On the Pollen Grain and the Fertilization Mechanism of Pseudotsuga menziesii (Mirbel) Franco var. viridis Schwer.

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As reported by H. BARNER and the present author in an article: "The Formation of Pollen, the Pollination Mechanism, and the Determination of the Most Favourable Time for Controlled Pollination in Pseudotsuga menziesii", published in Silvae Genetica 11, 4, 1962 (2), the pollinatiori mechanism of P. menziesii differs in several respects from what has hitherto been known.

Part of the results of the described investigation could however not at that time be satisfactorily explained, and we have in the course of the last 4–5 years tried to fill in these gaps. The main points of doubt were: the number of pollen grain mitoses, the passage of the male cells from pollen grain to egg cell, and the reason why the pollen grains of P. menziesii could not be germinated in vitro.

The results of the present investigation seem to confirm our previous findings of two pollengrain mitoses plus the division forming the male cells, but the result is not final.

It appears that the fertilization in P. menziesii is carried out by spermatozoids, which, remaining inside the membrane enclosing the body cell complex, are moving from the pollen grain into the apex of the nucellus through a short (pollen) tube.

Motile spermatozoids were seen leaving a pollen grain of P. menziesii forced in a hanging drop about a week before normal pollen germination.

The peculiar fertilization mechanism probably explains why all attempts to germinate pollen of P. menziesii in vitro have hitherto been without results.

Material and Methods

Good crop years in Pseudotsuga are rare in Denmark, and 1967 was the first one since 1962. True, there was some scant flowering in between, but in such years pollen in the micropylar canals, although seemingly normal during the first 2–3 weeks after pollination, often proved empty about germination time. Sometimes only one clone was flowering in a given place, and suspecting that self-pollination might be the cause of the empty pollen grains, we took the precaution to bag and cross-pollinate an adequate number of female inflorescences to be used in the investigation. Whether or not self-pollination actually was the cause cannot be said with certainty, but in 1967 80% of the micropylar canals of the healthy ovules of 5 trees, designated for the investigation by the State Forestry's Tree Improvement Station and pollinated by them with foreign pollen, contained from 2 to 10 pollen grains per canal, and almost all of them were normal. In unbagged inflorescences left for free pollination only few pollen in few canals were found. It may be mentioned in this connection that usually we found that out of 100 ovuliferous scales of P. menziesii only 30 had 2 healthy ovules, 58 had 1 healthy and 1 shrunk and collapsed ovule, 12 had no healthy ovules at all. Whether this was due to hereditary, climatic or other causes is unknown, but it increases considerably the work connected with procurement of pollen from the micropylar canals; that the seed setting must be decreased is evident.

For study of the fertilization mechanism pollen grains were taken from the micropylar canals during the last three weeks of june. The forcing period in vitro for germination was thus reduced from 4–5 weeks to a few days.

For germination studies the pollen grains were taken directly from the micropylar canal of a fresh ovule to a "hanging drop" consisting either of different sugar solutions or of fluid squeezed from ovules. The cultures were placed in a humidity chamber at room temperature (about 23° C). In order to avoid the risk of damaging the pollen grains no disinfectants were used.

For studies of anatomy and stages of development the pollen grains were placed in a drop of not too dense acetic orcein and examined without cover glas or with small pieces of broken glas under two or three edges of the cover glass; otherwise the weight of the glass would flatten the elongated pollen grains. For examination a Zeiss WL phase-contrast microscope and a Wild M. 5 preparation microscope were used.

During the investigation all plates of lasting interest were photographed with a Zeiss Ikonta camera mounted on the microscope. For technique see (2), page 89—90.

The Development of the Pollen Grain

As mentioned in (2) meiosis in P. menziesii takes place in March. Thereafter, during the following 2–3 weeks, dependent on the temperature, the mitotic divisions occur. Pollination takes place during the first half part of May. At pollination time the pollen grains are globular, but on the stigmatic flap or in the micropylar canal they elongate during the next 3—4 weeks attaining at the end of that period a length of 5—600 μ. During the said period the embryonal cell (the body cell) moves toward the middle of the pollen grain but keeps up the connection with the pore by means of 4 tube connections, which elongate together with the pollen grain ([2], fig. 32). At germination time (usually about medio June) the body cell dissolves, and 2 male cells are seen ([2], fig. 35, 38 and the present fig. 4, 4 *a*).

