SOME OBSERVATIONS ON GERMINATION OF PSEUDOTSUGA MENZIESII (MIRB.) FRANCO (DOUGLAS-FIR) POLLEN IN VITRO

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INTRODUCTION

Although Pseudotsuga menziesii (Mirb.) Franco (Douglas-fir) is one of the most important species on the Pacific Coast of North America, information on pollen morphology and germination of pollen grain is still not clearly described.

Lawson, as early as 1909, gave the details of the development of globular pollen grains of Douglas-fir (grown in vivo) from the time of pollination to the three-celled stages (stalk cell, body cell, and tube cell). Allen. (1943) and Ching and Ching (1959) presented photographs of the pollen grains at the three-celled stage, but the actual division of the body cell to two sperm cells was not observed. Allen (1943) also indicated that fertilization of the egg nucleus occurred about nine weeks after pollination. The four-celled stage and formation of the pollen tubes of Douglas-fir pollen grains in vivo were detected by Barner and Christiansen (1962). They also said that the actual germination of the pollen in vitro proved unattainable.

Since the beginning of this century, the question whether or not the pollen tubes in vivo and in vitro utilize externally supplied nutrients has attracted much attention on quite a few other species (Bose, 1959; Konar, 1958; O'Kelley, 1955, 1957). Information on the possible use of supplied nutrients in insuring a uniformly high rate of pollination, and fertilization of Douglas-fir in its reproductive phase, seems to be scanty. It is, therefore, considered necessary to determine the types of substances that stimulate germination and the rate of elongation of the pollen in vitro. Such stimulation would have practical value in insuring a uniformly high rate of filled seed production in the Douglas-fir tree improvement program already underway in the Pacific Northwest.

MATERIALS AND METHODS

Microsporangiate strobili of Douglas-fir were collected from trees at Caycuse clone bank of B. C. Forest Products Limited, Vancouver Island, on May 2, 1967. Pollen was extracted at room temperature and stored in a vial at 0 C to 2 C.

Dry and turgid pollen grains were investigated under the microscope to observe the pollen shape. Identification of the pollen pore was carried out using basic fuchsin glycerine jelly techniques (Wodehouse, 1935).

For the germination test, a series of solutions were prepared: 0.01, 0.1, 1, 5, 10, 100, and 1,000 ppm of boric acid, calcium nitrate, potassium salt of gibberellic acid (10% GA), indoleacetic acid (IAA), indolebutyric acid (IBA), naphthaleneacetic acid (NAA), and thiamin. Sucrose solution was tested to the osmotic milieu of pollen grains in a series concentration of 1, 5, 8, 10, 15, and 20 percent. A stock solution as described by Brewbaker and Kwack (1963), was used. A fungicide (Arasan) and a bactericide (Fortimycin) in different concentrations were introduced to the

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culture medium. The combination of dilute stock solution, 10 ppm IAA, and a series of sucrose (5, 8, 10, 15, and 20 percent) solutions were prepared for the germination test. The pH of the culture solution was adjusted to 7.

Petri dishes and two-cavity slides were soaked for twelve hours in 3N HC1, rinsed five times in distilled water, and then covered to protect them from dust. All the instruments were sterilized by autoclave, or by 70 percent alcohol. Filter paper was placed in the petri dishes and a thin layer of distilled water was added. Two or three drops of cultural medium were added to the slide cavities. Pollen grains were immediately dusted on to the medium. They were not immersed in the medium or covered with a cover slip, as free access to air is necessary for germination. Then the petri dishes were kept at room temperature.

RESULTS

The average dimensions of the total 240 pollen grains were 94.32 A - 5.72 by 128.99+16.32 microns. Dry pollen grains appeared in cup shape (fig. 1). The turgid grains were spherical or elliptical, without a trace of bladders, or furrows (fig. 2). The exine was thin and smooth, measuring only about 2microns inthickness. The intine measured about 8 microns in thickness and was of uniform hyaline appearance. In basic fuchsin glycerine jelly technique, a pore of about 2 microns could be observed.

Pollen germination was enhanced and was proportional to the concentration of trace elements, boron and calcium. The potassium salt of GA also increased growth of pollen. IAA, IBA, and NAA increased the pollen germination and elongation in low concentrations, but completely inhibited both in high concentration, 1,000 ppm. Thiamin gave the same effects as GA.

Sucrose gave the pollen grains both nutrients and osmotic milieu. Osmotic pressure of the medium was very important during germination. As to the concentration for the culture, 15 percent gave the best when only sucrose was used.

Stock solution media gave the highest germination percentage (56.95) and longest elongation (186.14 +27.73 microns; 24 hours incubation). High Arasan concentrations (more than 500 ppm) inhibited the germination and growth of pollen. The medium with the lower concentration of Arasan did not have much effect on the germination and growth. Fortimycin enhanced the germination and growth of pollen grains in low concentrations, but decreased when the concentration was more than 166 IU (international units). The concentrations which did not inhibit the germination and growth of pollen grains could not prevent contamination by fungi and bacteria.

