SOME OBSERVATIONS ON GERMINATION OF *PSEUDOTSUGA MENZIESII* (MIRB.) FRANCO POLLEN IN VITRO

by

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ABSTRACT

Pollen morphology, pollen germination, and the effects of nutrients during pollen germination of Pseudotsuga menziesii (Mirb.) were studied.

Pollen extraction was done at room temperature and the pollen morphology was studied using a staining technique. Several types of substances were introduced to the medium (double-distilled water) to culture the pollen. The substances were boric acid, calcium nitrate, potassium salt of gibberellic acid, indoleacetic acid, indolebutyric acid, naphthaleneacetic acid, thiamin, water extracts of Douglas-fir seeds, sucrose and stock solution. Incubation of pollen grains was carried out in the growth chamber where light intensity was about 3500 foot-candles during the 12-hour light period each day and the temperature was maintained at 20°C at night and 25°C during the day time. The relative humidity was kept at about 40 percent. Pollen grains were checked daily.

Mature pollen grains were at the two-celled stage with two degenerated prothallial cells. When dry, the grains were cup-shaped, while turgid grains were spherical or elliptical without any furrows or sacs. The exine was thin being about 2 microns, and quite smooth. The intine was about 8 microns thick and was of uniform hyaline appearance. The pores in the exine were about 2 microns in diameter; those in the intine were enclosed with a membrane. Boron and

calcium ions were very important to the pollen germination and elongation. Pollen germination was stimulated by growth-promoting substances, but was inhibited by fungicide and bactericide. Sucrose solution of 10 to 15 percent was recommended for the osmotic milieu and for nutrient purposes. Stock solution (boron 0.1 g., calcium nitrate 0.3 g., and double-distilled water 100 ml.) is the best for pollen germination and elongation. Pollen grains cultured in the medium containing stock solution B, 10 ppm IAA, and sucrose were found in four-celled stages (tube cell, two sperm cells and stalk cell) after five days. The actual germination of the Douglas-fir pollen in vitro was accomplished in this study. These may be of practical value in ensuring a uniformly high rate of seed production in Douglas-fir seed orchards, but field studies (artificial pollination) are needed to obtain further information.

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INTRODUCTION

The development of male and female gametophyte and fertilization of Pseudotsuga menziesii Mirb. Franco (Douglas-fir) have been described by Lawson (1909). He gave details of pollen grown in vivo and presented diagrams of the development of globular pollen grains from the time of pollination to the three-nucleate stage (stalk cell, body cell and tube cell). But the actual division of the body cell to two sperm cells was not observed; it seems this division took place before the tip of the pollen tube reached the nucellus. According to Allen (1943), the pollen remains in the micropyle about three weeks before it germinates. After five weeks' germination, the tip of the pollen tube reaches the nucellus, and a week later it penetrates the archegonium. Fertilization of the egg nucleus occurs about nine weeks after pollination. Allen also presented photographs of the pollen grain at the three-nucleate stage but not the formation of the sperm cells. Ching and Ching (1959) studied extracts of Douglas-fir pollen and effects of gibberellic acid on its germination. Total pollen production was highest under standard room condition. Treatment with the potassium salt of gibberellic acid caused significantly more pollen tube growth and indicated the possibility of increasing seed setting by such a treatment. The three-nucleate stage of the pollen grain occurred after 46 hour incubation under 100 ppm

gibberellic acid treatment. The four-nucleate stage and formation of the pollen tubes of Douglas-fir pollen grains in vivo were presented in photographs by Barner and Christiansen (1962), when the pollen grain comes into contact with the nucellus top, the body cell divides forming two male cells. Simultaneously, a pollen tube is formed at the pole of the pollen grain in contact with the nucellus.

Since the beginning of this century, the question whether the pollen tubes in vivo and in vitro utilize externally supplied nutrients or not, has attracted much attention. The growth of pollen tubes of gymnosperms is extremely slow. The pollen grains of gymnosperms usually have a low vitamin content, whereas those of angiosperms are rich in vitamins of the B-group (Lunden, 1954). The importance of auxins, vitamins and trace elements on the growth of plants and pollen tube elongation is now generally recognized (Stiles, 1964; Schopfer, 1949; Audus, 1953; Leopold, 1955). However the evidence in regard to the possible use of such substances in ensuring a uniformly high rate of pollination and fertilization of Douglas-fir in reproductive phase appears to be scanty. It is, therefore, considered necessary to study the germination of Douglas-fir pollen and to determine the type of substances either auxins, vitamins, trace elements or antibiotics, that stimulate germination and the rate of elongation of the pollen tube

in vitro. Such stimulation may be of practical value in ensuring a uniformly high rate of filled seed production in Douglas-fir seed orchards.

MATERIAL AND METHODS

Male cones of Douglas-fir were collected from trees at Caycuse clone bank of B. C. Forest Products Ltd., Vancouver Island on May 2, 1967. Pollen extraction was done at room temperature. The pollen was stored in a vial at $0^{o}C$ to $2^{o}C$ in the refrigerator.

Pollen grains collected from eight trees (Tree No. BCFP 25, 37, 44, 133, 135, 166 and 189) were placed in distilled water for about 2 minutes. 30 grains were measured in random field under the microscope from each tree.

Dry pollen was directly fixed and stained with fast green and observed under the microscope to study pollen shape. Identification of the pollen pore was carried out using basic fuchsin glycerine jelly technique (Wodehouse, 1959). In this technique, dry and turgid pollen grains was used. The procedure was as follows:

1. Prepare basic fuchsin glycerine jelly according to Wodehouse (1959).
2. Place pollen on the slide.
3. Add one to three drops of 100 per cent alcohol and allow to partially evaporate. As the alcohol evaporates, it spreads out and leaves an oil ring.
4. Wipe off the oil ring with cotton

5. Add one drop hot basic fuchsin glycerine jelly and stir.
6. Pass a cover glass through a flame several times and cover the slide.
7. Cool (or seal with lacquer for permanence).

Pollen grains collected from tree BCFP 189 were chosen for the germination test. Five kinds of medium were prepared as follows:

1. A series of solutions were prepared: 0.01, 0.1, 1, 5, 10, 100, and 1000 ppm of boric acid, calcium nitrate, potassium salt of gibberellic acid (10% GA), indoleacetic acid (IAA), indolebutyric acid (IBA), naphthaleneacetic acid (NAA), and thiamin.
2. Water extract was prepared from seeds from which the seed coats were peeled. Crushed seeds were boiled in distilled water for half an hour. One preparation is one gram per 100 ml. of distilled water; the other 10 gram per 100 ml. of distilled water.
3. Sucrose solution was tested to the osmotic milieu of pollen grains in a concentration series: 1, 5, 8, 10, 15 and 20 percent.
4. A stock solution, basically described by Brewbaker and Kwack (1963), was prepared as follows:

Stock solution A	H_3BO_3	0.1 g.
	$Ca(NO_3)_2.4H_2O$	0.3 g.

	$MgSO_4 \cdot 7H_2O$	0.2 g.
	KNO_3	0.1 g.
	in distilled water	100 ml.
Stock solution B	stock solution A	1 ml.
	distilled water	9 ml.

5. Fungicide (Arasan: active ingredient-tiram "tetramethyl-thiuramdisulfide", 75%; inert ingredient, 25%), and bactericide (fortimycin) in different concentrations were introduced to the culture medium.

The pH of the culture solution was adjusted to about 7. Details of procedure are as follows:

1. There are three possible methods for sterilizing the Petri dishes and two cavity slides:
   (a) Soaking for 12 hours in 3N HCl, rinsing 5 times in distilled water, and then covering to protect from dust.
   (b) Wiping with 70% alcohol.
   (c) Autoclaving under 15 lb pressure for 20 minutes.
2. The experimental stand was wiped with 70% alcohol.
3. Filter paper was placed in the Petri dishes and a thin layer of distilled water was added.
4. Slide with two-cavity was placed in the Petri dish.
5. Two or three drops of cultural medium were added to the slide cavities.

6. Pollen grains were immediately dusted on the medium. They were not immersed in the medium or covered with a cover slip, as free access to air is necessary for germination.
7. All above steps were done in the transfer chamber.
8. Then the Petri dishes were kept in the growth chamber where light intensity was about 3500 foot-candles during the 12-hour light period each day. The temperature was 20°C at night and 25°C during the day time, and relative humidity about 40%.
9. After 24 hours' incubation, pollen grains were picked up with a sterile micro spatula, transferred to the slides and then covered with a cover slip. Sometimes fixation and staining were used after a cover slip was put on the slide.
10. Pollen grains were measured under the microscope. Over one thousand pollen grains were counted under each experimental condition. Fifty or more, randomly selected, were measured.

From pollination to fertilization, it takes about nine weeks (Allen 1943). Pollen grains grow very slowly in vivo. In vitro it should take a long time of culture to get the 4-nucleate stage. The solution for this culture is prepared from the above information which shows elongation and growth. The culture solution is combined with stock solution B,

10 ppm IAA and a series of 5, 8, 10, 15 and 20 per cent sucrose.

Dry pollen grains were irradiated with ultraviolet light frome one hour to eight hours and then tested for germination rate in stock solution B. A 30 watt ultraviolet light source was used at a distance of 50 cm from the pollen. Incubation and measurement were done under the same conditions as mentioned above.

When pollen grains begin to elongate, pressure is exerted on the exine. When the exine can no longer sustain the tension, it breaks and is cast off gradually or quickly. Methods of breakage were observed and counted to establish which was the more frequent method.

RESULTS

Measurements of the sizes of turgid pollen from the eight selected trees are summarized in Table 1.

Table 1

Mean and standard deviation of the sizes of turgid pollen grains from eight selected trees.

Tree	No.	Width (micron)	Length (micron)
BCFP	25	94.71± 4.79	119.65±12.35
	37	93.99±16.90	146.91±25.25
	44	95.70± 3.48	129.09±11.39
	55	95.59± 4.93	131.92±19.82
	133	95.85± 3.52	128.27±16.31
	135	94.40± 1.91	143.25±16.64
	166	91.13± 4.06	127.79±12.74
	189	93.22± 6.14	105.02±16.05
Average		94.32± 5.74	128.99±16.32

Pollen ranged from 91.13±4.06 to 95.85±3.52 microns in width and from 105.02±16.05 to 146.91±25.25 microns in length. The average of the total 240 pollen grains was 94.32±5.72 by 128.99±16.32 microns. The variation of each pollen grain in width and in length is from 80.24 to 106.02 microns, and from 82.60 to 194.70 microns, respectively (Fig. 1).

Dry pollen grains appeared to be cup shaped (Figs. 2, 3). The pollen grains are round from the polar view with

the nucleus in the middle (Fig. 3). From the bottom view, there are two shaded areas around the dry pollen and a light part is between them. A nucleus can be found in the middle of the light area. The shaded area is due to the part of cup-shaped pollen grain shadowed on it (Fig. 4). The turgid grains are spherical or elliptical, without a trace of bladders or furrow. The exine is thin, measuring only about 2 microns in thickness. The surface is quite smooth (Fig. 5), with no sculpturing. The intine measures about 8 microns in thickness and is of uniform hyaline appearance (Wodehouse, 1959) (Fig. 6). A protuberance at the proximal pole (Fig. 7) where two degenerated prothallial cells are placed can sometimes be observed. The leptoma (the area with thin exine) at the distal pole described by Erdtman (1957) was not found in this study.

In the basic fuchsin glycerine jelly technique only the exine of the pollen grains stains, leaving the intine and cell contents uncolored. The pore (Figs. 8, 19) of about 2 microns can be observed when the focus is being adjusted.

Data obtained from pollen germination in auxins, vitamins and trace elements are compiled in Table 2. It is shown that pollen germination in trace elements, boric acid and calcium nitrate, is proportional to concentration. From "t" Table for significance test, both of them stimulate the pollen germination at 1000 ppm. Boric acid proportionally increases the pollen growth, but calcium nitrate does not

significantly affect the pollen growth. GA enchances both pollen germination and elongation. IAA, IBA, and NAA increases the pollen germination and elongation in low concentration, but completely inhibits both at high concentration, 1000 ppm. Thiamin stimulates the pollen germination to a considerable extent at 10 ppm, but slightly inhibits it at concentration above 100 ppm. With regard to the effect of thiamin on pollen elongation, it seems that high concentration (1000 ppm) still promotes pollen elongation.

