EFFECT OF N, N′-DINITROETHYLENEDIAMIDE ON POLLEN GERMINATION AND NITROGEN METABOLISM DURING POLLEN TUBE ELONGATION[1]

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Abstract: The nitrogen metabolism associated with pollen germination in ***Crinum asiatica*** var. ***sinicum*** Bak. was studied. A synthetic plant growth stimulant, EDNA (N, N′-dinitroethylenediamide), was added to the culture medium in order to detect whether or not this chemical can effect pollen germination and nitrogen metabolism during pollen germination and pollen tube elongation.

In the 180 minute experimental period, the amino acid content in germinating pollen increased about 52% and a remarkable amount of amino acids were released into the culture medium. The protein content of ungerminated pollen contained 18.3% of dry weight, and increased to its maximum quantites in 15 minutes after imbibition in the culture medium, and then decreased gradually. Using ^{14}C-leucine as a tracer to study protein synthesis, we found about 50% of total newly synthesized protein occurred in the first 30 minutes of the germinating period. The RNA content in germinating pollen increased steadily for 150 minutes germinating period, and then maintained at the same level.

EDNA at the 2.5 ppm can stimulate the pollen tube elongation, but when its concentration was 10 ppm or higher in the culture medium, a strong inhibited effect on pollen germination and pollen tube elongation was observed. At this optimal concentration of EDNA (2.5-5 ppm), ^{14}C-leucine incorporation into protein was also stimulated. But beyond this concentration, the higher the concentration of EDNA present in the culture, the stronger was the inhibition effect on protein synthesis. From the ^{14}C-uridine incorporation studies, EDNA did not stimulate the RNA synthesis. Although the inhibition mechanism of EDNA on pollen germination and pollen tube elongation are not clear, yet EDNA can inhibit protein synthesis which may be the important effect of this chemical on pollen germination and pollen tube elongation.

INTRODUCTION

N, N′-dinitroethylenediamide (EDNA) is a synthetic plant growth regulator, a new product of the Dow Company, U. S. A.. In recent years, many kinds of synthetic herbicides, and plant growth regulators have been used in agriculture and horticulture, however none of these chemicals have been used to examine its effect on pollen germination and pollen tube elongation. Horticulture and plant breeders often fail to get fertile seeds during artificial pollination. The main cause of failure of seed setting is due to the result of slow growth of the pollen tube or its

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early degeneration in the style. So that it is worthwhile to study the effect of these chemicals on pollen germination and pollen tube growth.

The purpose of this experiment was primarily to observe various protein and RNA syntheses on pollen germination in order to get an understanding of the relationship between the change in nitrogen metabolism and pollen germination and pollen tube elongation. Secondarily, to observe the effect of EDNA on pollen germination and pollen tube elongation in order to get some information as to the possible future application of this drug in agriculture and horticulture.

MATERIALS AND METHODS

A. Collection and storage.

Anthers of *Crinum asiaticum* var. *sinicum* Bak. were collected in July. The anthers were placed in a desciccator at room temperature for 24 hours, and the pollen was then collected and stored at 4°C.

B. Condition for pollen germination.

For pollen germination, a culture medium was used as defined by Brewbaker and Kwack (1963) containing 10% sucrose; 100 ppm KNO_3; 100 ppm H_3BO_3; 300 ppm $Ca(NO_3)_2 \cdot 4H_2O$; 200 ppm $MgSO_4 \cdot 7H_2O$. The pH value was adjusted to 5.2 and maintained at 30°C.

C. Germination percentage and average tube length.

Pollen was removed from a shaking culture at desired intervals, and fixed with a drop of 3% I_2KI on a slide. They were then examined under a light microscope. The germination percentage was determined from 150 to 200 pollen grains. The average tube length was obtained from 100 pollen tubes with the aid of a micrometer.

D. Chemical analysis.

20 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. Pollen was centrifuged at the end of incubation period and samples were taken for analysis. The quantity of free amino acids was extracted by 80% ethanol and then was determined by the Moore and Stein modified ninhydrin method (1954). The protein contents were measured by Lowry *et al.* method (1951). RNA was extracted according to Smille and Krotkov method (1960) and the amount of RNA was measured by the orcinol method, and yeast RNA was used as the standard. The optical density was determined at 660 mμ.

