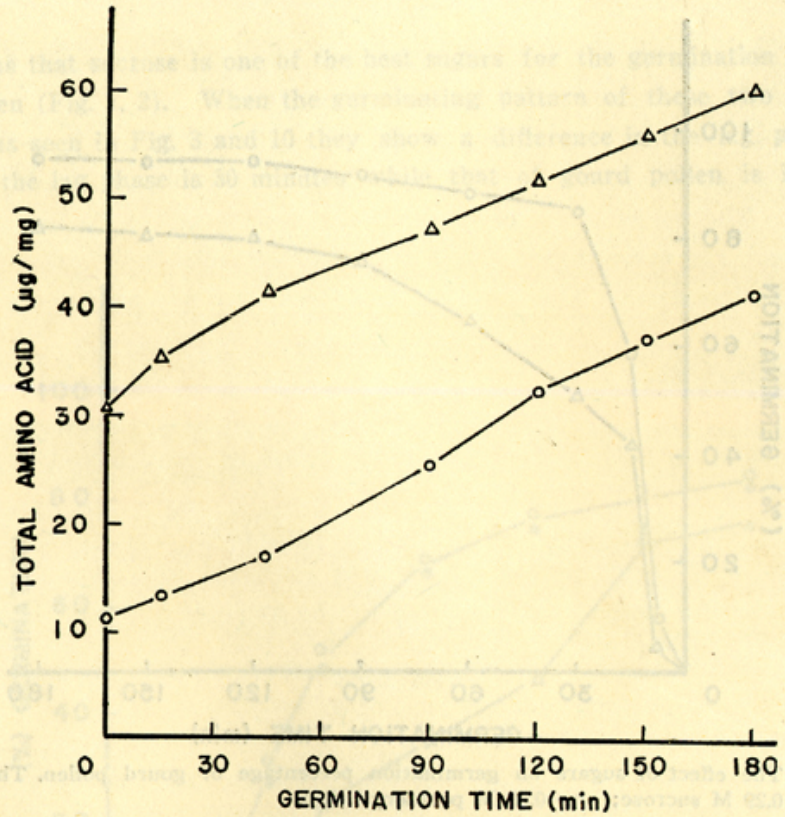

Fig. 4. Free amino acid content in pollen germination. -△- gourd pollen (2240±120 pollen grains per mg. of pollen); -○- lily pollen (4140±200 pollen grains per mg. of pollen).

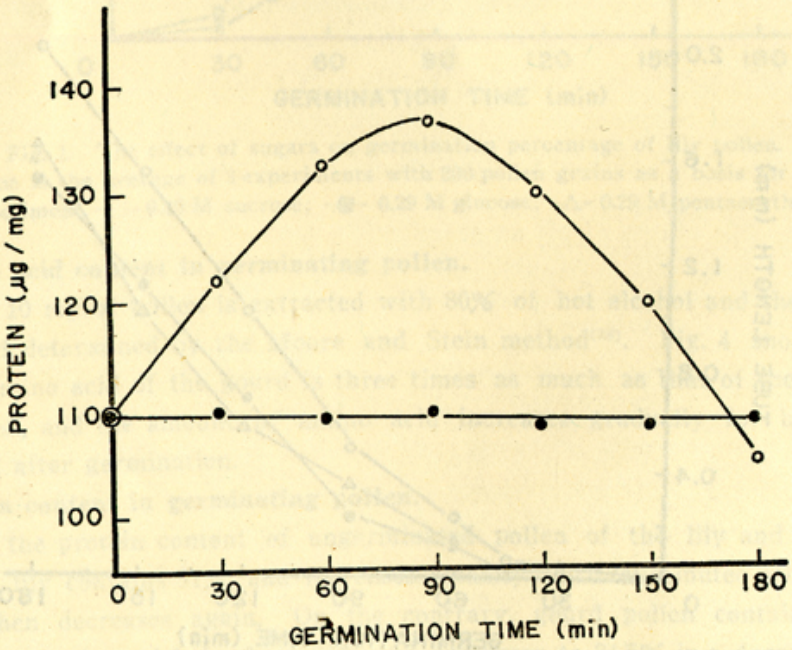

Fig. 5. The changes of protein content of lily pollen for a period of 180 min. germination. Lily pollen was incubated at optimal conditions. -○- control; -●- 2 μg/ml of CH was added at the beginning of the germination.