The structure of the cell *and* the mitotic divisions *of* the pollen grain are extremely difficult to analyse. The divisions in different pollen grains are not synchronized, the graines are packed with opaque granules, the divisions are intranuclear and, furthermore, there seems to be an extra nuclear membrane with a dense texture which additionally obstruct the vision. For these reasons the divisions may easily be overlooked, and their sequence is difficult to ascertain. We have as yet not been able to solve these problems satisfactorily, but it is hoped that the following observations may elucidate at least part of them.

The Nucleus

During metaphase I it was observed, that besides the 13 bivalents there was in the pollen mother cell a number of

organelles strongly resembling prophase chromosomes; they appeared black in phase contrast but barely discernable in ordinary light *(fig. 8)*; their position suggests that they, together with the bivalents, had been enclosed in a membrane, which was ruptured and cast off by the squashing. Similar organelles were observed in meiotic prophase and metaphase II and in mitotic prophase, and they are probably also present at other stages. The shape of the nucleus is unusual; it is more or less cylindrical with a constriction and a "head" at one end ([2], fig. 12, 15, root tip); at resting stages the "head" is covered by a cap (the present *fig. 1, 12, 14*). The peculiar shape of the nucleus with the cap is also seen in T. II ([11] fig. 25), and in meiotic prophase (present *fig. 7, 7 a*). The organelles (and chromosomes) are embedded in nucleoplasm which, as seen in phase contrast, has another refractive index than the cytoplasm. The impression is that in a "nucleus" f. inst. like the one shown in (2) fig. 15, the nucleus containing the chromosomes is hidden by nucleoplasm and organelles, and that the function of the latter is to form a structure or frame to keep up the shape. That the "head" has a framework or organelles is clearly seen in (2) fig. 15. The nature of the organelles is so far unknown, but taking into consideration that the spermatozoid of *P. menziesii* evidently is a unicellular, multiciliated organism with a powerfull locomotor apparatus, a neuromotor system and organs for orientation etc., it seems probable that it may have a sort of skeletal system and possibly also a special envelope, a pellicle. That structures of the spermatozoid should be discernible already at the meiotic stage seems quite possible; it is more remarkable that they also seem to be present in the nuclei of somatic cells. This and other problems mentioned above need, however, further investigation.

The cytoplasm of the cells, meiotic as well as mitotic, including the body cell and the spermatozoids, is densely filled with minute bodies, which appear black in phase contrast, faint in ordinary light. The most characteristic form of these bodies is that of a two-pronged fork, which sometimes has a certain resemblance to a mitotic chromosome just before splitting, others have the shape of craters (the present *fig. 1, 5, 6, 12, 14* [arrows], also [2] fig. 10 [The zone near the cell membrane], 18, 19 and 38). In the body cell (f. inst. the present *fig. 1)* the spectacular central part has sometimes been mistaken for a prophase nucleus; what is actually seen is evidently the cytoplasm and nuclear cap of a spermatozoid (perhaps spermatozoids). The nature of these bodies is unknown, but the spermatozoids being ciliate, the bodies may perhaps, in places where cilias are formed, be connected with the formation of such organs.

The formation of the prothallium cells is relatively easy to determine, although the mitotic divisions have not been seen. The number of cut off, degenerated prothallium cells observed is two, which seems to be generally accepted ([2] p. 94 and [9]).

The origin of the tube nucleus is still undetermined, but there is evidence (the present *fig. 6, t)* suggesting that this nucleus, like the stalk cell, is a unit in the nutritive system of pollen grain and a centre, at which the tubular system of the new cytoplasm, formed in the prolonged part of the elongated pollen grain, converges. The nucleus consists of a circular part surrounded by a ring of small sections in which the cytoplasmatic tubules terminate; its structure does not seem to indicate that it could be a daughter nucleus of the embryonal nucleus. As shown later it seems most likely that the short pollen tube in *P. menziesii* is formed by a spermatozoid forcing a tube of inner intine out through the outer intine of the pollen grain, and the possibility of the nucleus in question having any connection with the formation of the pollen tube seems therefore remote. It seems most likely that the "tube nucleus" was formed during meiosis.