Seed extracts were used to culture the pollen. Two concentrations were introduced as media. The results are presented in Table 3. It is shown that extracts not only increased the elongation but also increased the pollen germination.

Sucrose supplies the pollen grains both nutrients and osmotic milieu. Osmotic pressure of the medium is very important during germination. If it is not balanced, the pollen bursts or shrinks. Extent of germination in sucrose solution is summarized in Table 4 and extent of viability in Table 5. Sucrose aids pollen germination, but not pollen growth. As to the concentration for the culture, it is recommended that 15% is the best when only sucrose is used.

Pollen is cultured in the two stock solutions A and B. Table 6 shows that both solutions give the highest germination percentage and longest elongation of all media used. Also stock solution effectively prevents pollen grains from bursting or shrinking.

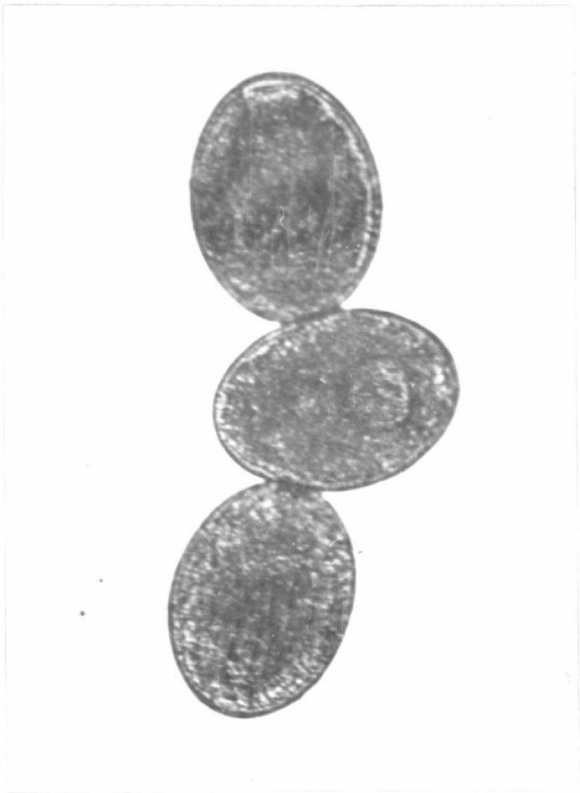


Fig. 1.
Turgid pollen grains
from BCFP 189.
(250X).

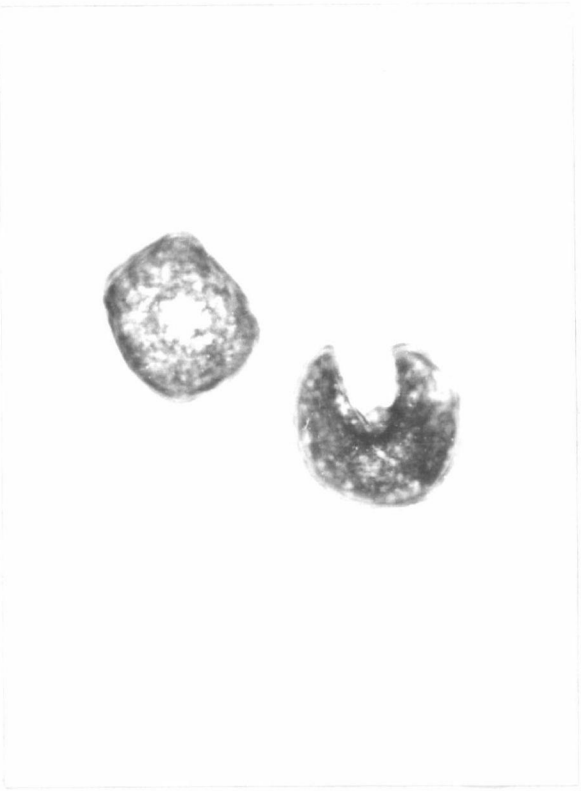


Fig. 2.
Dry pollen grains
(cup shape).
(200X).

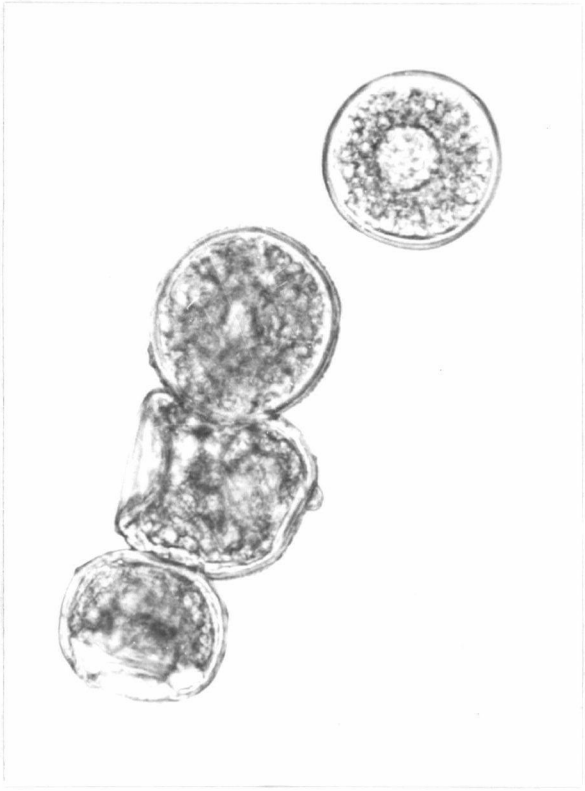


Fig. 3.
Polar view (upper two grains) and side view (lower two) of dry pollen grains.
(240X).

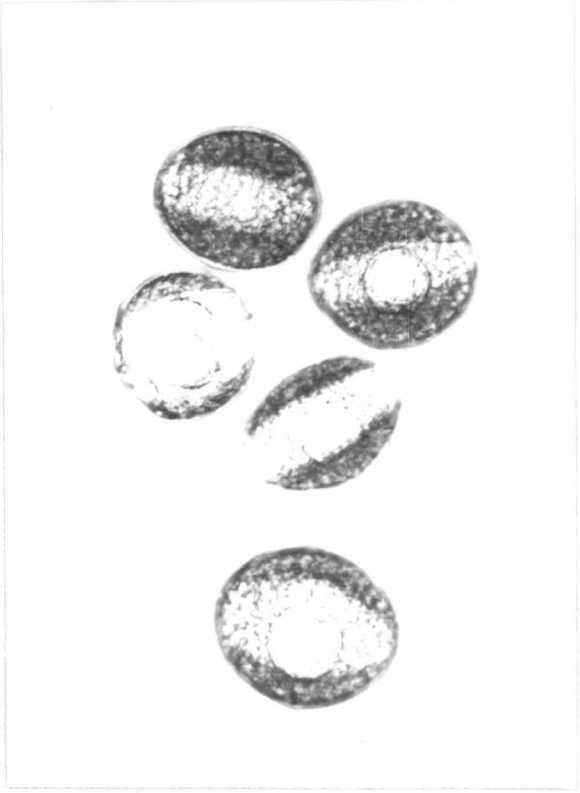


Fig. 4.
Bottom view of dry pollen grains.
(200X).

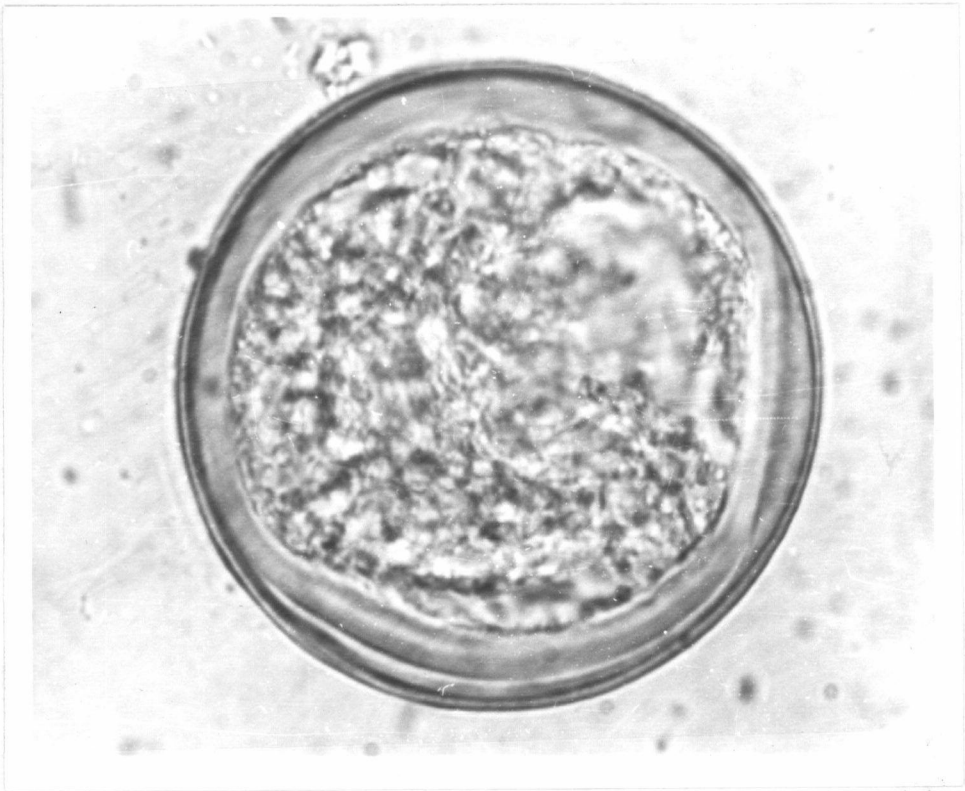


Fig. 5. Smooth surface of exine. (1000X).

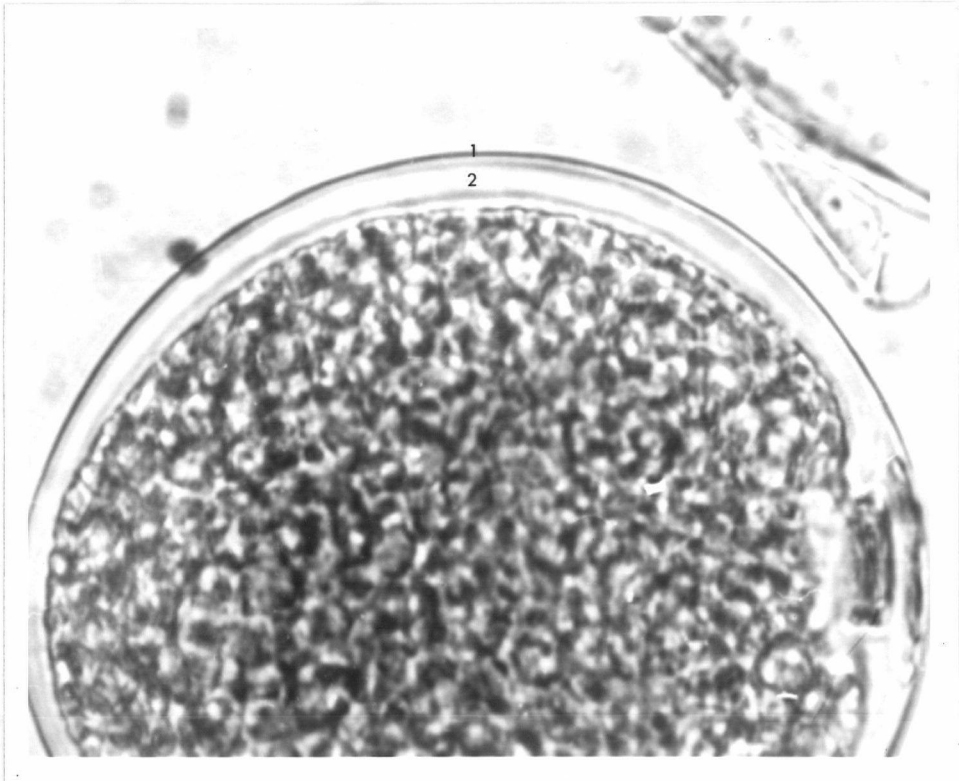


Fig. 6. (1) thin exine, about 2 microns
(2) thick, uniform hyaline intine, about 8 microns. (3000X).