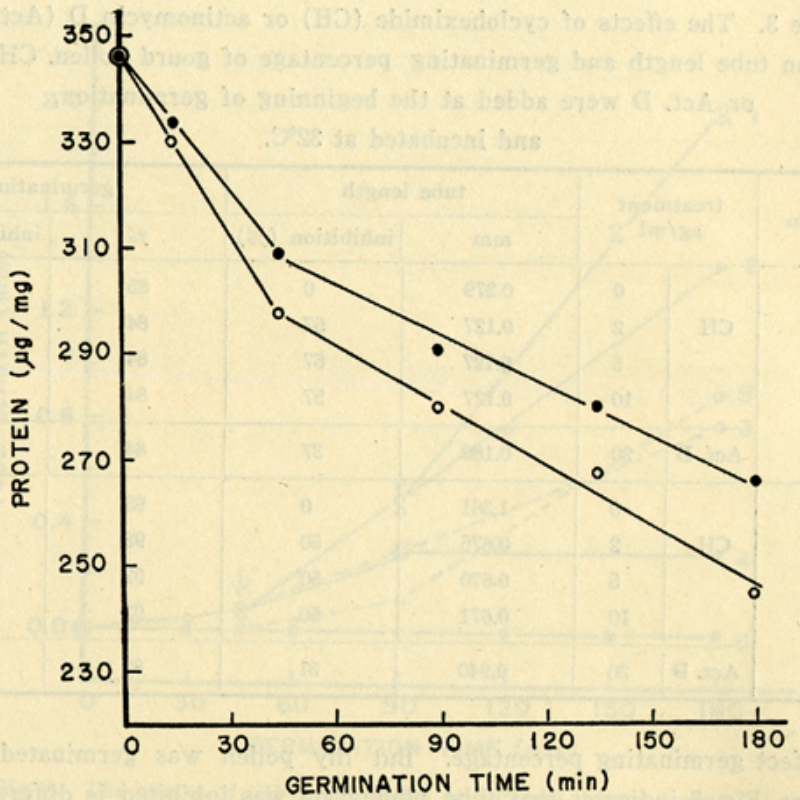

Fig. 6. The changes of protein content of gourd pollen for a period of 180 min. germination. Gourd pollen was incubated under optimal conditions. -○- control; -●- 2μg/ml of CH was added at the beginning of the germination.

4. The effect of a protein synthesized inhibitor on tube elongation.

When a protein synthesized inhibitor such as cyclohexamide (CH) or chloramphenical (CA) is added to the culture medium, Table 2 and 3 show that the inhibitors

Table 2. The effects of cycloheximide (CH) and actinomycin D (Act. D) on germination percentage and tube length of lily. CH or Act. D was added at the beginning of germination and pollen was incubated at 30°C for 150 minutes.

μg/ml		tube length		germination	
		mm	inhibition (%)	%	inhibition (%)
CH	0.0	1.4670	0	72	0
	0.1	0.2050	86	71	< 1
	1.0	0.0190	99	70	< 2
	2.0	0.0197	99	71	< 1
	5.0	0.0197	99	70	< 2
	10.0	0.0196	97	70	< 2
Act. D	20.0	1.1600	21	70	< 2

Table 3. The effects of cycloheximide (CH) or actinomycin D (Act. D) on tube length and germinating percentage of gourd pollen. CH or Act. D were added at the beginning of germination, and incubated at 32°C.

time of germination (min)	treatment μg/ml		tube length		germination	
			mm	inhibition (%)	%	inhibition (%)
30	CH	0	0.279	0	85	0
		2	0.127	57	84	< 1
		5	0.127	57	84	< 1
		10	0.127	57	84	< 1
	Act. D	20	0.180	37	84	< 1
120	CH	0	1.361	0	93	< 1
		2	0.676	50	92	< 1
		5	0.670	50	92	< 1
		10	0.671	50	92	< 1
	Act. D	20	0.940	31	92	< 1

did not affect germinating percentage. But lily pollen was germinated in CH for 150 minutes, Fig. 7 indicates that tube elongation was inhibited is different degrees by various concentrations of CH. When CH was added at various stages, Fig. 8 shows that CH inhibited the tube elongation of lily pollen in early stages (0–90′) rather than at latter stages. It seems that protein synthesis is necessary for tube elongation. Since gourd pollen possessed enough protein for tube elongation, tube elongation continued even though CH was added at an early stage (Fig. 9 and 10). If CH is replaced by CA, Fig. 8 shows that it had no effect on lily tube elongation even after 3 hrs. of treatment. Table 4 shows that in general, tube elongation of flowers with short life (less than one day) such as *Luffa cylindrica* Raem., *Momordica charantia* Linn., *Arachis hypogaca* Linn., *Duranta repens* Linn. or *Tradescantia* sp. were not affected by CH (10 μg/ml) whereas, flowers with longer life (longer than two days) such as *Lilium longiflorum* Thunb., *Erythrina corallodendron* Linn., *Carica papaya* Linn., *Allamanda cathartica* Linn., *Crinum asiatica* var. *sinicum* Bak., and *Zephyranthes carinata* Herb. were sensitive to CH and sometimes the inhibition reached 95%. This again proves the necessity of protein for tube elongation.