Fig. 1: Pore-end of elongated pollen grain, June 2nd. Body cell complex has started movement toward middle of PG. In the complex are visible: cap of nucleus of spermatozoids (nc), cytoplasm of spermatozoids (cy. 2), old cytoplasm af PG (cy. 1); the complex is surrounded by new cytoplasm of pollen grain (cy. 3); organelles (see text) are seen in cytoplasm (arrow). ×460. —

Fig. 2: Part of damaged, elongated PG, June 19. B is near middle of PG enveloped in at least two membranes; m: piece of broken membrane with dense granules. In B organelles (see segment of cytoplasm, *fig. 5*). ×180. —

Fig. 3: Spheroidal PG. medio May, flattened to show structure and connections between body cell, stalk cell and tube cell. Tube nucleus (t) is pressed out of position; normally it is situated at dotted circle. A wide tube leads from pore (p) through stalk cell and body cell to rosette (R) on top of B which is connected with t. Stalk cell (St) is connected with cytoplasm, seemingly by cytoplasmatic tubes at least at 1 and 2. B is connected with cytoplasm at least by tubes 3 and 4. t seems included in the cytoplasmatic tube systems and thus in connection with 1, 2, 3, 4. On top of B are seen 2 oblong cells (sp), probably spermatozoids. (cf. text and diagram *fig. 3 a*). ×450. —

Fig. 3 a: Diagram of probable structure and connections of body cell, stalk cell and tube cell shown in *fig. 3*. —

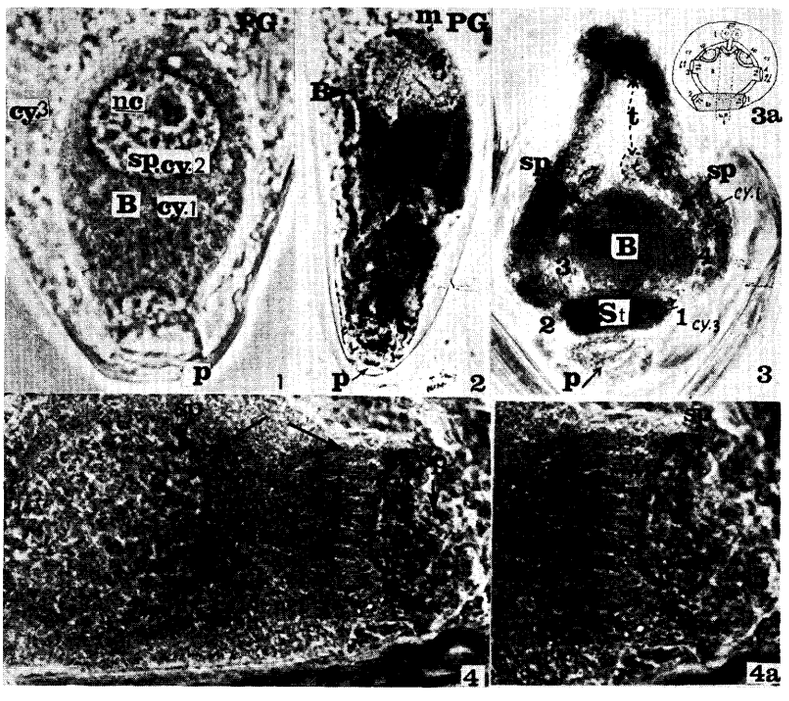


Fig. 4: Two spermatozoids seemingly with "spindles" (arrows) after mitotic division, actually the "spindles" are probably tubular fibres torn apart by the sp leaving their base. ×450. —

Fig. 4 a: Enlarged section of right side of *fig. 4*. ×735. —

Abbreviations: B = body cell; cy. 1 = cytoplasm of tube cell; cy. 2 = cytoplasm of spermatozoids; cy. 3 = new cytoplasm; m = membrane; nc = nuclear cap; p = pore; PG = pollengrain; R = rosette; sp = spermatozoids; St = stalk cell; t = tube cell.