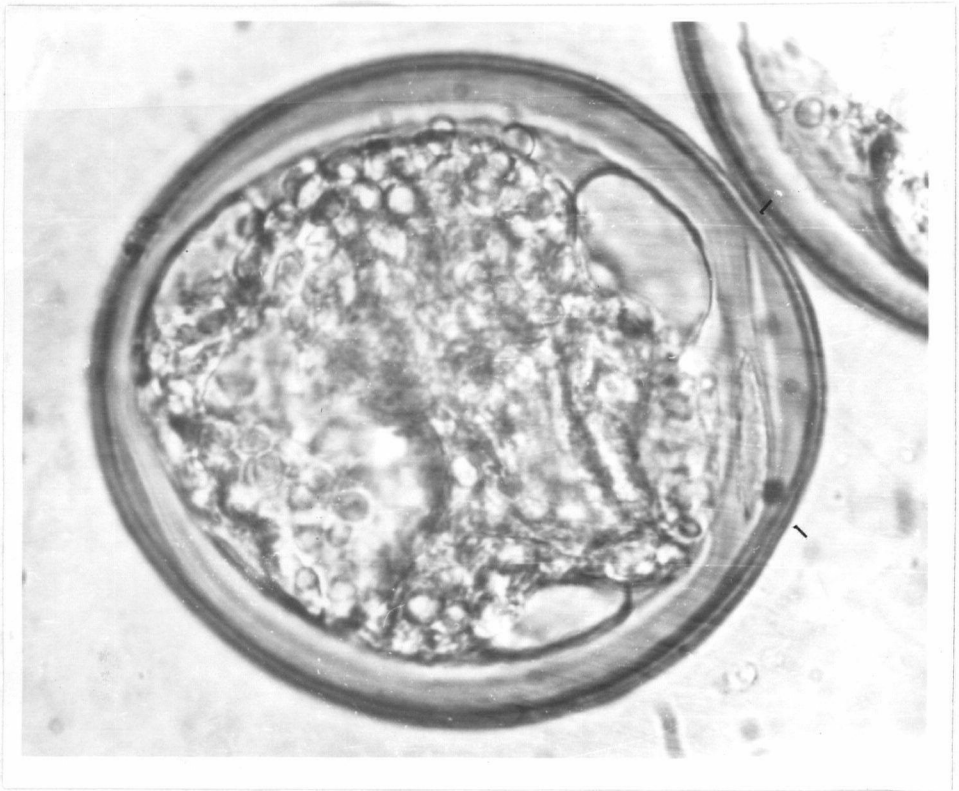


Fig. 7. Protuberance at the proximal pole where one prothallial cell is placed. (1000X).

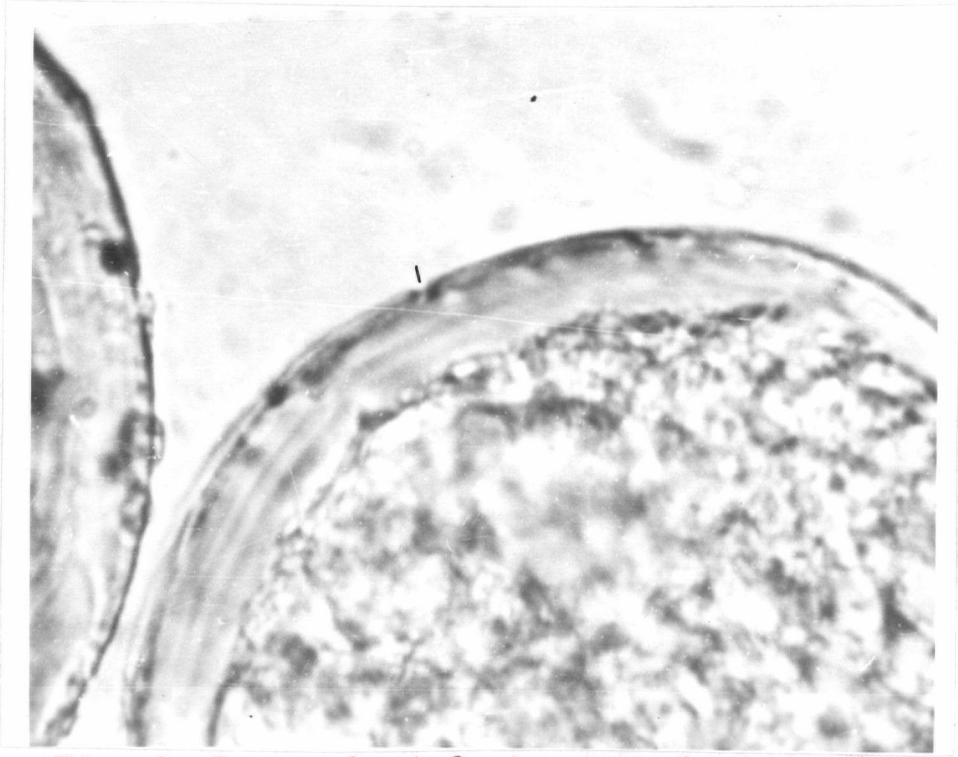


Fig. 8. Pore, about 2 microns, shown in the exine of pollen grain. (2000X).

Table 2

Per cent of germination and the size (mean and standard deviation) of width and length of elongated pollen grains in microns.

Boric Acid	%	Width	Length
0.01 ppm	38.85	85.90±4.21	160.13±16.48
0.1	40.11	86.73±3.98	165.88±16.74
1.0	42.97	86.85±2.63	174.29±15.23
5.0	43.43	88.03±2.12	170.63±17.44
10.0	47.89	85.31±2.54	179.92±16.23
100.0	48.70	87.44±2.46	183.34±15.48
1000.0	40.76	88.13±3.12	177.12±20.31
Calcium Nitrate			
0.01 ppm	41.97	87.44±1.43	160.27±14.28
0.1	38.18	88.03±5.24	159.77±12.21
1.0	42.86	88.62±3.29	153.05±18.32
5.0	43.21	85.67±6.71	160.24±17.79
10.0	43.08	86.61±2.18	158.59±17.55
100.0	47.52	86.26±3.52	165.59±16.84
1000.0	39.62	87.36±3.68	179.36±21.49
GA			
0.01 ppm	40.32	86.62±2.47	151.39±14.12
0.1	44.94	87.44±2.36	150.09±10.02
1.0	49.03	88.85±4.51	160.01±13.27
5.0	51.34	87.32±3.69	162.84±13.45

(Table 2 continued)

GA	%	Width	Length
10.0	53.33	89.44±5.01	161.66±12.09
100.00	50.77	87.56±2.43	153.40±19.83
1000.00	44.79	86.73±2.96	140.03±23.11
IAA			
0.01 ppm	45.80	91.33±3.03	167.91±16.49
0.1	47.01	88.62±2.38	164.41±17.56
1.0	55.46	90.51±2.16	154.11±19.94
5.0	46.72	88.03±5.21	168.86±19.33
10.0	49.30	87.67±5.40	157.94±25.83
100.0	46.96	88.06±3.24	151.40±21.14
1000.0	8.03	----------	----------
IBA			
0.01 ppm	37.21	87.20±3.45	160.83±14.78
0.1	50.95	88.62±2.93	161.66±14.42
1.0	46.40	90.30±2.89	156.38±15.63
5.0	45.73	86.85±3.56	170.75±26.31
10.0	45.00	89.09±3.74	157.32±20.05
100.0	50.53	89.21±3.80	154.34±19.92
1000.0	4.31	----------	----------
NAA			
0.01 ppn	34.74	85.00±6.83	158.86±14.65
0.1	48.76	87.08±2.52	169.33±17.59
1.0	48.95	89.21±3.09	161.37±17.77
5.0	54.14	92.16±2.21	169.21±16.12

(Table 2 continued)

NAA	%	Width	Length
10.0	45.04	86.61±7.35	159.89±18.12
100.0	30.58	87.08±5.20	160.95±20.13
1000.0	4.49	----------	----------
Thiamin			
0.01 ppm	46.97	88.38±3.70	157.18±15.43
0.1	44.04	90.15±2.98	162.20±16.30
1.0	49.06	88.97±2.69	168.03±16.47
5.0	49.83	88.15±2.34	157.06±19.83
10.0	51.33	88.74±5.21	158.59±21.04
100.0	37.00	86.49±8.27	157.41±21.33
1000.0	35.67	87.30±5.36	174.32±24.23
Control			
0.00 ppm	38.17	87.30±1.17	150.48±10.58

Table 3

Per cent of germination and the size (mean and standard deviation) of width and length of elongated pollen grains in microns in seed extracts.

Concentration	%	Width	Length
1 g./100 ml.	42.36	86.74±3.77	160.62±25.48
10 g./100 ml.	45.61	89.20±8.02	167.43±28.55

Arasan which is usually used to sterilize the seeds was introduced to the medium for the pollen germination test. The results are shown in Tables 7 and 8. In high concentrations (more than 500 ppm), Arasan inhibits the germination and growth of pollen. The lower concentrations (less than 500 ppm) of Arasan do not have much effect on germination and on growth. Stock solution B with Arasan makes no difference to the pollen germination and elongation as compared to stock solution only.

Fortimycin (133333 IU) was diluted to various concentrations for ther germination test. The results are shown in Tables 9 and 10. In low concentrations, it seems fortimycin does enhance the germination and growth of pollen, but high concentration decreases the germination and growth. After 54 hours of incubation, it is shown that fortimycin inhibits the growth of pollen. When stock solution B is combined with a low concentration of fortimycin, it enhances pollen germination and growth improvement.

Pollen grains were incubated in a solution containing stock solution B, 10.0 ppm IAA, and a series of 5, 8, 10, 15, 20 per cent sucrose. The results are shown in Table 11. After 24 hours' incubation, the two cell stage could be found: the generative cell and the tube cell (Fig. 9). After two days, the three cell stage, i.e., the tube cell, body cell and stalk cell could be seen in the elongated pollen where the body cell is between the other

two cells. Stalk cell is at the end of the pollen where two degenerated prothallial cells are cut off (Fig. 10). In five days' incubation, the body cell divides to form two sperm cells where two overlap and remain in the original position (Fig. 11).

Ultraviolet radiated pollen grains were incubated in stock solution B for the germination test. It seems ultraviolet light (UV) does not give any vital effects under 8 hours' irradiation. It is shown in Table 12.

When the grains are moistened, they swell and begin to germinate; the exines split open and are subsequently thrown off gradually or all at once. The exine usually splits in the following two appearances:

1) the exine separates into two cups, equal or one larger than the other (Fig. 12);
2) the exine splits wide open (Fig. 13, 14), the cast off exines have a tendency to curl tightly inward. The possibility of dehiscence in the two ways in 15% sucrose medium is compiled as follows:

	No. of Pollen Counted	%
Exine separates in two	777	75.73
Exine splits wide open	249	24.27
Total	1026	100.00

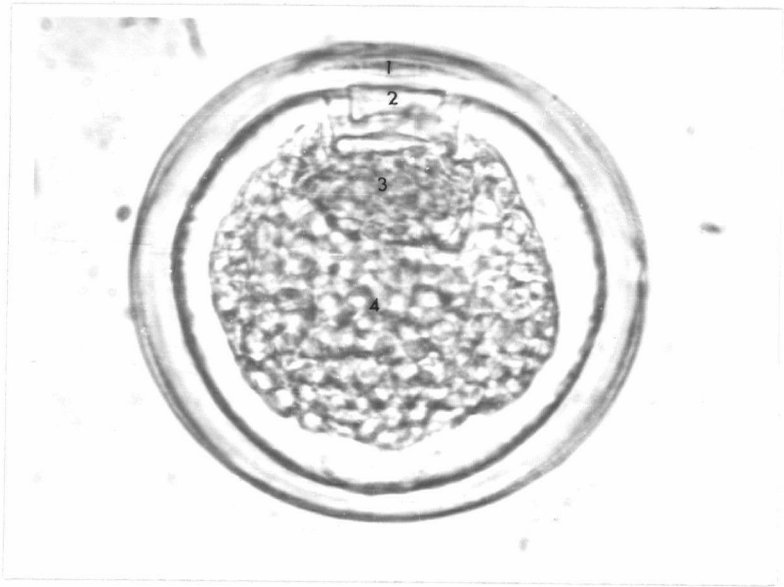


Fig. 9. Two-nucleate stage. (1) first prothallial cell (2) second prothallial cell (3) generative cell (4) tube cell. (600X).