5. The changes of total RNA content during pollen germination.

RNA content of lily pollen during germination increased steadily for 90 minutes and then increased at a slower rate (Fig. 11). For gourd pollen (Fig. 12), the RNA content increased rapidly during the first 15 minutes but after germination it maintained the same level even when incubated for a longer period.

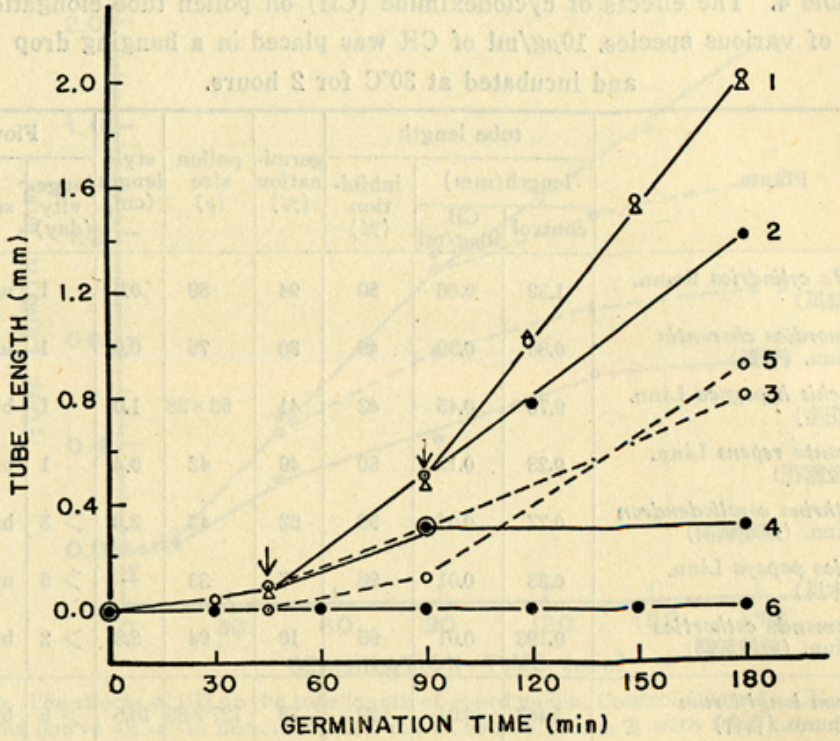

Fig. 8. The effects of CH or CA added at different stages during lily tube elongation. Sample was removed from treated medium at desired time and washed with normal culture medium and incubated for a total period of 180 min.. 1. control (-○-) and CA treatment (-△-); 2. CH was added and incubated 90-180 min.; 3. CH was added and incubated 45-90 min.; 4. CH was added and incubated 45-180 min.; 5. CH was added and incubated 0-45 min; 6. CH was added at the beginning of germination (0 min) and CH was not removed from the medium.

6. The effect of a RNA synthesized inhibitor on tube elongation.

In general, pollen tube elongation is inhibited by a RNA synthesized inhibitor such as Actinomycin D (Act. D.). If Act. D. (20 μg/ml) is added before germination, and incubated for 100 to 150 minutes, tube elongation is inhibited about 25% (Fig. 12 & 13). When Act. D. was added following 90 minutes of germination, the tube length was about 90% of the control (Fig. 13). Fig. 11 shows that the amount of RNA content is inhibited about 22% by the Act. D. treatment. The effect of Bromouracil (100 μg/ml) is similar to that of Act. D.

DISCUSSION AND CONCLUSION

Some ions are necessary for various species of pollen grain germination for example, calcium and boron are very important for tube elongation, calcium is an essential component of the the middle lamella, and is the activator of some metallic enzymes[1]. Vasil[25], Gauch and Dugger[8] suggested that boron may form a sugar-

Table 4. The effects of cycloheximide (CH) on pollen tube elongation of various species. 10μg/ml of CH was placed in a hanging drop and incubated at 30°C for 2 hours.