The following procedures were used in the studies on ^{14}C-leucine incorporation into protein. 30 mg of pollen which had been cultured in 5 ml culture medium containing 0.5 μc of ^{14}C-leucine for desired intervals was centrifuged at 20,000 xg for 10 minutes. The germinating pollen was collected and then was homogenized in a glass homogenizer at 2°C in Tris-HCl buffer (pH 7.0, 0.05 M). The homogenates were centrifuged at 20,000 xg for 10 minutes. The debris was washed with Tris buffer three times (2 ml each time) and all the washings were combined to the homogenate supernatant. An aliquot of 2 ml was removed and mixed with 2 ml of 10% trichloroacetic acid. After mixing and standing for 30 minutes in an ice bucket, the precipitate was filtered with 0.45 μ millipore. The precipitate on the millipore was washed five times with 5% trichloroacetic acid and twice with 95% ethanol, dried and then counted in a gas-flow counter (Nuclear Chicago. Model 470) equipped with a micromil window. The debris was suspended in 6 ml Tris-HCl buffer and an aliquot of 0.1 ml was removed to a glass plate and was counted by the above

method.

The procedure used in studies on ^{14}C-uridine incorporation into RNA was as follows: 30 mg of pollen grains which had been cultured in 5 ml culture medium containing 1 μc ^{14}C-uridine for desired intervals was centrifuged and the pelletized pollen was homogenized and centrifuged by the prescribed method. An aliquot of 2 ml was removed and 2 ml of 0.6 M perchloroacetic acid was added. The precipitated RNA then was filtered with 0.45 μ millipore paper. The precipitation was rinsed five times with 5% trichloroacetic acid, dried and then counted.

Uridine-2-^{14}C and ^{14}C-leucine were purchased from Calbiochem Corp. with a specific activity of 20 mc/mM and 216 mc/mM respectively.

RESULTS

1. Nitrogen metabolism in germinating pollen.

The time-course determination of amino acids, protein, and RNA in germinating pollen are shown in Figs. 1, 2 and 3, respectively. In the 180 minute experimental

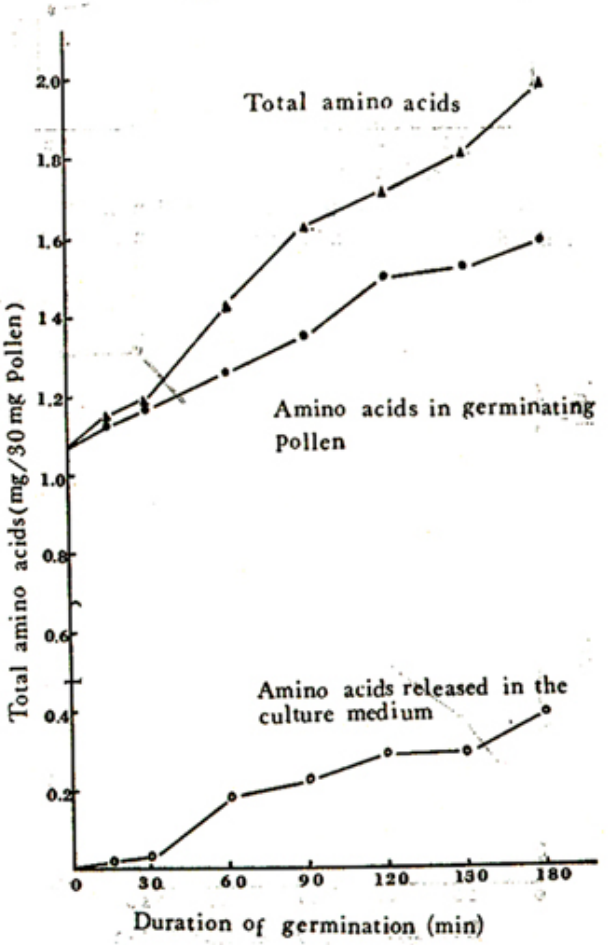

Fig. 1. Change of amino acid content in the pollen grains of *Crinum asiatica* during the 180 minute germination period.