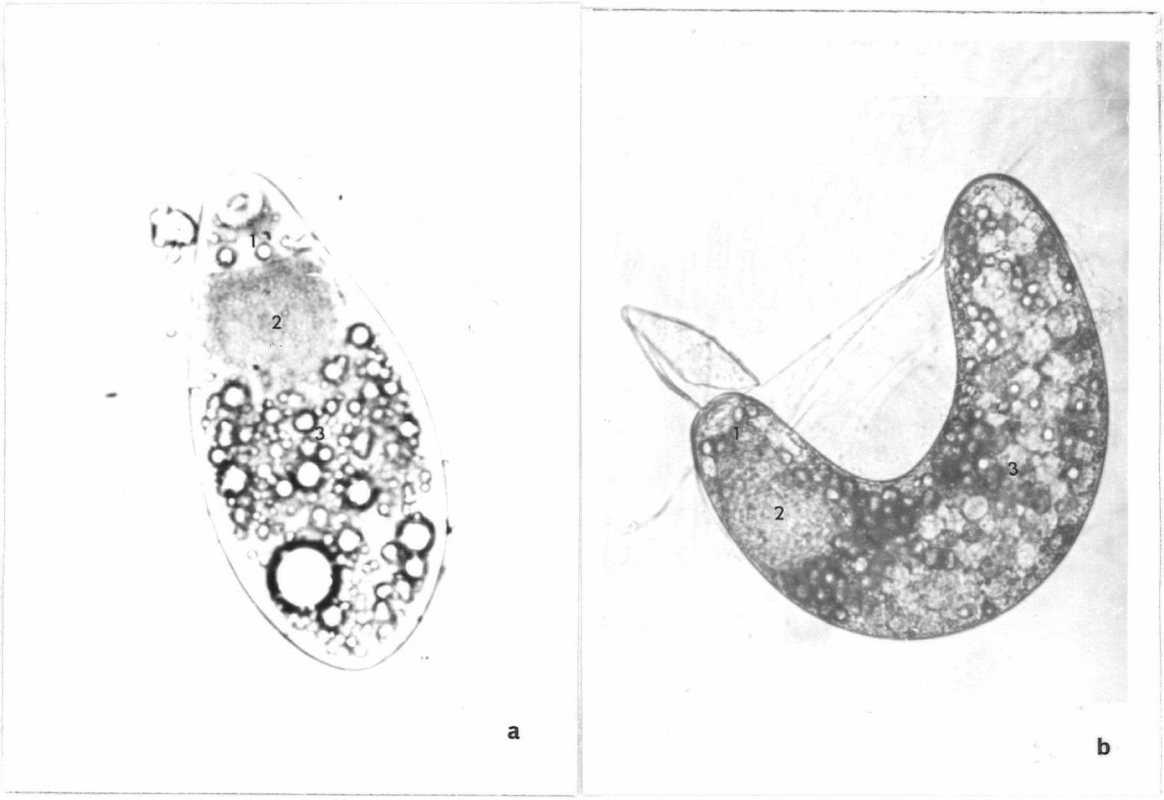


Fig. 10. Three nucleate stage. (1) stalk cell (2) body cell (3) tube cell. (250X).

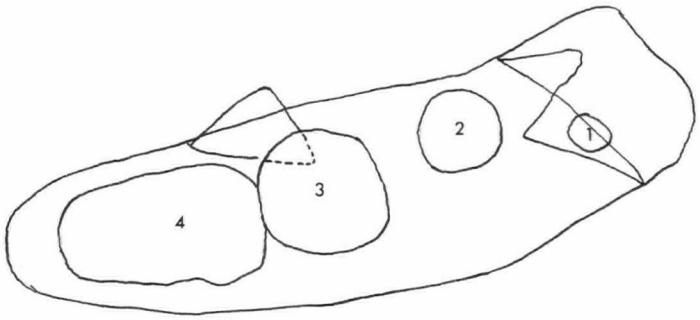
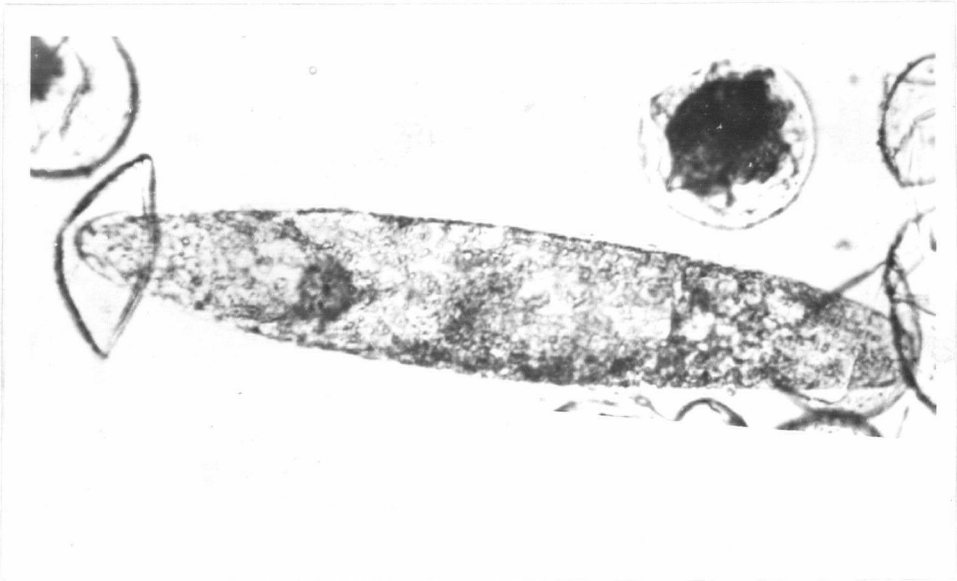


Fig. 11-1. (200X).

Fig. 11. Four nucleate stage. (1) stalk cell
(2) (3) sperm cells. (4) tube cell
(5) first prothallial cell.
(6) second prothallial cell.
(N) nucleus.



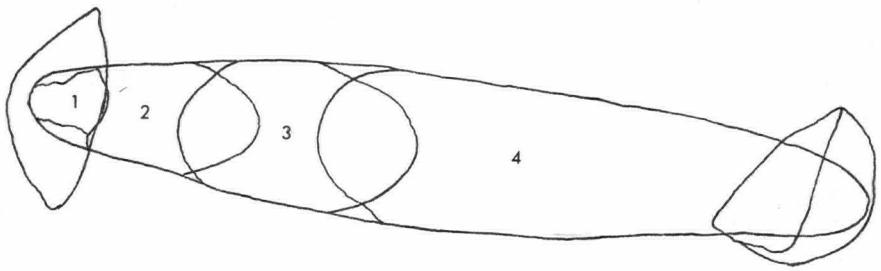
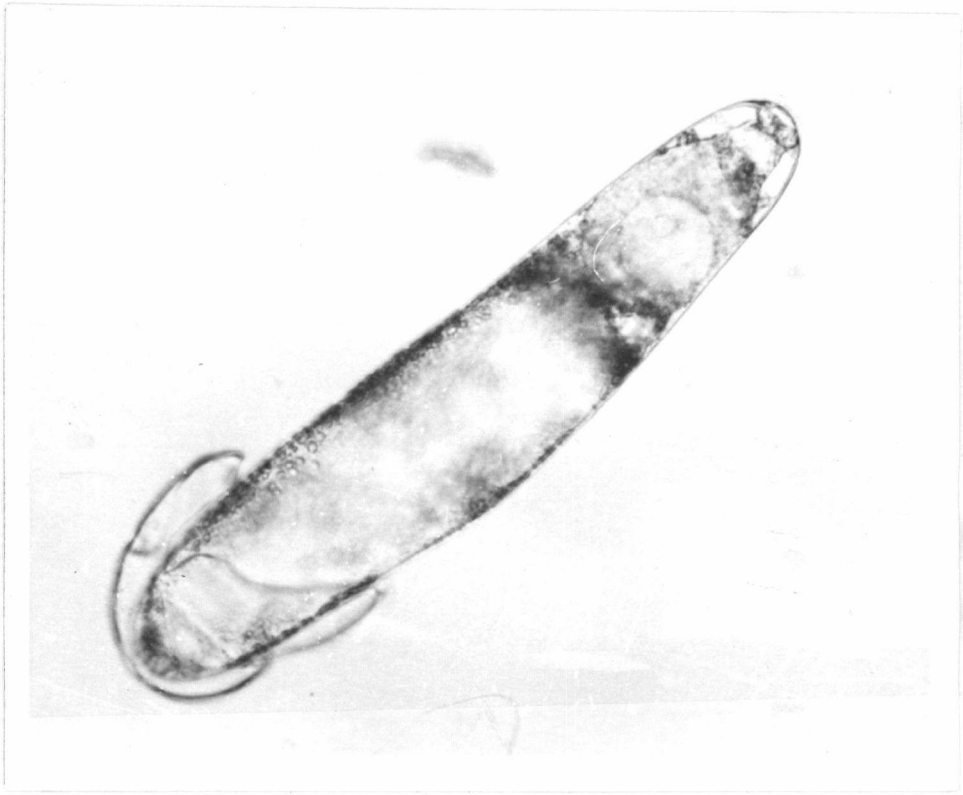


Fig. 11-2.
(250X).



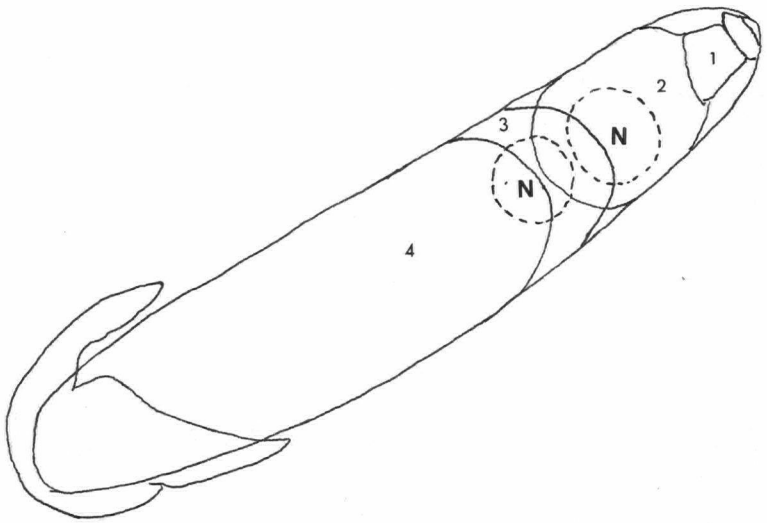


Fig. 11-3.
(300X).

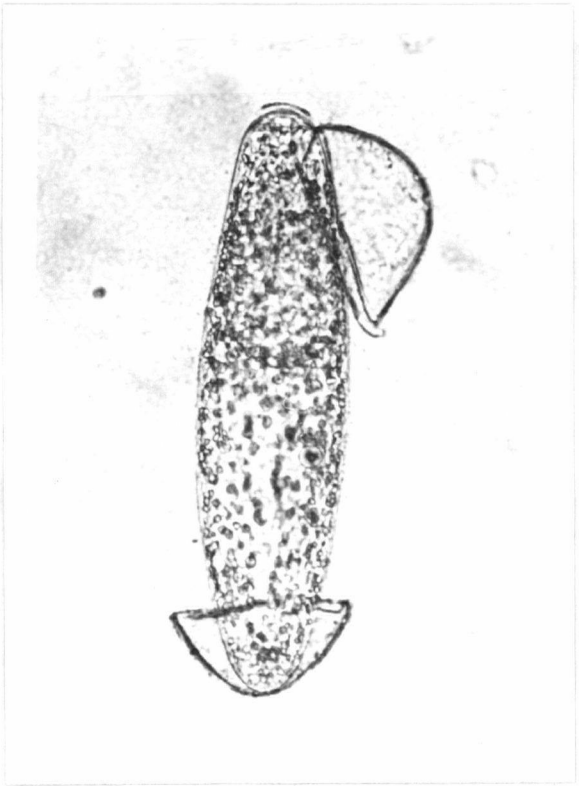


Fig. 12.
Exine separates in two parts. (250X).