	Plants	tube length			germination (%)	pollen size (μ)	style length (cm)	Flower	
		length (mm)		inhibition (%)				longevity (day)	sexuality
		control	CH 10μg/ml						
雙子葉植物	*Luffa cylindrica* Raem. (絲瓜)	1.32	0.66	50	94	89	0.5	1	unisexual
	Momordica charantia Linn. (苦瓜)	0.80	0.39	49	80	75	0.5	1	unisexual
	Arachis hypogaca Linn. (花生)	0.78	0.45	42	41	53×28	1.0	1	bisexual
	Duranta repens Linn. (金露花)	0.23	0.12	50	49	42	0.4	1	bisexual
	Erythrina corallodendron Linn. (珊瑚刺桐)	0.77	0.01	99	52	42	2.0	> 3	bisexual
	Carica papaya Linn. (木瓜)	0.23	0.01	96	7	33	—	> 5	unisexual
	Allamanda cathartica Linn. (軟枝黃蟬)	0.193	0.01	95	10	94	3.8	> 2	bisexual
單子葉植物	*Lilium longiflorum* Thunb (百合)	1.467	0.01	99	78	127×89	10.5	> 5	bisexual
	Crinum asiatica L. var. *sinicum* Bak (白花文珠蘭)	1.28	0.01	99	92	66	12.0	> 3	bisexual
	Zephyranthes carinata Herb. (紅花文珠蘭)	1.27	0.01	99	90	94×56	7.2	> 3	bisexual
	Tradescantia sp. (紫鴨跖草)	2.01	1.41	30	50	53×28	1.3	< 1	bisexual

borate complex, which will enhance the absorption and metabolism of sugar, and increase the uptake of oxygen, and benefit cell wall synthsis. Larson and Lewis[15] used the electron-microscope to observe and prove that new cell walls are synthesized during pollen tube elongation, therefore, calcium and boron are both necessary substances in the elongating process of pollen tubes.

To the germinating pollen, the sugar in the medium not only can maintain its osmotic pressure (to prevent pollen tubes from bursting), but can also serve as a nutrient. Recently, Dickinson[6] produced evidence that lily pollen cannot utilize pentaerythritol in the place of sugar, in which case the length of pollen tube decreases (Fig. 3). The optimal conc. of the sugar is about 10%.

If CH is added to lily pollen at the beginning, tube elongation is completely inhibited, this strong inhibiting phenomenon is analogous to the results obtained on the germination of urediospores studied by Dunkle and his coworkers[7], whereas gourd pollen tubes can still elongate (Fig. 9). From this, we know that the mechanism of the first step of the elongation of lily pollen tube is apparently different

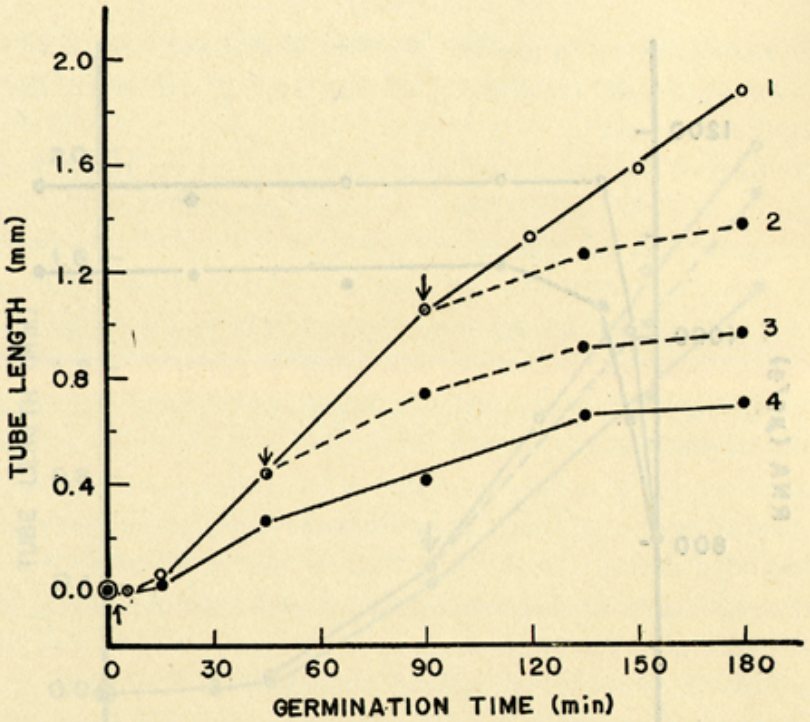

Fig. 10. The effects of CH on the tube length of gourd pollen. Control (curve 1); CH was added at zero time (curve 4); at 45 min. (curve 3); and at 90 min. (curve 2) with final conc. at 2μg/ml,