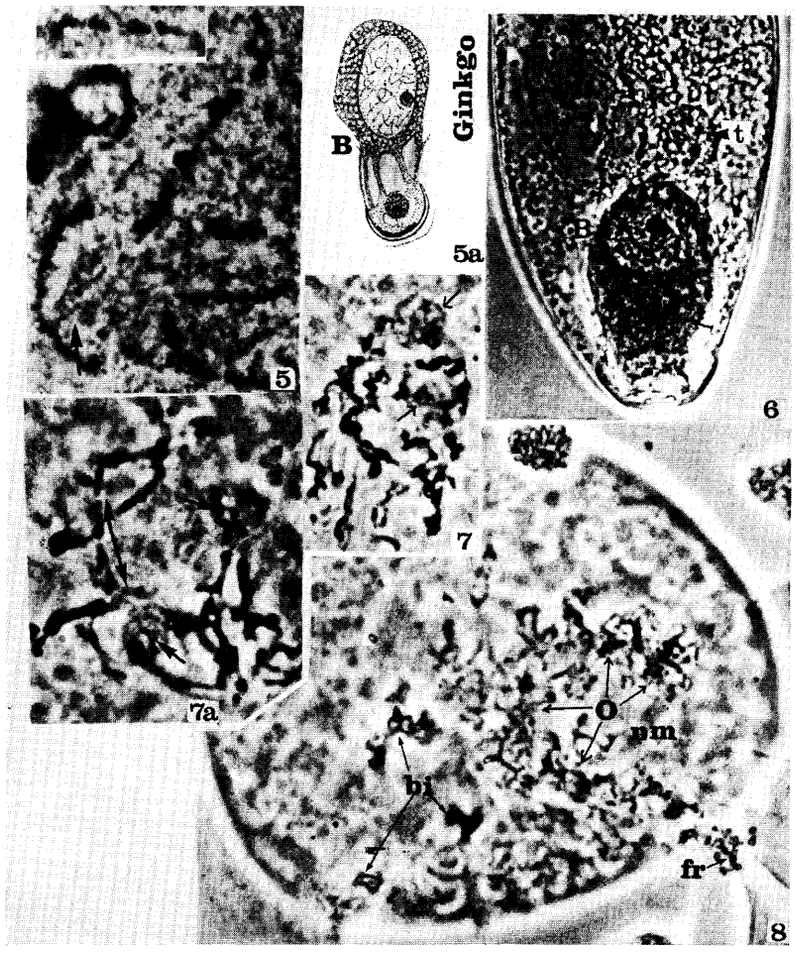


Fig. 5: Sector of cytoplasm of spermatozoids from body cell of *P. menziesii*, June 19 *(fig. 2);* chains of organelles, often following grooves in cytoplasm, are suggestive of young cilia, perhaps in a special membrane. Inset, top: similar organelles from another cell. ×1280. —

Fig. 5 a: View of cytoplasm of body cell complex of *Ginkgo biloba;* (after FAVRE-DUCHARTRE [3]), note the resemblance to *fig. 2 and 5.*

Fig. 6: Shows connection between body cell (B) and "tube nucleus" (t) and between the latter and the new cytoplasmatic reticulum in elongated part of PG. ×350. —

Fig. 7: Meiotic prophase with two nuclear caps (arrows). Black organelles are probably not chromosomes but structural units. ×575. —

Fig. 7 a: Segment of late meiotic prophase with 2 nuclear caps (arrows) and organelles much like inset *fig. 5* (double arrows) (cf. *fig. 7* and text). ×600. —

Fig. 8: Metaphase I. 13 bivalents (bi) are squeezed out of (nuclear) membrane (nm) containing organelles (o) resembling those shown *fig. 7, 7 a;* below at (fr.) fragment suggestive of ciliar band. ×435. —

Abbreviations: B = body cell; fr = fragment; nm = nuclear membrane; o = organelles; t = "tube nucleus".

The Stalk Cell

The present *fig. 3* shows evidence that the stalk cell is a unit in the nutritive system of the pollen grain and, particularly, of the body cell, and as such it seems likely that it was formed together with the pollen grain during meiosis.