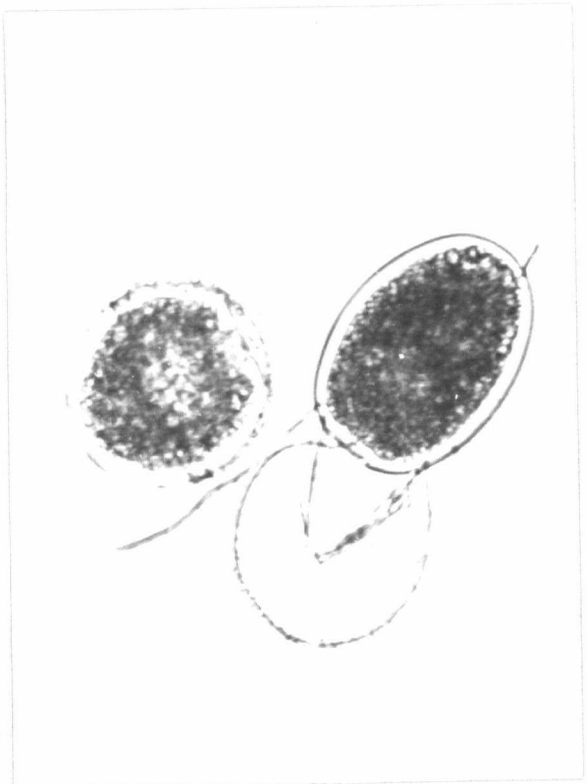


Fig. 13.
Exine splits wide open at one end. (200X).

Table 4

Per cent of germination and the size (mean and standard deviation) of width and length of elongated pollen grains in microns in sucrose solution.

Concentration	%	Width	Length
5%	57.02	87.67±5.78	145.18±20.53
8%	56.65	87.79±4.60	146.79±23.24
10%	57.75	88.21±4.48	145.24±30.90
15%	56.08	86.33±3.12	152.22±15.22
20%	63.83	88.25±2.86	150.29±18.17
Average	58.26	87.86±4.28	147.53±23.75
Control	40.56	89.45±6.34	158.35±24.42

Table 5

Per cent of viability of pollen grains in sucrose solution.

Concentration	non-germination	Germination		
		viable	bursting	shrinkage
8%	43.35	24.84	24.29	7.25
10%	36.49	28.19	25.12	10.09
15%	43.91	48.87	0.75	6.47
20%	36.17	52.26	0.35	11.04

Table 6

Per cent of germination and the size (mean and standard deviation) of width and length of elongated pollen grains in microns in stock solution.

Stock Solution	%	Width	Length
A	59.82	87.67±4.01	183.61±27.84
B	54.08	85.90±6.84	188.68±27.61
Average	56.95	86.78±5.42	186.14±27.73
Control	34.85	87.79±6.31	157.06±14.93

Table 7

Per cent of germination and the size (mean and standard deviation) of width and length of elongated pollen grains in microns in Arasan solution.

Concentration	%	Width	Length
5 ppm	40.80	85.67±3.41	152.14±23.36
10	35.19	85.90±5.02	158.88±19.47
50	38.29	86.73±4.18	157.47±17.46
100	40.25	88.50±2.25	153.87±17.81
500	36.01	87.33±2.13	147.11±19.47
1000	28.04	88.64±4.32	142.63±16.75
Control	37.96	87.79±6.38	152.37±20.17

Table 8

Per cent of germination and the size (mean and standard deviation) of width and length of elongated pollen grains in microns in stock solution with Arasan.

Concentration	%	Width	Length
1 ppm+stock B	60.59	87.44±2.39	161.07±17.34
5 +stock B	66.47	85.67±4.41	164.46±15.28
10 +stock B	58.94	85.90±4.38	179.12±18.40
50 +stock B	60.10	86.73±4.63	180.18±25.70
100 +stock B	60.20	88.50±3.12	159.30±30.08
Control	39.36	89.04±7.67	157.77±16.52

Table 9

Per cent of germination and the size (mean and standard deviation) of width and length of elongated pollen grains in microns in fortimycin solution.

Concentration	%	Width	Length	
			24 Hrs	54 Hrs
10.44 IU	42.67	86.14±4.15	160.95±17.43	171.34±21.52
20.83	44.95	87.56±2.45	169.21±14.59	170.51±20.13
41.66	43.96	89.44±2.76	156.33±15.48	163.56±23.45
83.33	43.77	88.26±3.51	146.08±16.78	152.78±21.03
166.66	47.30	88.77±1.83	142.93±15.62	155.64±18.48
333.33	37.47	89.56±2.03	142.31±20.43	142.24±15.23
666.66	32.60	88.26±4.21	132.08±19.21	143.83±14.45
1333.33	30.02	88.85±2.53	136.33±19.35	134.93±15.85

Table 10

Per cent of germination and the size (mean and standard deviation) of width and length of elongated pollen grains in microns in stock solution B with fortimycin.

Concentration	%	Width	Length
10.44 IU+stock B	59.91	82.84±6.24	192.10±20.36
20.83 +stock B	60.00	84.24±4.19	175.58±14.98
41.66 +stock B	54.96	88.50±2.38	200.60±25.48
83.33 +stock B	55.25	86.14±5.06	168.27±14.42
166.66 +stock B	56.95	90.39±2.41	176.06±21.13

Table 11

Per cent of germination and the size (mean and standard deviation) of width and length of elongated pollen grains in microns in the following solution.

Contents of Solution	24 Hours %	24 Hours Length	48 Hours %	48 Hours Length
5% sucrose 10 ppm IAA stock B	67.31	174.99±26.58	66.54	205.44±19.48
8% sucrose 10 ppm IAA stock B	65.48	185.99±22.61	67.69	207.68±27.55
10% sucrose 10 ppm IAA stock B	63.13	168.74±18.43	70.56	218.30±23.39
15% sucrose 10 ppm IAA stock B	66.88	183.59±17.68	70.86	208.61±29.34
20% sucrose 10 ppm IAA stock B	69.57	173.39±25.74	68.74	217.31±20.27
Control	38.92	168.49±15.38	40.95	189.29±20.31

The number of cases in which the exine splits wide open is one third of those in which the exine separates in two. There are also two other methods in the dehiscence of exine but they can seldom be found under the microscope.

1) Two small ends of exine are cut off and the remaining ribbon-like exine is around the middle of the growing pollen (Fig. 15).
2) One end of exine is cut off and the germinating pollen seems to be squeezed out from the exine (Fig. 16). Sometimes the exine splits open and gives way to the growing pollen (Fig. 17).

Table 12

Per cent of germination and the size (mean and standard deviation) of width and length of elongated pollen grains irradiated by UV in microns in stock solution B.

Time of Irradiation	%	Width	Length
1 hr	66.08	86.85±4.60	158.71±15.73
2	57.57	88.15±3.50	157.06±14.15
3	59.89	86.97±4.25	164.59±16.31
4	64.53	86.26±2.58	156.11±15.48
5	61.16	87.56±3.01	157.88±16.73
6	58.33	87.56±3.21	167.79±20.18
7	64.31	87.79±2.35	173.97±11.23
8	64.31	85.19±4.12	163.19±14.58
Control	36.62	87.44±6.46	154.95±18.67

Fig. 14.
Exine splits wide open in the middle of the pollen and then the growing pollen tears the exine apart.
(250X).

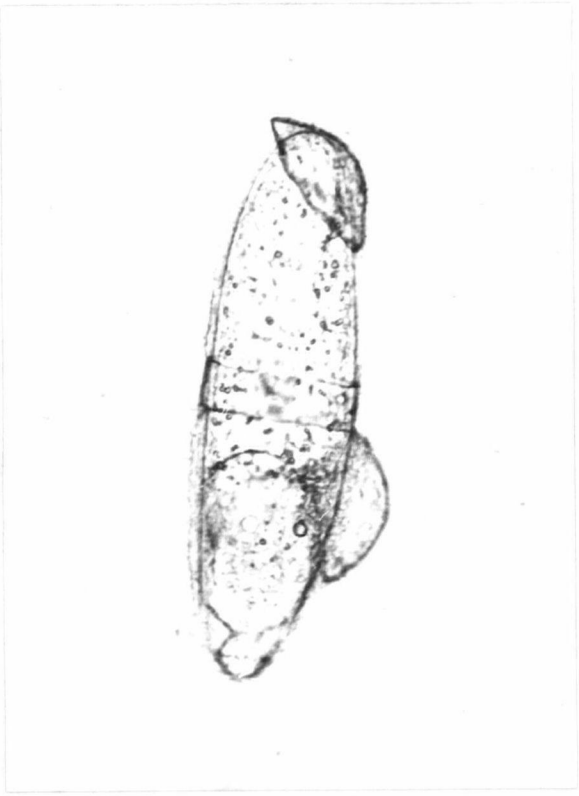


Fig. 15.
Two ends of exine were cut off and one ribbon-like part of exine formed a ring around the pollen.
(200X).

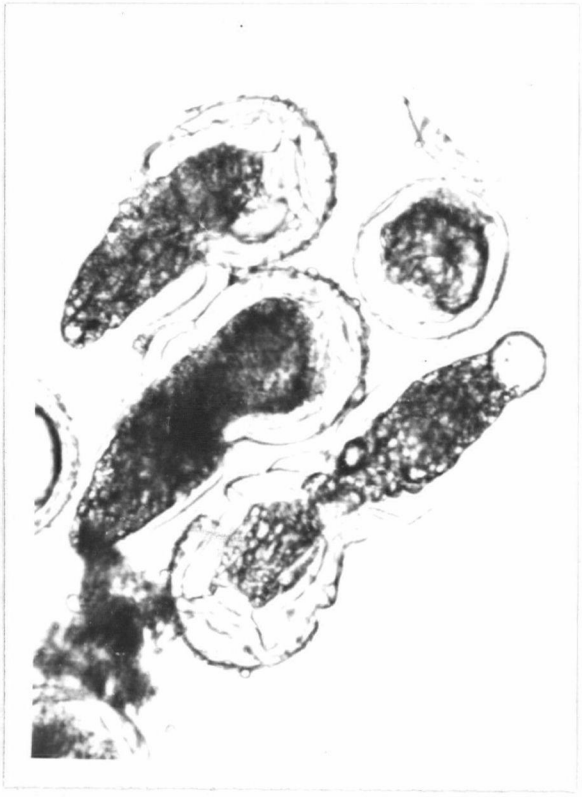


Fig. 16.
Abortive pollen grains. One end of exine is cut off and pollen grains are squeezed out. (200X).

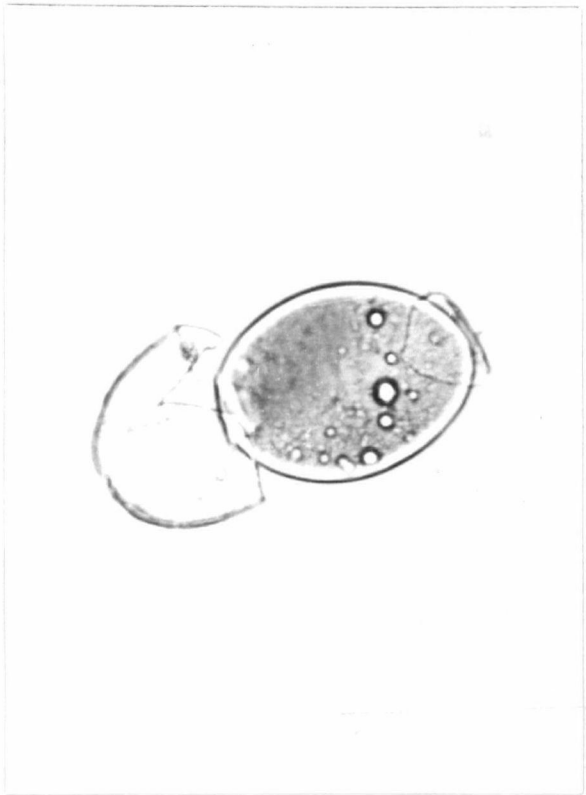


Fig. 17.
One end of exine is cut off and the part left splits open. (250X).