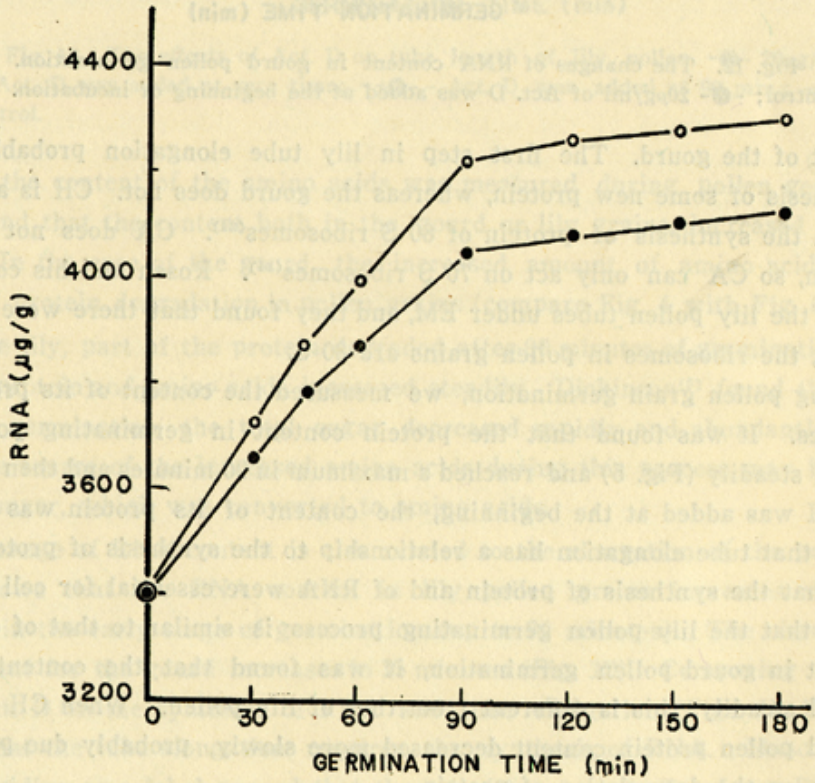

Fig. 11. The changes of total RNA content in lily pollen germination. -○- control; -●- 20μg/ml of Act. D was added at the beginning of incubation.

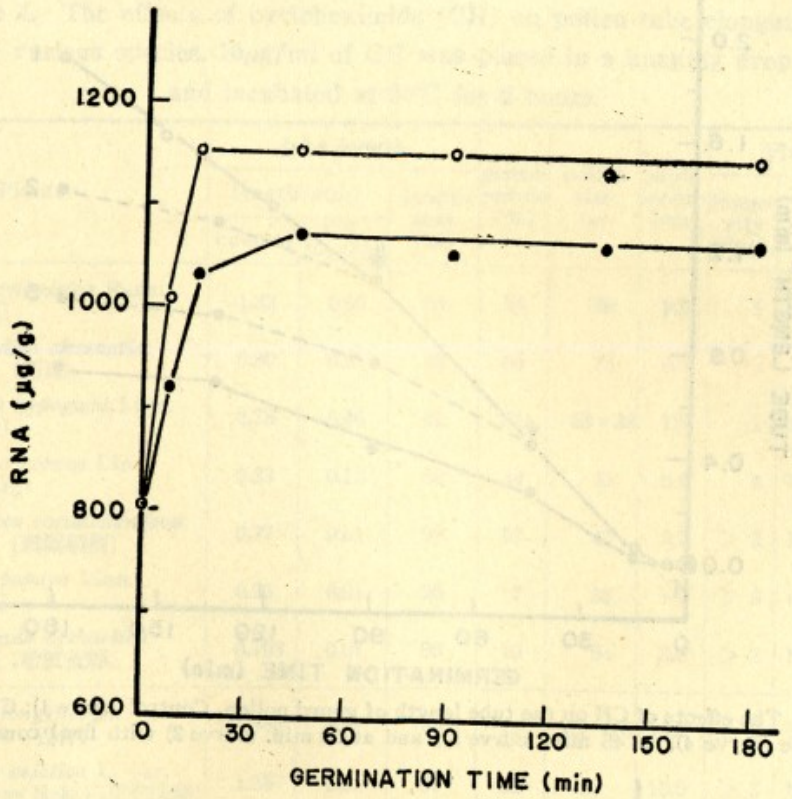

Fig. 12. The cnanges of RNA content in gourd pollen germination. -○- control; -●- 20μg/ml of Act. D was added at the beginning of incubation.

from that of the gourd. The first step in lily tube elongation probably requires the synthesis of some new protein, whereas the gourd does not. CH is an inhibitor acting on the synthesis of protein of 80 S ribosomes[24]. CA does not affect tube elongation, so CA can only act on 70 S ribosomes[24]. Rosen and his coworkers[21] observed the lily pollen tubes under EM, and they found that there were ribosomes, therefore, the ribosomes in pollen grains are 80 S.