The results of the incubation of pollen grains in the combined solution which contained stock solution, 10 ppm IAA, and the series of sucrose solution (5, 8, 10, 15, and 20 percent) are shown in table 1.

After 24 hours incubation, the two-cell stage could be found, these being the generative cell and the tube cell. After two days, three-celled stage, i.e., tube cell, body cell, and stalk cell, could be seen in the elongate pollen, where the body cell was between the other two cells. After five days' incubation, the body cell divided to form two sperm cells, but the two overlapped and remained in the original position (fig. 3, 4).

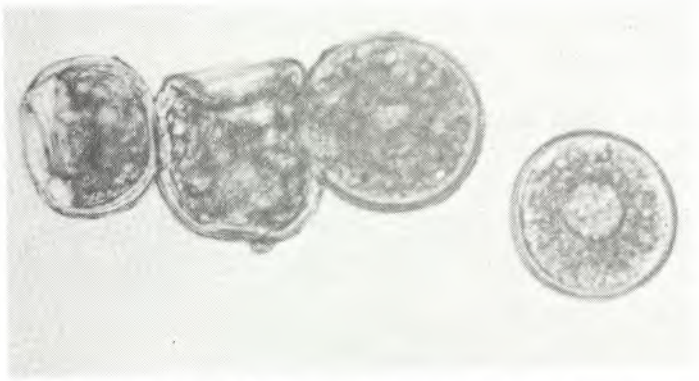

Fig. 1. -- Polar view (right two grains) and side view (left two) of dry pollen grains. (x 240).

Fig. 2. -- Turgid pollen grains. (x 240).

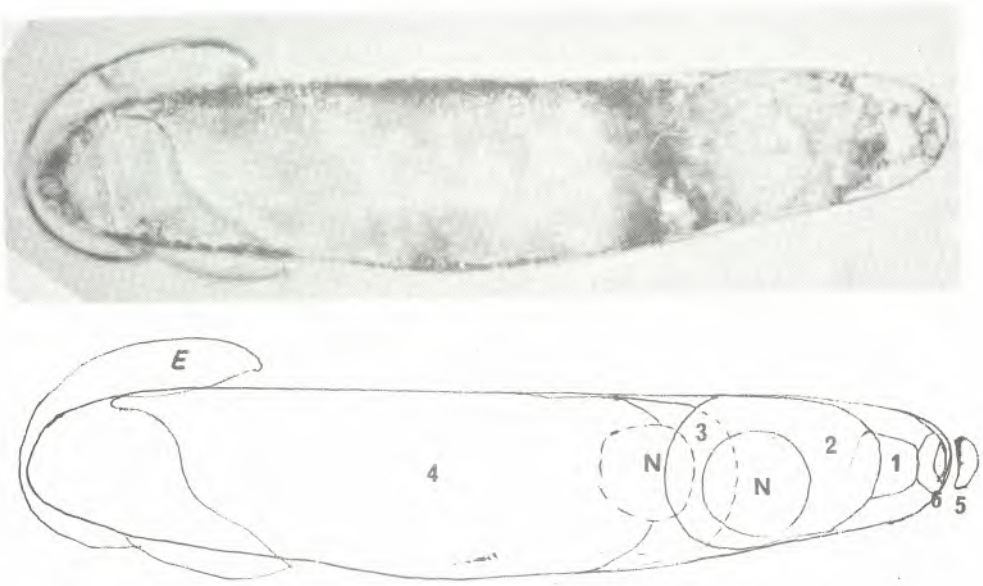

Fig. 3. -- Four nucleate stage. (1) stalk cell. (2) (3) sperm cells. (4) tube cell. (5) first prothallial cell. (6) second prothallial cell. (N) nucleus. (E) exine. (x 200).

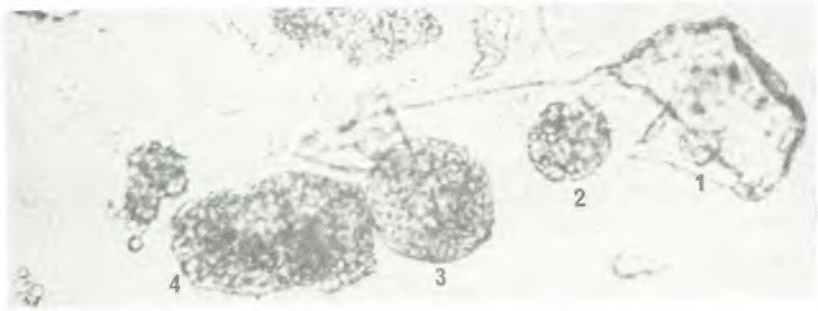

Fig. 4. -- Four nucleate stage. (1) stalk cell. (2) (3) sperm cells. (4) tube cell. (x 200).

The dehiscence of the exine took place in one of the following ways: (a) the exine separated into two cups, equal halves or one larger than the other; (b) the exine broke wide open, the cast-off exines had a tendency to curl tightly inward; (c) two ends of exine were cut off and one ribbon-like portion of exine was cut off and the germinating pollen seemed to be squeezed out from the exine.