DISCUSSION

A. Pollen Size

The pollen grains of the Pseudotsuga species have been described by several authors. The measurements of diameter are summarized in Table 13.

Table 13

The diameter of Pseudotsuga pollen grain sizes.

Authors	Years	Species	Size (microns)
Bunnell	1965	P. menziesii	91.07
Eisenhut	1961	P. menziesii	84.8x81.1
Erdtman	1943	P. menziesii	80x93
Sziklai	1964	P. menziesii	91.08-99.19
Van Campo	1950	P. menziesii	140
Wodehouse	1935	P. mucronata	90-100
Erdtman	1965	P. rhederi	75-115
Ueno	1958	P. japonica	85
Yamazaki	1962	P. japonica	80-85

The average size reported by Wodehouse (1935) utilizing stained preparation was 90 to 100 microns. Van Campo Duplan obtained an average size of 140 microns from fresh Douglas-fir pollen.. Sziklai (1964) reported the diameter of pollen grains varied considerably between four trees investigated, from 91.08 to 99.19 microns. The average diameter calculated from 1000 measurements by Bunnell (1965) was 91.07 microns, ranged from 67.7 to 137.8 microns. Turgid pollen appears spherical or oval, usually an oval. Therefore the

measurement of width and length is necessary. In this study, the average of fresh pollen from 240 grains was 94.32±5.72 by 128.99±16.32 microns ranging from 80.24 to 106.20 microns in width, and from 82.60 to 194.70 microns in length. The minimum and maximum values of width appeared to be 70.80 and 108.56 microns, respectively.

From the above information, the measurement of pollen size varied one from the others. Wodehouse gave the lowest values but Van Campo Duplan, the highest one. The reason Wodehouse got the lowest values was due to use of stained preparation. In this study, it was found the pollen grains of stained preparation gave values of about 20 microns lower than that of fresh preparation. Van Campo Duplan obtained an average 140 microns in fresh preparation. It is believed that this is the average length of pollen grains, since in the present study, the length of pollen grains of BCFP 37 and 135 is over 140 microns.

B. Dry and Turgid Pollen Grains

The dry, living pollen grains which were collected at room temperature and stored in a vial at 0°C to 2°C in the refrigerator are relatively small. The exine is contracted and the furrow appears to make the grain cup-shaped (Fig. 2, 3). The exine of dry pollen grains is in network-like appearance (Fig. 18).

It is believed that in recent investigations the so-called apertures are not really open but are covered by extremely thin parts of exine (Larson and Lewis, 1961). Eisenhut (1961) reported the presence of an extraordinarily large pore in Douglas-fir pollen and supported his claim with photographs. He described the typical or average pore as being somewhat oval-shaped, 56 by 70 microns in size and 18.6 microns deep. Wodehouse (1959) presented a schematic optical cross-section of a Douglas-fir pollen grain without an aperture.

In this study Eisenhut's claim was not supported and it was found that Douglas-fir pollen has pores in the exine. The average pollen size is 94.32 ± 5.72 by 128.99 ± 16.32 microns and thickness of exine and intine is about 10 microns. But it is impossible that Douglas-fir pollen has an oval-shaped pore, 56 by 70 microns in size, and 18.6 microns deep, almost the same size as pollen. However, the thickness of exine and intine is about 10 microns. Eisenhut presented a photograph of Douglas-fir pollen in which the pollen grains seem to be in a dry state, the same as in Fig. 3 (the two lower pollens). The furrow of dry pollen in cup shape seems to be a pore from the side view. Maybe Eisenhut had mistaken the furrow of dry pollen as his so-called pore.

The exine consists of one of the most extraordinarily resistant material known in this organic world. The more differentiated, thick exine shows various layers and

consists of sporollenin, a high-molecular terpene (Zetschke, 1928, 1932). Also the exine channels remain evident as do the cytoplasmatic strands through the intine. These channels are suggested to be the route for material used during the development of exine moving from the protoplasm to the exine (Larson, 1961). In a relatively dry state the pollen grain invaginates into the proximal part producing a convex or cup shape. However, the turgid grain appears spherical or oval. A pore can be seen by using basic fuchsin glycerine jelly techniques (Fig. 8, 19). Comparing two apertures in Fig. 19, we can easily recognize that one is the breakage of exine of germination pollen, the other is the pollen pore. It was found that Douglas-fir pollen does not grow by pollen tube but pollen elongation. So it seems that the pores in the pollen are the points where exines split.

With regard to the pore in the intine, I can confirm Barner and Christiansen's statement (1962). There is an aperture at the proximal pole of the pollen grains (Fig. 20, 23), where two small discs - the two degenerated prothallial cells, a flat one and the other concave are found near the pore. It seems that the orifice of the pore is enclosed by a membrane (Fig. 21).

The turgid pollen is spherical or elliptical, usually in an elliptical shape, which is free of sacs or

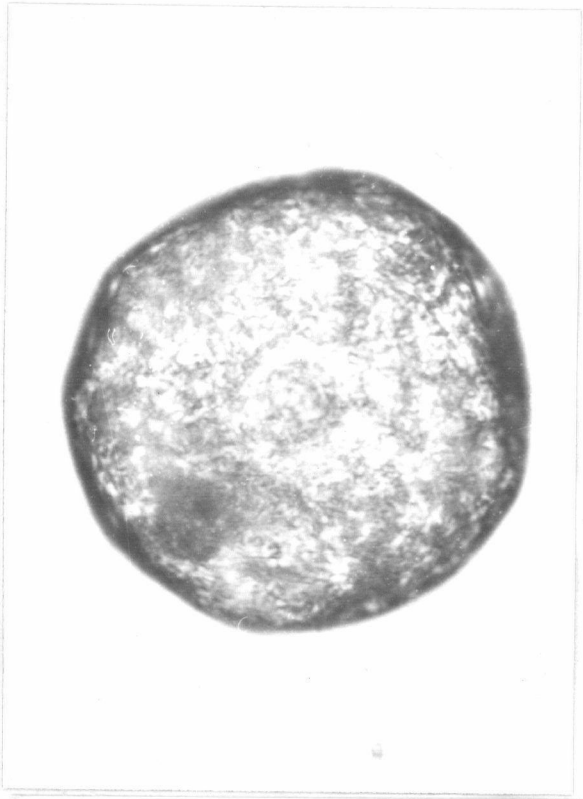


Fig. 18.
The network-like exine of dry pollen grain in polar view.
(750X).

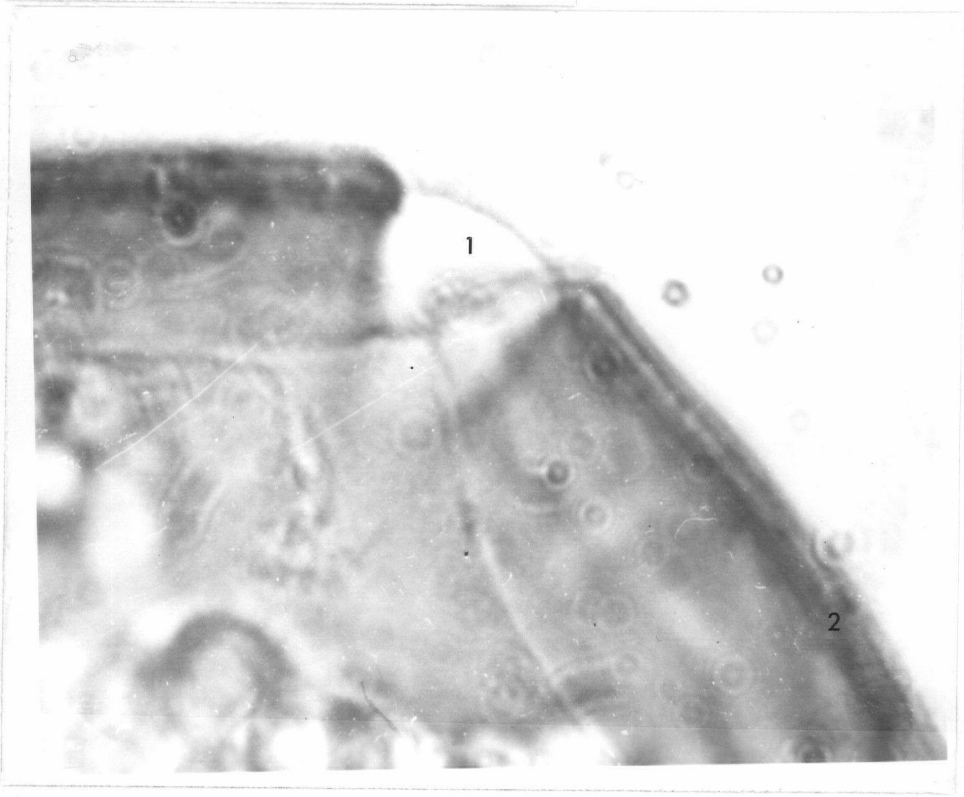


Fig. 19. (1) The breakage of the exine of germinating pollen grain.
(2) The pore in the exine of pollen grain.
(3000X).

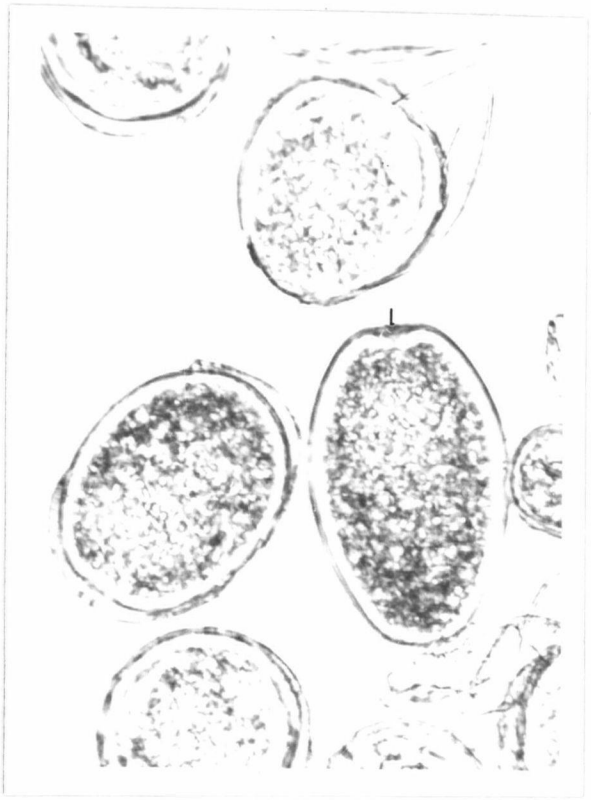


Fig. 20.
The pore in the intine of pollen grain. (labeled). (250X).

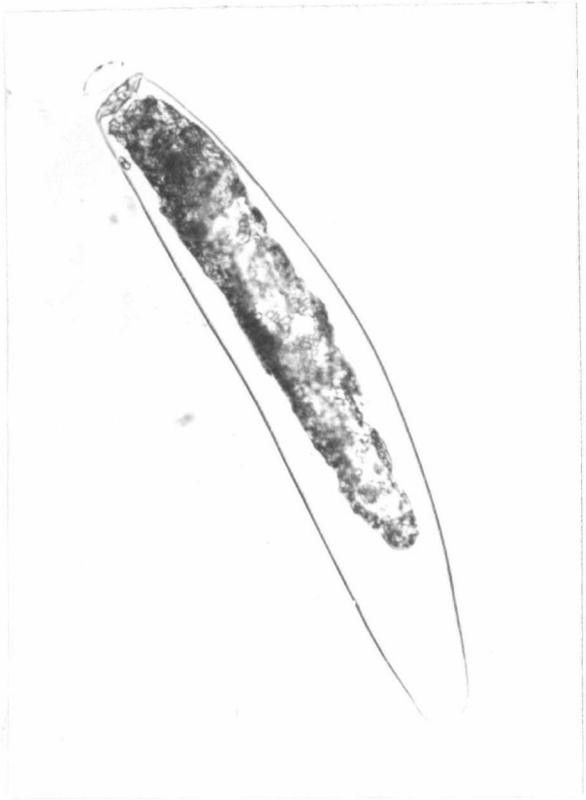


Fig. 21.
Shrinkage of elongating pollen grain. (200X).

furrows. The exine is thin and quite smooth, about 2 microns thick. The intine is thick and is of uniform hyaline appearance, about 8 microns thick (Fig. 5, 6).