During pollen grain germination, we measured the content of its protein every 30 minutes. It was found that the protein content in germinating pollen of lily increased steadily (Fig. 5) and reached a maximum in 90 minutes and then decreased; but if CH was added at the beginning, the content of its protein was unchanged. It seems that tube elongation has a relationship to the synthesis of protein. Key[13] proved that the synthesis of protein and of RNA were essential for cell elongation. It seems that the lily pollen germinating process is similar to that of cell elongation. But in gourd pollen germination, it was found that the content of protein decreased steadily, this is different from that of lily pollen. When CH was added, the gourd pollen protein content decreased more slowly, probably due primarily to inhibition on the degradation of protein.

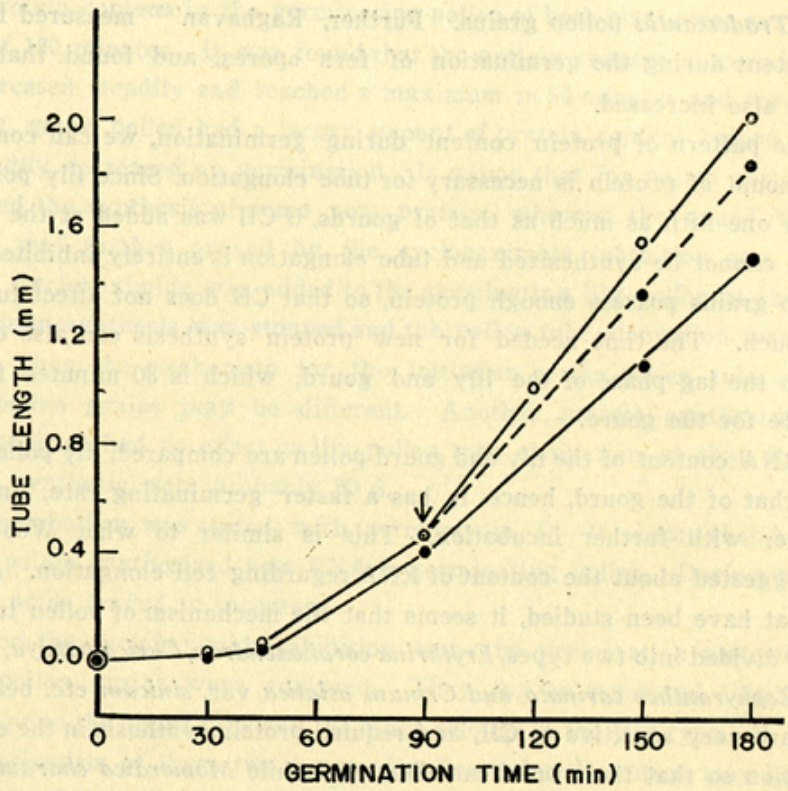

Fig. 13. The effects of Act. D on tube length of lily pollen. -●- 20μg/ml of Act. D was added at zero time; --●-- Act. D was added at 90 min.; -○- control.

When the content of the amino acids was measured during pollen germination, it was found that the content both in the gourd or lily grains, increased with time (Fig. 4). In the case of the gourd, the increased amount of amino acids was the product of protein degradation in pollen grains (compare Fig. 4 with Fig. 6). In the case of the lily, part of the protein degraded after 90 minutes of germination, before that, both protein and amino acids increased steadily. Dickinson[6] found that during lily pollen germination, the total sugar decreased rapidly and abundantly. Hence part of the source of the increased amino acids during this process may have come from the sugar, which was converted to amino acids.

The change of RNA content is also related to the elongation of the pollen tube. Fig. 11 shows that the RNA content in lily pollen grains increases with time, especially in the early stage of germination (up to 90 minutes). The change of the RNA content for the gourd increases in 15 minutes (Fig. 12). Comparing the inhibition of Act. D on the synthesis of RNA and tube elongation (Figs. 11, 12 and 13), it seems that the tube elongation requires the synthesis of RNA. Mascasenhas[17] using ^{3}H-uridine as a label proved that there had been synthesis of new RNA in the

germinting *Tradescantia* pollen grains. Further, Raghavan[20] measured the change of RNA content during the germination of fern spores, and found that the RNA content was also increased.

From the pattern of protein content during germination, we can conclude that a certain amount of protein is necessary for tube elongation. Since lily pollen grains contain only one fifth as much as that of gourds, if CH was added at the beginning, new protein cannot be synthesized and tube elongation is entirely inhibited, whereas gourd pollen grains possess enough protein, so that CH does not affect tube elongation very much. The time needed for new protein synthesis can also explain the difference in the lag phase of the lily and gourd, which is 30 minutes for the lily and 5 minute for the gourd.