Table 1. -- Pollen germination.

Culture media	After 24 hours		After 48 hours	
	germination (%)	length (μ)	germination (%)	length (μ)
5% sucrose 10 ppm IAA	67.31	174.99 ±26.58	66.54	205.44 ±19.48
8% sucrose 10 ppm IAA	65.48	185.99 ±22.61	67.69	207.68 ±27.55
10% sucrose 10 ppm IAA	63.13	168.74 ±18.43	70.56	218.30 ±23.39
15% sucrose 10 ppm IAA	66.88	183.59 ±17.68	70.86	208.61 ±29.34
20% sucrose 10 ppm IAA	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

DISCUSSION

The average size of Douglas-fir pollen grains reported by Wodehouse (1935) utilizing stained preparation is 90 to 100 microns. Van Campo Duplan (1950) obtained an average size of 140 microns from fresh Douglas-fir pollen. Sziklai (1964) reported that the diameter of pollen grains varied considerably between trees, from 91.08 microns to 99.19 microns. The average diameter calculated after 1,000 measurements by Bunnel (1965) is 91.07 microns. The turgid pollen appeared spherical or oval, usually oval. It is believed that two measurements are needed. In this study, the average of 240 grains is 94.32+5.72 by 128.99+16,32 microns.

Dry pollen is contracted and the furrow appeared to make the grain cup-shaped. The appearance of the exine of dry pollen grains is netlike. Wodehouse (1935) reported Douglas-fir pollen to be completely without an aperture, whereas Eisenhut (1961) described the typical or average pore of Douglas-fir as being somewhat oval-shaped, 56 by 70 microns in size, and 18.6 microns deep. The results of this study support Eisenhut's claim that Douglas-fir pollen has a pore but the size of the pore was found to be much less, about 24. It seems the pores in the pollen are the points where exine splits. With regard to the pore in the intine, we could confirm Barner and Christiansen's observations (1962) that there was an aperture at the proximal pole of the pollen grains. It seems that the orifice of the pore is enclosed by a membrane.

Boron increased not only the germination percentage but also the length of the pollen tube. The increase in concentration of calcium gave higher germination, but elongation of the pollen tube was not effected. Stock solution containing boric acid, calcium nitrate, magnesium sulphate and potassium nitrate not only increased the germination percentage and pollen tube elongation, but also prevented the pollen grains from bursting.

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. At 1,000 ppm, IAA, IBA, and NAA completely inhibited the germination of pollen.

Pollen grains do not contain chlorophyll and are dependent on internal and external sources for the supply of essential nutrients. Sucrose served as a nutrient and also played a part in the creation of a favorable osmotic condition for germination and growth. From both these aspects, Douglas-fir pollen developed best in a 15 percent sucrose solution. 11

Dry pollen can be readily recognized as having one degenerated prothallial cell, lying close to the spore, and the other one about to divide. It is evident that after the reduction division of the pollen mother cell and the formation of four pollen grains, another division begins and produces the degenerated prothallial cells. Lawson (1909) found that within the pollen two degenerated prothallial cells were recognizable. Allen (1943) observed no division of the prothallial cells in Douglas-fir. This study supports Lawson's claim.

Following the germination of pollen, the exine dehisced and the first and second prothallial cells were divided from the pollen grains. At this stage the pollen cell has divided to form the tube cell and the generative cell. The diameter of the generative cell is about 45 microns, and that of tube nucleus about 13 microns under carmin staining. The tube cell is at the opposite end to the abortive prothallial cells. After two days' incubation, the generative cell has divided to form two distinct cells, the body cell and the stalk cell. The body cell is between the tube cell and the stalk cell. The tube cell is considerably larger than those two cells at this time, but the diameter of the tube nucleus is almost the same as the stalk nucleus. In five days, four cells are visible (tube cell, two sperm cells, and stalk cell) within the elongated pollen on the medium culture of stock solution, 10 ppm IAA with sucrose. Each cell is completely surrounded by a thin but sharply defined cell membrane. Two sperm cells appear in elongating Douglas-fir pollen, but not two nuclei which occur in some gymnosperm species. 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. The protrusion of the elongating pollen always takes place opposite the two degenerated prothallial cells,

Lawson (1909) observed that there was an early disintegration of the tissues of the apex of the nucleus correlated with the position of pollen tubes in Pseudotsuga. Allen (1946) stated that nothing substantiated Lawson's account of a breakdown of the nuclear apex in advance of the pollen tube. Earner and Christiansen (1962) confirmed Allen's statement and reported the pollen was deposited on the nucellus top. Where the pollen grains came into contact with the nucellus top, the body cell divided forming two male cells. Simultaneously a pollen tube is

is formed either from the membrane of the body cell or from an inner layer of intine. The pollen tube is always formed at that end of the pollen grain which is in contact with the nucellus.

In this study in vitro, a four nucleate stage was obtained but pollen tube protrusion was not observed. Although pollen tube protrusion was not detected in this study, the division to the four nuclei stage indicates that germination of pollen grains is possible in vitro.

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