The color of Douglas-fir pollen is yellow or yellowish. It is caused by pigment matter localized in the caves and holes of exine in the form of oil and fat, (Freytag, 1959). Changes in the color of the oil during storage seem to be accompanied by a loss of germination power (Pfeiffer, 1955). Protection against ultraviolet radiation is a function of this pigment (Asbeck, 1954).

C. Effects of Nutrients During Pollen Germination

a. Roles of Boron and Calcium

On the basis of present evidence, the role of boron in pollen germination and pollen tube growth may be three-fold (Vasil, 1960):

"(a) it promotes absorption and metabolism of sugars by forming sugar-borate complexes,
(b) it increases oxygen uptake, and
(c) it is involved in the synthesis of pectic material from the wall of the activity of the elongating pollen".

In this experiment, boron increased not only the germination percentage but also the pollen elongation. It stimulated the germination and growth to a considerable extent. The stimulation of pollen is due to the presence of boron which increases it viability and prevents bursting. It plays a very important role in the metabolism.

Pollen grains are low in calcium content, averaging about 0.03% (Todd and Bretherick, 1942). Gymnosperm pollen appears to have lower calcium contents than angiosperm pollen, ranging between 0.03% and 0.04% for the three species studied by Todd and Brethrick. Calcium plays many important roles in plant growth to which pectin seems to be the most pertinent to the pollen studies. Calcium relates primarily to the binding of calcium ion to pectate carboxyl groups along the pollen wall which is incorporated non-metabolically, in exchangeable form (Brewbaker and Kwack, 1963). It was found that the factor to poor germination and growth in small pollen populations, but excellent in large pollen populations, is shown to be the calcium ions (Brewbaker and Kwack, 1963). Also deficiency of calcium may play an important role in natural pollen mutation rates, as well as in the increasing rates of mutation during prolonged pollen storage (Brewbaker and Kwack, 1963). This experiment shows the population effect can be overcome by the calcium ions. Germination and growth of small pollen populations in solution with calcium ions are similar to that of the large population. Also the relation of calcium to the germination rate is determined by its concentration. The higher the concentration the better the germination, but not the pollen elongation. The length of pollen elongation is not affected very much, by the calcium concentration.

For preventing pollen from bursting, boric acid

works more efficiently than calcium. Calcium improves the germination percentage and elongation, but not the viability. Boric acid does not exactly prevent the pollen grains from bursting, but the percentage of bursting inversely relates to the concentration. Rapid elongation and bursting at the tip under osmotic stress, responds markedly to exogenous calcium ion and borate and gives no convincing response to auxins.

Stock solution containing boric acid, calcium nitrate, magnesium sulfate and potassium nitrate not only increases the germination percentage and pollen elongation, but also effectively prevents the pollen grains from bursting. Pollen germination percentage is 56.95 in stock solution and pollen length, 186.14 microns. The balance of osmotic pressure between the cytoplasm in the pollen and the outside medium depends upon stock solution. There are three possible roles that boric and calcium ions have played, (a) the borate could react with sugar and pass through the cellular membranes as the ionized sugar-borate complexes until such time as a cell utilizes this complex and liberates the boron ion, (b) the borate associated with the cellular membranes react chemically with sugar molecular facilitating its passage through the membrane. The sugar is released inside the cell by a second reaction (Ganch and Duggar, 1953), (c) boric acid ions may play a

role to membrane pore by controlling the water and ions in and out the cellular membrane. Borate does increase the rate of calcium ion uptake and in an exchangeable form. Cationic equilibrium involving calcium is requisite to the normal permeability and selectivity properties. In addition, the problem of population effect does not exist in the stock solution.

b. Roles of Growth-Promoting Substances

Pollen germination was stimulated by growth-promoting substances, but pollen elongation was not markedly accelerated. The germination percentage was directly related to the concentration of GA, IAA, IBA, NAA and thiamin up to a certain concentration after which a decrease became evident.

The concentration of GA and thiamin at 1000.0 ppm gave a little inhibition to the pollen germination, while IAA, IBA and NAA completely inhibited the germination of pollen. A few pollen grains germinated but burst afterwards. No viable pollen could be measured. Growth-promoting substances stimulated the germination and improved the growth, but could not prevent the pollen from bursting. The viability of pollen is not high.

c. Roles of Sucrose

Pollen grains do not contain chlorophyll and are dependent on internal and external sources for the supply of essential nutrients. They only contain some reserve food

which is utilized during the initial stages of germination, but is not enough for continued growth. The reserve food may support growth until it is consumed. However, in the culture medium, the nutrients stimulate and improve the germination and elongation of pollen grains.

When pollen grains of Douglas-fir were cultured in deionized water for 24 hours, the germination percentage was 40.56 and the size was 89.45±6.34 by 154.35±24.42 microns. If some sucrose was added, the average germination percentage increased to 58.26 and the size decreased to 87.86±4.28 by 147.53±23.75 microns. Even though the range of pollen length in the sucrose medium was lower than the control, the germination percentage of the former was rather higher than that of the latter. The externally supplied sucrose in the medium improved the germination of pollen.

Pollen grains are cultured in a series of sucrose medium 8, 10, 15 and 20 per cent for viability test. It was found that 15 percent sucrose was the best for Douglas-fir pollen for either nutrient purposes or osmotic milieu. When the concentration of sucrose was over 15 percent, the cytoplasm in the elongated pollen shrank and was misshapen (Fig. 21). However when the concentration was below 15 percent the osmotic pressure of medium was so low that the elongated pollen burst after germination (Fig. 22). It served as a nutrient material for the growing pollen.

Apart from the nutrient purpose, it creates favourable osmotic condition for germination and growth.

Using pollen grains, incubated in stock solution plus 10 ppm IAA and a series of sucrose for two days, it was found that solutions containing 15 percent sucrose were optional for germination, growth and osmotic milieu. The osmotic role was performed by sucrose, stock solution, and IAA. After one-day culture in stock solution plus 10 ppm IAA and 15 percent sucrose, the germination percentage was 66.88, and length 183.59±17.68 microns; two-day culture, the extent of germination was 70.96 percent and length, 208.62± 29.34 microns. Bursting in 10 percent sucrose and shrinkage in 20 percent sucrose solutions were higher than those in 15 percent sucrose solution.

Ching and Ching (1959) germinated Douglas-fir pollen on a basic medium containing 10 percent sucrose and one percent agar. He got a high germination percentage of 88.4 and growth, 209.09 microns in length for 46 hours. Therefore, 10 to 15 percent sucrose will be recommended to make pollen suspension solution for artificial pollination.

d. Effects of Fungicide and Bactericide

Arasan was tested in this experiment. In low concentration, Arasan did not inhibit the germination and growth of pollen, but could not keep the pollen from bursting. In high concentration, Arasan inhibits not only the germination percentage but the elongation of pollen. When

Arasan combined with stock solution B, it had no effect on pollen germination and growth. Johnson (1963) reported that lab studies showed that viability decreased with increasing concentration of insecticide (Guthion and Sevin) and duration of immersion.

A series of fortimycin was tested in pollen germination. Concentration higher than 333.33 IU caused the decrease of pollen germination and elongation. Concentration under 166.66 IU seemed to enhance both. This may be due to fortimycin containing penicillin which is contaminated with other chemicals. It is known that stretomycin, contaminated with phenylacetic acid in the medium, improves the germination and growth of pollen (Pulvertaft, 1946; Vasil, 1958, 1960; Sen, 1960).

Douglas-fir pollen grows very slowly. As a result we face the contamination of fungi and bacteria in long-term cultivation in vitro. All of the sterilized instruments became contaminated. This is due to the pollen itself. The best way to obtain germinating pollen without microbial contamination is by the method described by Petru et al. (1964), or to add fungicide and bactericide to culture medium to prevent contamination. But, low concentration of fungicide and bactericide did not prevent contamination of micro-organism and bursting of pollen. In high concentration it inhibited the germination and growth. Use of fungicide and bactericide in culture medium in physiological experiments requiring long-term cultivation of pollen under

artificial conditions is not recommended.

e. Effects of Seed Extracts

Water extraction of seed raised the germination percentage and growth. However, it did not show any considerable improvement but had the same effects of boron and other growth-promoting substances. The effect of extracts from one gram of dry seeds per 100 ml. of water were not much different from that of 10 gram per 100 ml. of water on germination and elongation. The latter did retain the balance of osmotic pressure in and out of the pollen cytoplasm and kept the pollen from bursting.

f. Effects of Ultraviolet Radiation

It is known that ultraviolet radiation for as little as 8 hours is sufficient to cause an important decrease of germination capacity (Asbeck, 1954, 1955). It can be shown that a relative change in the color of pollen takes place depending on the intensity of ultraviolet radiation. The pigment protects against ultraviolet radiation. According to the chemical nature of the pigment, this filter works either by chromatic absorption alone or with fluorescence (Asbeck, 1954, 1955). Douglas-fir pollen was irradiated by 30 watt ultraviolet light at a distance of 50 cm. for as long as 8 hours and then incubated in the stock solution B. It gave the same results as the pollen without irradiation. It seems that the pigment in exine serves

quite well as ultraviolet filter

D. Germination and Growth of Pollen Grain

Dry pollen stained with basic fuchsin glycerine jelly can readily be detected in that some have one degenerated prothallial cell lying close to the spore, and the other about to divide (Fig. 9, 23). Some have no degenerated prothallial cell with the dividing one. This gives evidence that after the reduction division of the pollen mother cell and the formation of four pollen grains, another division begins and gives the first and second degenerated prothallial cell. Lawson (1909) found that two degenerated prothallial cells were recognizable within the pollen. Allen (1943) observed no division of the prothallial cells in the Douglas-fir pollen. In this study Lawson's claim was supported that two degenerated cells were detected.

After the pollen becomes turgid, growth begins. The exines become tense first and begin to break. Eisenhut (1961) described one way of dehiscence of pollen exine similar to Larix and gave the diagram of the castoff exine. In this study, the dehiscence of exine splits in four ways. Separation of exine in two (Fig. 12) is the more frequent method; two halves are on the ends of pollen grains or thrown off gradually or at once. The second method is that the exine splits wide open on one end (Fig. 13) or in the middle of the pollen grain and the exine is not cast off,

the elongating pollen grain will tear the exine apart and leave a ribbonlike exine across it (Fig. 14). The third method is seldom found. The two ends of the exine are cut off and one ribbon-like exine is left around the middle of the germinating pollen (Fig. 15). The last method shows one tip of exine cut off and the germinating pollen seems to be squeezed out of the remaining exine (Fig. 16). Pollen which germinates in this way is inviable. The reason is that the exine mechanically inhibits the growth of pollen. Sometimes the remaining exine splits open and follows the second method (Fig. 17).

Following the split of exine, the first and second prothallial cells were divided from the pollen (Fig. 23). The first prothallial cell was cast off immediately, the second one gradually. Sometimes the second prothallial cell stocks to the pollen grain and does not leave the elongating pollen. At this stage the pollen cell has divided to form the tube cell and the generative cell (Fig. 9). The length of elongated pollen is about 160 microns. The diameter of the generative cell is about 45 microns, and that of the tube nucleus, about 13 microns under carmine staining. The tube nucleus is at the opposite end to the abortive prothallial cells. After two days, three cells (Fig. 10) i.e., tube cell, body cell and stalk cell, can be found in the elongated pollen. The generative cell has divided to form two distinct cells, presumably the body cell and the stalk cell.