If the RNA content of the lily and gourd pollen are compared, lily pollen contains more than that of the gourd, hence it has a faster germinating rate, and the tube grows longer with further incubation. This is similar to what Woodstock and Skoog[26] suggested about the content of RNA regarding cell elongation. Among the varieties that have been studied, it seems that the mechanism of pollen tube elongation may be divided into two types, *Erythrina corallodendron, Carica papaya, Allamanda cathartica, Zephyranthes carinata and Crinum asiatica* var. *sinicum* etc. belong to lily type, they are very sensitive to CH, and require protein synthesis in the early stages of germination so that their tubes can elongate; while *Momordica charantia, Arachis hypogaca, Duranta repens* and *Tradescantia* etc. are similar to the gourd, and are insensitive to CH, the necessary protein for their pollen tube elongation, has probably already been stored in the ungerminated pollen. These two mechanisms have no relation to the number of cotyledons, the size of pollen grains or the sexulity of the flowers but may be related to the longevity of their flowers and the length of their styles.

SUMMARY

The germination of pollen grains collected from a long period blooming flower (White Trumpet Lily, *Lilium longiflorum*) and a short period blooming flower (Dish-Cloth Gourd, *Luffa cylindrica*) was studied. It was found that the germination pattern of the two plants was quite different. Since protein and RNA metabolism are active during pollen germination, the protein and RNA metablism during the pollen germination of the two plants was compared.

It was found that for pollen germination in vitro, pH 5.2 at 30°C and pH 6.0 at 32°C were the optimal conditions for lily pollen and gourd pollen respectively. 10% sucrose was the best nutrient and Ca^{++} and BO_3^{---} were found to be necessary. After three hours of incubation, the length of the pollen tubes of these two kinds of pollen reached 2.0 mm. The lily pollen required a lag phase of 30 minutes while the gourd pollen germinated readily after 5 minutes of incubation.

The protein content in the germinating pollen of both plants was analyzed within a period of 180 minutes. It was found that the protein content in germinting pollen of lily increased steadily and reached a maximum in 90 minutes and then decreased. In contrast, gourd pollen had a larger amount of protein content before germination which steadily decreased on germination. It seems that for pollen germination, the lily required the synthesis of some new protein, whereas the gourd did not. The hypothesis was further proved by the cycloheximide inhibition test for protein synthesis. Cycloheximide was added to the germinating lily pollen at the beginning, and the proten synthesis was stopped and the pollen tube elongation was completely inhibited. Thus, the mechanism for the initiation of the pollen tube of these two kinds of pollen grains may be different. Another protein synthesized inhibitor (chloramphenicol) had no effect on lily pollen tube elongation, so that, its ribosomes for protein synthesis were probably 80 S.

RNA metabolism was tested with actinomycin D. It seems that both the lily and gourd pollen synthesized new RNA in germinating pollen. During germination, free amino acids tended to incerease.

Based on the cycloheximide inhibition test, the germination patterns of eleven species of pollen grains were analyzed. They can be separated into two groups, one is similar to the gourd type and the other belongs to the lily type. It is proposed that the mechanism of these two types of pollen tube elongation may be related to the longevity of their flowers and the length of their styles.