The Generative Cell

No such cell has been observed, and if the above assumption viz. that the tube cell and stalk cell are not daughter cells of the embryonal cell, there seems, as a matter of fact, no room for it.

The Body Cell

If no generative cell is formed, the body cell and the second prothallium cell are sister cells. The body cell has a unique structure, particularly at the later stages of development. At pollination time, when the pollen grain is still spheroidal, the body cell has the appearance shown in (2), fig. 24, and in the present *fig. 3,* (3 a). In *fig. 3* the body cell and "stalk cell" are pressed apart, and the cone-shaped lower part of the body cell is seen. The cone-shaped lower part of the body cell fits into the concave upper part of the stalk cell. A large tube ([2] fig. 28 "tu") leads from the pore through the centre of the "stalk" and body cells to the "tube cell". Other tubes connect the middle of the body cell with the cytoplasm of the pollen grain. — Judging from the evidence at hand, the upper half of the body cell carries two male cells, the lower half carries at later stages the four tube connections between body cell and stalk cell ([2], fig. 32). The body cell is at the stage shown in *fig. 1* surrounded by the old cytoplasm of the pollen grain and both are surrounded by at least two membranes densely filled with small bodies *(fig. 2)* which render inspection extremely difficult, particularly at later stages of development. The whole body cell complex is surrounded by newly formed cytoplasm.

During the period between pollination and germination, the body cell moves from the pore to about the middle of the elongated pollen grain. Near germination time the membranes — or at least some of them — rupture, and the two spermatozoids sometimes become visible ([2], fig. 35).

The body cell, and the elongated pollen grain resemble in several respects those of *Ginkgo* and *Cycads,* but as far as it has been possible to judge from literature on the subject, the body cell of the two last mentioned genera remain nearer the pore end of the pollen grain than does the body cell of *P. menziesii.*

During the period between pollination and the separation of the spermatozoids no mitotic action was observed in the body cell, but it flattened and enlarged considerably. It was not, at any time before the separation (except perhaps, at the stage *fig. 3)* possible to distinguish two spermatozoids, but this circumstance may have been due to the spermatozoids lying very close together as they, according to SHIMAMURA (8), do in *Ginkgo.* However, 1—2 weeks before germination chains of black bodies appeared in the cytoplasm of the spermatozoids or in a special covering mem-