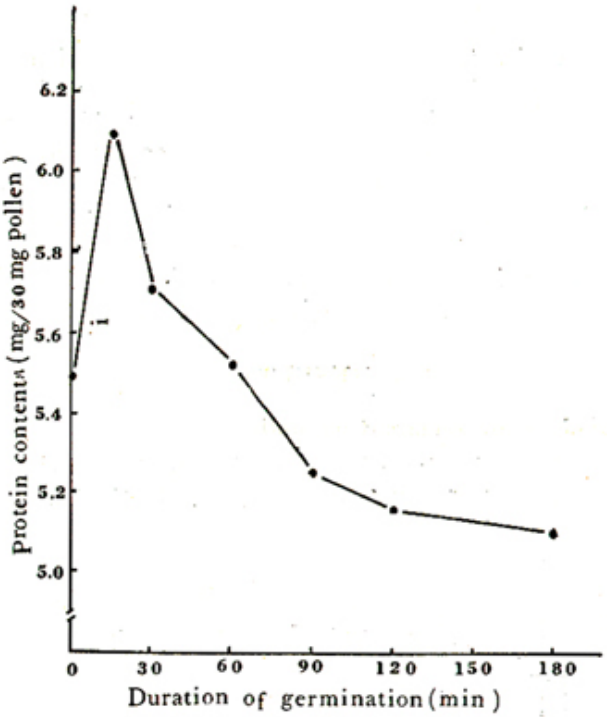

Fig. 2. Change of protein content in the pollen grains of *Crinum asiatica* during the 180 minute germination period.

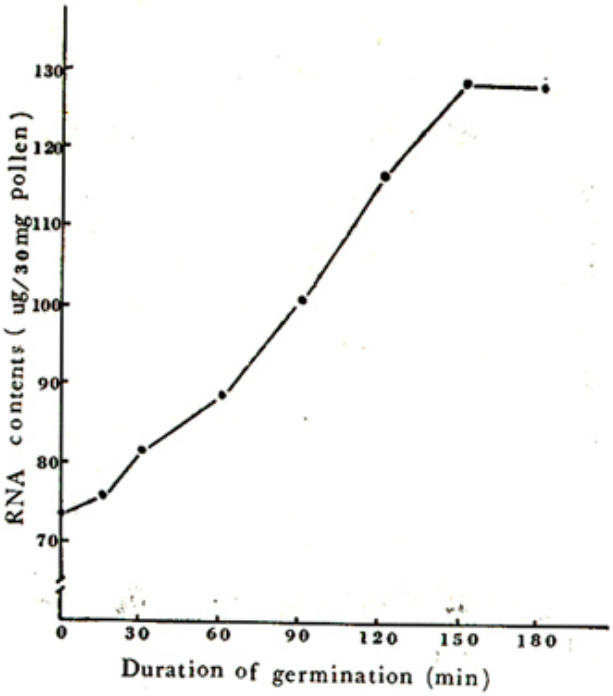

Fig. 3. Change of RNA content in the pollen grains of *Crinum asiatica* during the 180 minute germination period.

period, the ethanol-soluble amino acid content in the germinating pollen and the amino acids released in the culture medium had increased gradually. The ethanol-soluble amino acid content in germinating pollen had increased about 52% in the 180 minute incubation period (Fig. 1).

2. The effects of EDNA and cycloheximide on pollen germination and pollen tube elongation.

Pollen tube elongation was inhibited by cycloheximide when cycloheximide was added to the culture in the early stages of germination, especially at zero time (Fig. 4). When cycloheximide was added to the culture medium at zero time, the pollen tube length after 180 minutes incubation was only 2.4% of the control pollen tube length (Table 1). But when cycloheximide was added to the culture medium after 30 minutes imbibition, the pollen tube length at the end of 180 minute experimental period was 45% of the control. It seems that new protein synthesis at the early germination stage is necessary for pollen tube elongation.