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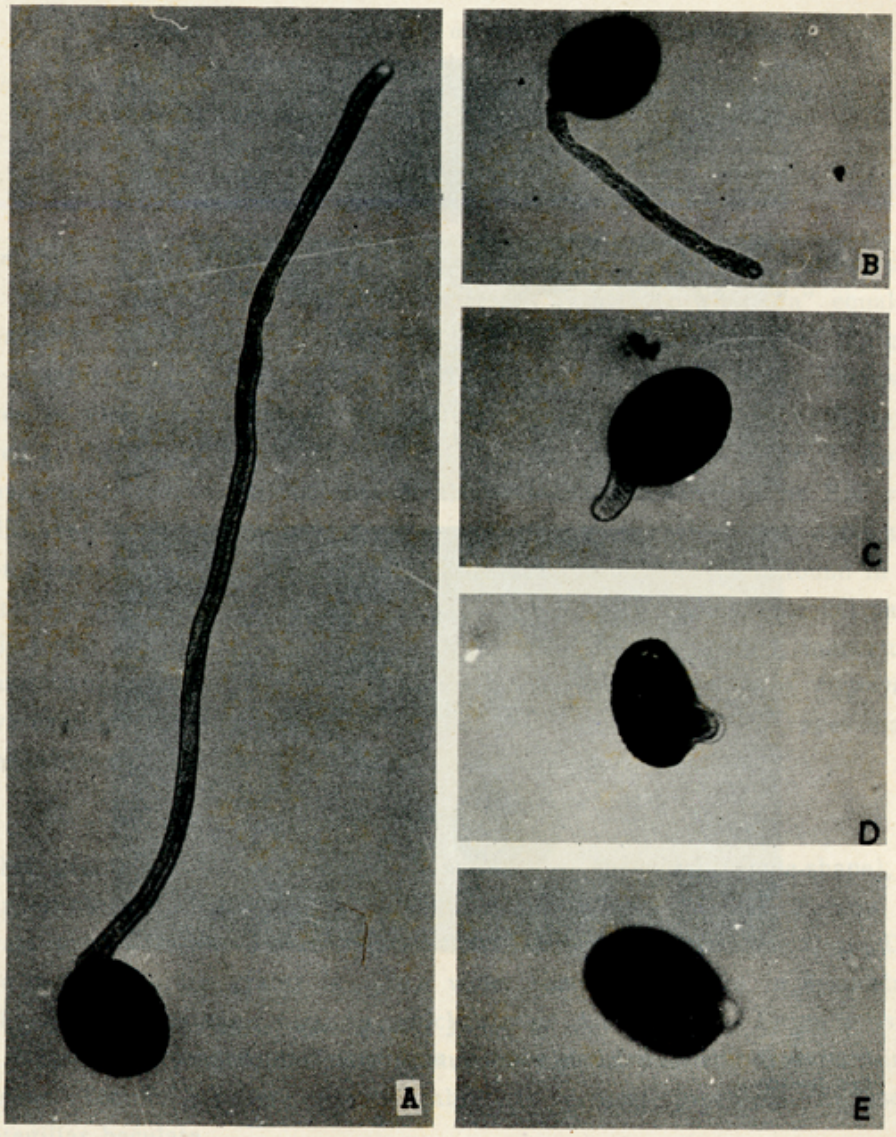

Fig. 7. Various conc. of CH affect the elongation of lily pollen tube. Sample was incubated under an optimal conditions for 150 min. A. control, B-E. CH was added at zero time (B, 0.1 μg/ml CH; C, 1 μg/ml CH; D, 2 μg/ml CH; E, 10 μg/ml CH). Photographs are magnifired by 100 x.

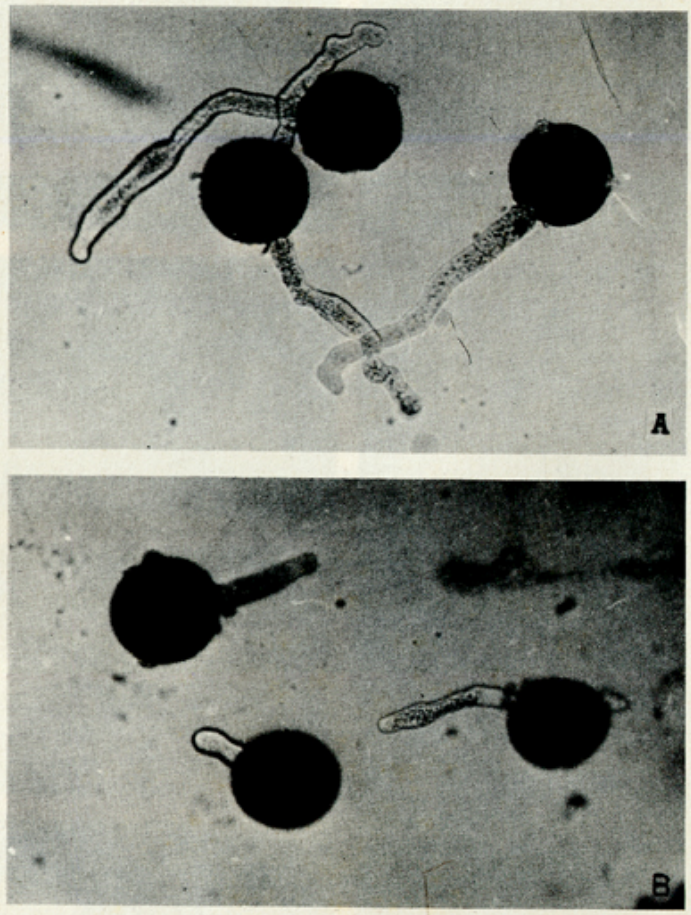

Fig. 9. The pollen tube of gourd. Sample was incubated for 45 min. A. control; B. CH (2μg/ml) was added at zero time. Both photographs are magnified by 100 x.