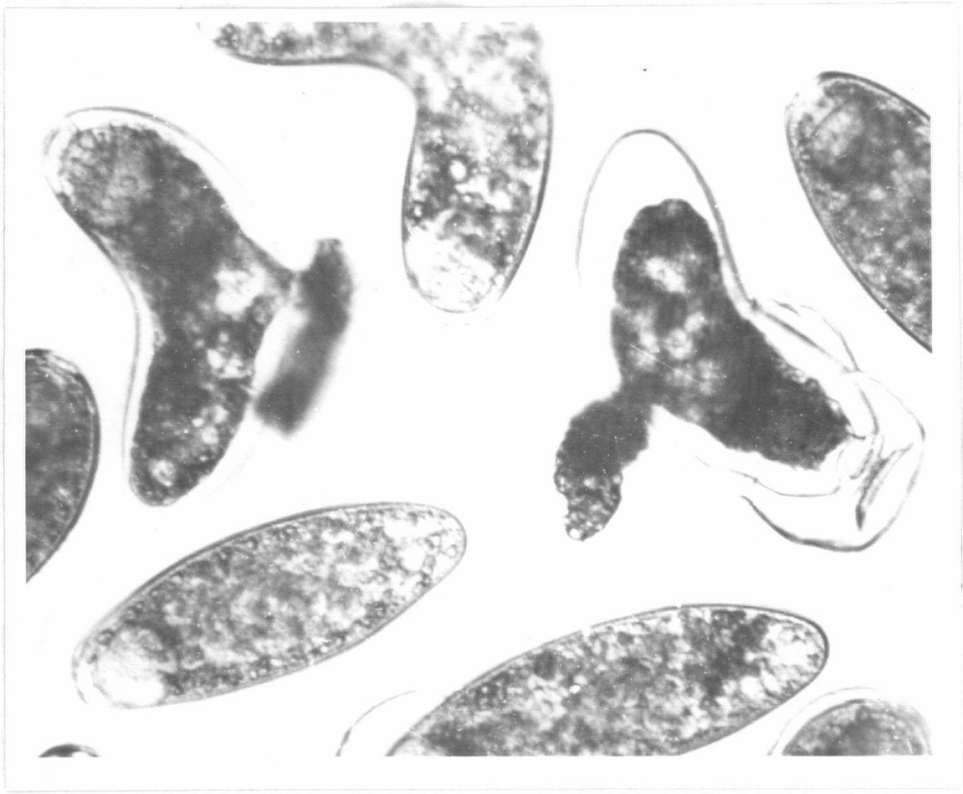


Fig. 22. Bursting of elongated pollen grain. (250X).

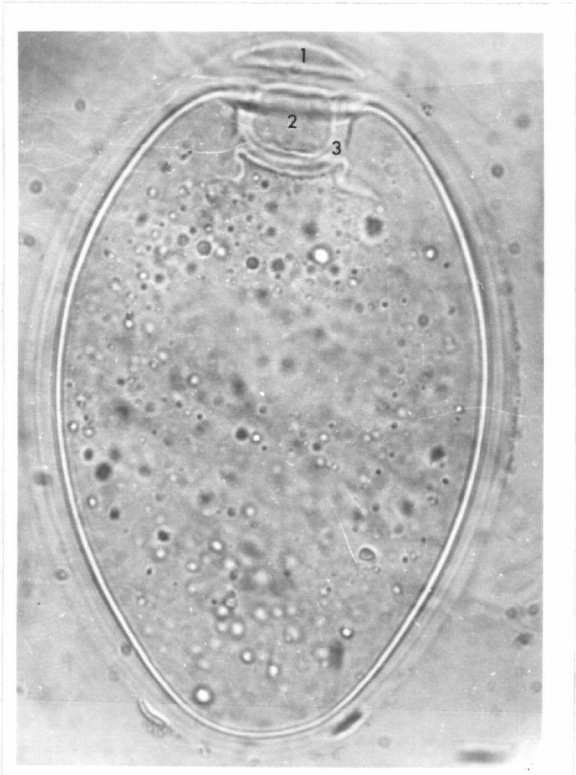


Fig. 23.
Pollen shows
(1) first prothallial cell
(2) second prothallial cell
(3) pore in the intine.
(500X).

The body cell is between the tube cell and the stalk cell. The body cell is larger than the stalk cell. The tube cell is considerably larger than these two cells at this stage. The diameter of the stalk nucleus is almost the same as the tube nucleus. In five days, it results in the organization of four cells, i.e., tube cell, two sperm cells, and stalk cell, within the elongated pollen from the culture medium of stock solution B, 10 ppm IAA and sucrose (Fig. 11). Each cell is completely surrounded by a thin but sharply defined cell membrane. Two sperm cells appear to be in the elongating Douglas-fir pollen, but not two nuclei which happens in some gymnosperm species. At this stage, the tube cell occupies almost two thirds of the elongated pollen. The two sperm cells are roughly the same size, but the stalk cell is far smaller than the other three cells. The two sperm cells and the stalk cell remain in their original positions while the tube cell descends with the tip of the growing pollen. In carmine staining, the whole sperm cell absorbs the stain, but only the nuclei of the stalk cell and the tube cell absorb the stain.

It was observed that the rupture of the exine and the protrusions of the elongating pollen always takes place at the side of the pollen grain directly opposite to that occupied by the two degenerated prothallial cells, i.e., on the side nearest the tube nucleus. A protuberance at the proximal pole (Fig. 7) where two degenerated prothallial

cells are placed can sometimes be observed. The thickness of the exine of the protuberant part is the same as the other part. Erdtman (1957) described that a protuberance was at the distal pole with tenuitas (called leptoma which is the thin sporoderm area, functioning as an aperture). This was not confirmed in this study.

When the pollen tube emerges or pollen elongates from the pollen it grows by addition of wall material at its apex (Schoch-Bodmer, 1945). The pollen tube wall contains cellulose and is cutinized (Frey-Wyssling, 1959). During the elongation of pollen grain, no extension of the width of pollen could be observed. Even though the pollen grains have elongated to 400 microns, there is no growth in width of pollen grain. It remains in the same state as when the pollen begins to germinate. Figure 15 shows that the two ends of the exine are cut off and one ribbon-like exine is left around the middle of the pollen. As the growth of the pollen continues, no tension is exerted on the elongating pollen grain by the remaining ribbon-like exine.

Protoplasmic streaming was not seen in the elongated pollen of Douglas-fir. In gymnosperms, where the growth of the pollen tube is extremely slow, the streaming of the cytoplasm has never been desmonstrated (Takeuchi, 1953). In contrast, the pollen tubes of most angiosperms show rapid protoplasmic streaming; the rate of protoplasmic streaming varies from plant to plant and is directly proportional to

the rate of elongation of the tube (Vasil, 1958, 1960).

During penetration of the nucellus tissue, the body cell divides to form the male nuclei or cells of unequal size. Fertilization of the egg nucleus occurs about nine weeks after pollination (Allen, 1943). The actual division of the body cell was not observed, but after a study of the chromatic and the cytoplasm immediately surrounding the nucleus, it seemed quite certain that this division takes place before the tip of the pollen tube reaches the nucellus (Lawson, 1909). Ching and Ching (1959) in their study on extraction of Douglas-fir pollen and effects of GA on its germination presented a picture of pollen grain containing three nuclei- the tube cell, the body cell and the stalk cell - with 100 ppm GA treatment after 46 hours' germination. At this stage no pollen tube protrusion is shown but there is the elongation of pollen. No one gives any details of the division of body cell to two sperm cells. Lawson (1909) observed there was an early disintegration of the tissue of the apex of the nucellus correlated with the position of pollen tubes in Pseudotsuga. The cells in this region of the nucellus separate from one another in places, and there appears to be a general breaking-down or dissolving of the tissue in advance of the descending pollen tubes. These latter structures, therefore find no obstructions in their path. There is no firm tissue to penetrate before reaching the archegonial chambers. At the time of fertilization, the

apex of the nucellus is completely broken down, and it takes an appearance very unlike that of Pinus and the majority of other Coniferales where the nucellus persists for some time of fertilization.

Allen (1946) stated that nothing substantiated Lawson's account of a breakdown of the nuclear apex (the nucellus top) in advance of the pollen tube; apart from the mucilaginous layer on the nucellus top, the tissue is normal. Barner and Christiansen (1962) confirmed Allen's statement and reported the pollen was deposited on the nucellus top. Wherever the pollen grains came into contact with the nucellus top, the body cell divided forming two male cells. The one nearest the distal pole was smaller than the other. Simultaneously, a pollen tube is formed either from the membrane of the body cell or from an inner layer of intine. The pollen tube is always formed at the top of the pollen grain which is in contact with the nucellus top. In this study in vitro, the four-nucleate stage was obtained but pollen tube protrusion was not observed, only the elongated pollen. Although Barner and Christiansen (1962) found actual germination of the pollen in vitro was unattainable it was accomplished in this study.

Barner and Christiansen (1962) report that the pollen tube is always formed at the top of the pollen grain which is in contact with the nucellus top (either distal or proximal pole). In this study the protrusion of the elon-

gating pollen always takes place at the side of the pollen grain directly opposite to that occupied by the two prothallial cells (distal pole). If the proximal pole were in contact with the nucellus top, how could the pollen tube be formed at this pole? I would suggest that the distal pole of the elongated pollen might curl down into the nucellus when the proximal pole was in contact with the nucellus top. This is due to chemotropism between the distal pole and the nucellus. In vitro the curling, elongated pollen (Fig. 10-b) can sometimes be observed.

The development of pollen germination of angiosperms is different from that of gymnosperms. In angiosperms, the pollen tube comes through the pore. In gymnosperms, the germinating pollen breaks the exine and protrudes the tube (?). Pollen grains of some species (Douglas-fir, larch) discard their exine, some (pine, spruce, hemlock etc.) do not. The germinated Douglas-fir pollen grains discarded their exine, and the elongated pollen was cigar-shaped. It seems that pollen produces no tube but a protrusion. If the germinated pine pollen cast off their exine, should we call it a pollen tube or a protrusion? We call the elongated protrusion of pine pollen a pollen tube. Then we call the elongated part of Douglas-fir pollen a pollen tube, but actually it is an elongated pollen grain (not a pollen tube). In Barner and Christiansen's report (1962), the photographs showed that the pollen tube was a thinner part of the distal

pole. Figure 11-2 showed their so-called pollen tube.

Laboratory studies showed that development of Douglas-fir pollen from two-nucleate stage to four-nucleate stage could be stimulated by trace elements and auxins. The actual germination of the pollen in vitro is possible. Also the above information tells us that stock solution is the best for pollen germination and elongation. It may be of practical value in ensuring a uniformly high rate of seed production in Douglas-fir seed orchards, but it needs field studies (artificial pollination) to get further information.

SUMMARY

A study was made of the pollen morphology and the pollen germination of Pseudotsuga menziesii (Mirb.) Franco (Douglas-fir) in vitro, and the effects of nutrients during pollen germination.

1. Pollen was extracted at room temperature. The width and length of pollen grains were measured under the microscope.
2. Dry pollen is cup shaped. Turgid pollen is spherical or oval, usually oval.
3. The exine is thin and smooth; the intine is thick and of uniform hyaline appearance.
4. Basic fuchsin glycerine jelly techniques were used to identify the pore in the pollen. Pores in exine and intine are visible.
5. Several types of substances were introduced to the medium to culture the pollen. It was found that boric acid and calcium ions were very important to the pollen germination and elongation. Pollen germination was stimulated by growth-substances, but pollen elongation was not markedly accelerated. 1000.0 ppm of IAA, IBA, and NAA completely inhibited the germination and growth of pollen. Fungicide and bactericide improved the germination and growth of pollen in low concentration, but not in high concentration.

6. 10 to 15 percent of sucrose is recommended to be used for the osmotic milieu and nutrient.
7. Ultraviolet radiation for 8 hours has not any effects on pollen germination in stock solution.
8. Breakage of the exine can occur by four methods. The exine separated in two. The exine split wide open. The two ends of exine were cut off and one ribbon-like exine left around the pollen. The last method with one tip of exine cut off and the germinating pollen seemed to be squeezed out of the remaining exine.
9. Pollen grains cultured in stock solution, 10 ppm IAA, and sucrose for one day were found in two nucleate stage (tube cell, generative cell) with two degenerated prothallial cells. The three nucleate stage (tube cell, body cell and stalk cell) could be found in two days' incubation. In five days, the body cell had divided to form two sperm cells. Therefore actual germination of the pollen in vitro proved attainable.

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