Various concentrations of EDNA were added to the culture medium at zero time. Table 1 shows that the concentration of EDNA at 2.5 ppm had a stimulating

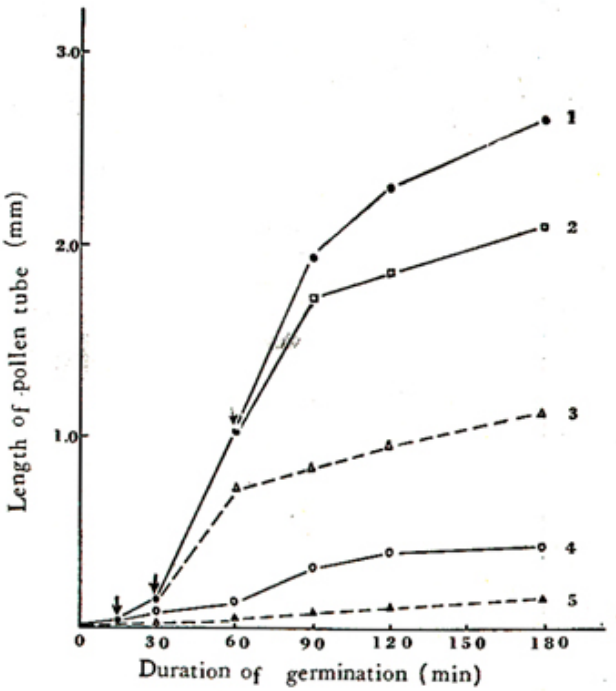

Fig. 4. Effect of cycloheximide and EDNA on the elongation of *Crinum asiatica* pollen tube. Cycloheximide (2 μg/ml) was added to the culture medium at various times and then incubated to the end of the 180 minute experimental period. The pollen tube length was measured as described in Material and Methods. 1. Control. 2. CH was added at 60 min. 3. CH was added at 30 min. 4. CH was added at 15 min. 5. CH was added at zero time. 6. EDNA (100 ppm) was added at zero time.

Table 1. Effects of EDNA on pollen germination and pollen tube elongation

Treatment	Pollen tube length		Germination (%)
	Tube length (mm)	Control (%)	
Control	2.661	100	90
2.5 ppm EDNA	2.963	111.0	90
5 ppm EDNA	2.712	100.2	90
10 ppm EDNA	1.712	64.3	—
25 ppm EDNA	1.209	45.3	—
50 ppm EDNA	0.200	7.5	31.7
100 ppm EDNA	0.020	0.7	12.9
Cycloheximide at 10 μg/ml	0.070	2.4	73.4

EDNA or cycloheximide was added to the culture at zero time and incubated for 180 minutes at 30°C. The measurement of pollen tube length and germination percentage were as described in Materials and Methods.

effect on pollen tube elongation. But the concentration of EDNA present in the culture medium of more than 10 ppm, shows that the higher concentration of EDNA present in the culture medium, the stronger was the inhibition effect on pollen tube elongation. EDNA at the concentration of 100 ppm inhibited the pollen germination.

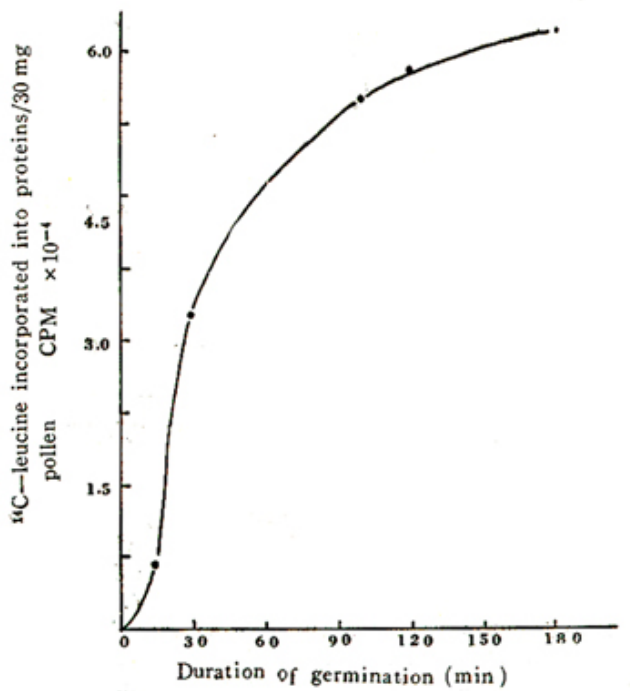

Fig. 5. Incorporation of ^{14}C-leucine into protein during the 180 minute germination period.

Table 2. Time course studies on ^{14}C-leucine incorporation into protein during pollen germination

Time (min)	Total incorporation (cpm)
0–15	6,990
15–30	25,020
30–60	14,000
60–120	12,676
120–180	1,272

30 mg of pollen which had been cultured in 5 ml culture medium containing 0.5 μc of ^{14}C-leucine for desired time and then the total incorporation of ^{14}C-leucine into protein was detected at desired intervals.

3. The time-course study on protein synthesis during pollen germination.

When ^{14}C-leucine was added to the culture medium at zero time to detect the total amount of ^{14}C-leucine incorporation into protein at the desired time, the total ^{14}C-leucine incorporation into protein increased with time course during the 180 minute experimental period (see Fig. 5 and Table 2). About 50% of the total ^{14}C-leucine incorporation into protein was observed at the beginning 30 minutes of germination, particulary in the initial fifteen minutes between 15 and 30 minutes after pollen imbibition in the culture medium. These data indicate that most of the new protein synthesis was synthesized at the early stage of pollen germination. So that when cycloheximide was added to the culture medium at zero time to 15 minutes, the inhibition effect on the pollen tube elongation was most effective.

Table 3. Effects of EDNA and cycloheximide on ^{14}C-leucine incorporation into protein during pollen germination

Treatment	Total incorporation (cpm)	Control (%)
Control	55,794	100
1.5 ppm EDNA	64,068	114.80
2.5 ppm EDNA	67,891	121.68
5 ppm EDNA	49,524	88.76
10 ppm EDNA	47,724	85.25
25 ppm EDNA	36,666	65.71
50 ppm EDNA	32,208	57.72
100 ppm EDNA	31,662	56.74
200 ppm EDNA	6,414	11.49
2 μg/ml CH	2,952	5.29
10 μg/ml CH	966	1.73

30 mg pollen which had been cultured in 5 ml culture medium containing 0.5 μc of ^{14}C-leucine and various concentrations of EDNA and cycloheximide (CH). At the end of 180 minute experimental period, the total incorporation of ^{14}C-leucine into protein was detected as described in "Materials and Methods".

4. Effect of EDNA on ^{14}C-leucine incorporation into protein during pollen germination.

When various concentrations of EDNA were added to the culture medium at zero time, and then cultured for 180 minutes in the presence of ^{14}C-leucine, the results are shown in Table 3. EDNA at the concentration of 1.5 ppm to 2.5 ppm has a stimulative effect on ^{14}C-leucine incorporation into protein. But when the concentration of EDNA is higher than 3 ppm, a noticeable inhibitive effect was observed. Table 3 also shows that cycloheximide at the concentration of 2 μg/ml has a strong inhibition effect on ^{14}C-leuine incorporation into protein.

5. Effect of EDNA on ^{14}C-uridine incorporation into RNA during pollen germination.

When EDNA at 2.5 ppm was added to the culture medium at zero time, the data indicated that EDNA had no stimulation on ^{14}C-uridine incorporation into RNA although at this concentration protein synthesis was enhanced. Actinomycin D, 5-florouracil, and cycloheximide inhibited RNA synthesis when these chemicals were added to the culture medium at zero time (Table 4).

Table 4. Effects of EDNA, actinomycin D, 5-florouracil, and cycloheximide on ^{14}C-uridine incorporation into RNA during pollen germination

Treatment	% of control
Control	100
EDNA, 1.5 ppm	96.7
EDNA, 100 ppm	56.3
Actinomycin D, 20 μg/ml	79.5
5-florouracil, 2.5 mM	67.9
Cycloheximide, 2 μg/ml.	49.9

30 mg pollen which had been cultured in 5 ml culture medium containing 1 μc of ^{14}C-uridine and presented EDNA and various inhibitors as shown in table. After 180 minutes incubation in culture medium and determined for the total incorporation into RNA. The total incorporation of ^{14}C-uridine into RNA in control is 905 cpm.

DISCUSSION

When the total amino acid content was measured in the germinating pollen, it increased with the time-course of germination (Fig. 1). These free amino acids may be from the hydrolysis products of reserve protein or from the conversion of carbohydrates. Dickinson (1967) found that in the germinating lily pollen, the total sugars decreased rapidly and abundantly, and in this period the free amino acids increased rapidly. Some of these free amino acids are released from the germinating pollen to the culture medium. Stanley and Linskens (1965) reported some protein may be also diffuse from the germinating pollen to the culture medium. These compounds diffuse out of the pollen, or on the pollen surface, can interact with the female tissue and avail the pollen tube growth in the ovary.

Cycloheximide is a protein synthesis inhibitor of 80 S ribosomes (Stutz and Noll, 1967). When cycloheximide was added to the culture medium at zero time,

the pollen tube elongation was completely inhibited. But cycloheximide only slightly inhibits the percentage of pollen germination. So that pollen germination and pollen tube elongation may be independent. The earlier addition of cycloheximide to the culture, the higher inhibition effect on pollen tube elongation was shown. This strong inhibiting phenomenon is analogous to the results obtained on the germination of urediospores reported by Dunkle *et al.* (1969) and the germination of lily and Dish-cloth gourd pollen reported by Lin *et al.* (1971). So that the first step in pollen tube elongation requires the synthesis of some new protein. By using ^{14}C-leucine incorporation to investigate the protein synthesis in pollen germination, it was found that about 50% of ^{14}C-leucine incorporation into protein was performed in the early stage of germination, especially between 15 to 30 minutes after pollen imbibition in the culture medium (Table 2). These data coincide with the results shown in Fig. 4 that when cycloheximide was present in the culture medium during the initial 15 minutes, the inhibition effect on pollen tube elongation was most effective.

In *Crinum*, the pollen tube elongation was stimulated by EDNA at the concentration of 2.5 ppm (Table 1). At this concentration EDNA also stimulated the protein synthesis. But at higher concentrations of EDNA in the culture medium there were inhibitive effects on both pollen tube elongation and ^{14}C-leucine incorporation into protein (Table 1 and 3). EDNA at the concentration of 100 ppm or more in the culture medium, inhibited not only the pollen tube elongation, but also inhibited pollen germination. This effect may be similar to the effect of auxin, which at the low concentrations can stimulate cell elongation and protein synthesis, but which at the high concentrations inhibits cell elongation and kills the plants. Although EDNA at the concentration of 2.5 ppm stimulated ^{14}C-leucine incorporation into proteins and pollen tube elongation, it did not stimulate ^{14}C-uridine incorporation into RNA. So that the new RNA synthesis is not required for the pollen tube elongation. The translation is more important than RNA transcription at the very beginning of pollen germination. Several investigators have shown that pollen germination and pollen tube elongation are independent of RNA synthesis (Linskens, 1967; Mascarenhas and Bell, 1969). Before pollen tube begins to form, the protein synthesis is initiated during the activation phase. The mature, ungerminated *Trandescantia* pollen grains contain all the ribosomes that are required for the growth of pollen tube, since no ribosomes are made during pollen tube growth (Mascarenhas and Bell, 1970). Linskens (1967) reported that the presence of ribonuclease-insensitive polysomes in the ungerminated lily pollen. He suggested that m-RNA possibly attached to the ribosomes is protected by some protein moiety against ribonuclease destruction. In this configuration, the polysome aggregation is inactive in protein synthesis. During germination and initiation of protein synthesis this protection is lost. This indicates a fraction of the ribosomes are metabolically inactive, but are prepackaged with m-RNA in the ungerminated pollen grain. The importance of this can be seen in the rapid activation of protein synthesis on imbibition (less than 2 minutes) and the rapid formation of a pollen tube. Key (1964) proved that the new synthesis of protein is essential for cell elongation. It seems that the pollen tube elongation process is similar to the plant cell elongation and requires the new synthesis of some protein.

Although we have not investigated EDNA at the optimal concentration wheather or not it can enhance the chromatin activity in the germinating pollen grain. But

in the etiolated soybean seedlings, the authors found that EDNA can not enhance the chromatin activity when the 4-day old seedlings were sprayed with 500 ppm EDNA for 24 hours (Chen, 1974).

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