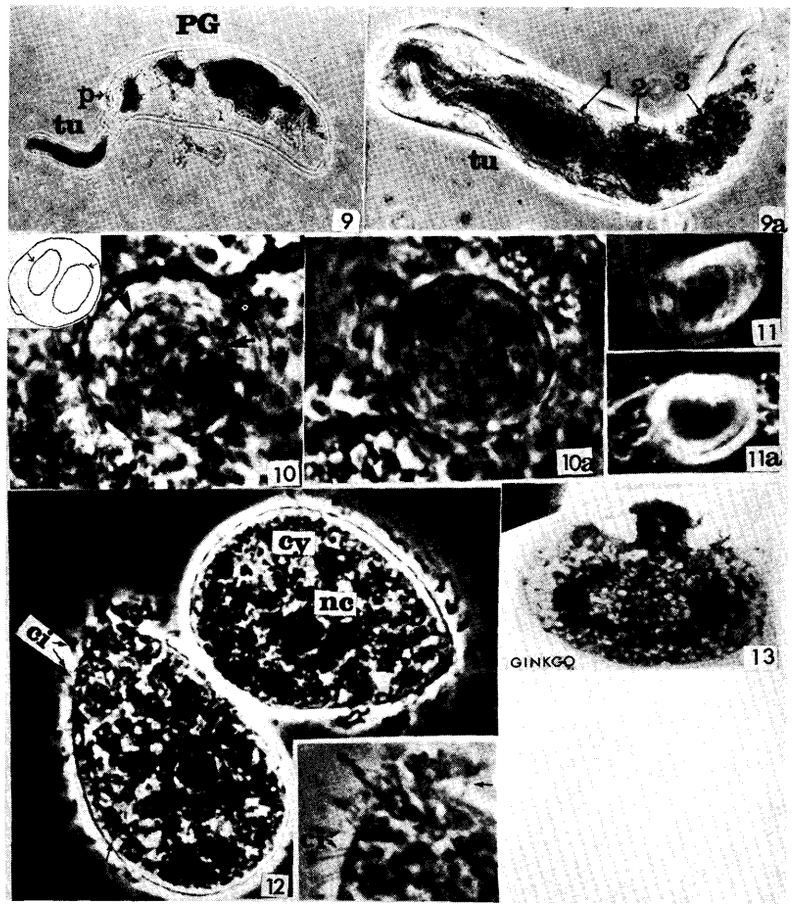


Fig. 9: Elongated PG with short pollen tube (tu) taken from nucellar apex June 27, probably decaying, contents of PG unidentifiable, cf. 9 a. ×106. —

Fig. 9 a: Pollen tube *fig. 9* enlarged; contents: tube connections (1), top of body cell (2), probably remnants of spermatozoids (3). ×390. —

Fig. 10: Body cell of PG forced in hanging drop (cf. p. 101), "rotating" prior to release of spermatozoids. Two compartments, probably vacuoles of spermatozoids, are faintly seen (arrows and inset). "Rotation' due to ciliar movements. Resembles corresponding, but somewhat earlier stage in *Ginkgo (fig. 13)*, cf. pp. 101 and 102. ×265. —

Fig. 10 a: Same cell as *fig. 10 with* increased speed of rotation. Left: nucellar cap (arrow). ×275. —

Fig. 11 and 11 a: The two spermatozoids shown in *Fig. 12:* Two spermatozoids (cf. text p. 100, 101) with by ciliar movement. ×350. —

Fig. 12: Two spermatozoids (cf. text p. 100, 101) with cytoplasm and organelles (arrow, left, bottom) as described p. 98, "cytoplasm"; inset below: enlargement of anterior part of left spermatozoid; arrow: structure of cilium: probably tuft of fibres with "muff" about middle. ×1100 and ×1850 (inset). —

Fig. 13: Corresponding to *fig. 10 a* (but earlier) stage in *Ginkgo* after FAVRE-DUCHARTRE (3), Pl. III, *fig. 5*, showing two spermatids and nuclear cap; note resemblance.

Abbreviations: ci = cilia; cy = cytoplasm; nc = nuclear cap; p = pore; PG = pollen grain; tu = short pollen tube.

brane; exact localization of the chains was not possible. These chains follow, as shown in *fig. 2, 5*, grooves in the cytoplasm, and they are suggestive of the ciliated bands of *Ginkgo* and *Cycads*. It is interesting to note that the appearance of the body cell at this stage to a high degree resembles the body cell of *Ginkgo* at the corresponding stage shown by FAVRE-DUCHARTRE (3), reproduced in *fig. 5 a*. According to M. FAVRE-DUCHARTRE the chains seen in *fig. 5 a* are chromatine threads indicating a forthcoming division of the body cell and formation of 2 spermatozoids. Although the corresponding chains in the body cell of *P. menziesii* do not seem to have much to do with chromosomes, the possibility that the situation in *Pseudotsuga* nevertheless may be similar to that in *Ginkgo* cannot of course be dismissed without further investigation.

The Spermatozoids

From the above descriptions of the cells of the pollen grain it appears, that the exact time of formation of the spermatozoids is unknown, but as mentioned before it seems likely that they are formed either by a third division in continuation of the two prothallium cell divisions in April or shortly before germination in June.

The appearance of the body cell shown in *fig. 3* seems in favour of the first mentioned alternative, the above comparison with *Ginkgo* in favour of the second. The male cells shown in (2), fig. 38, and the present *fig. 4, 4 a*, seem, on the face of it, to show mitotic divisions near germination time. On closer inspection, however, the genuineness of the "spindle fibres" is doubtful; they are probably tubules or fibres being severed by the spermatozoid in leaving its base. Only further investigation can solve this problem.

That two spermatozoids are produced is, however, beyond doubt, but sometimes one is smaller than the other or degenerating *(fig. 4)*, it has as yet not been possible to determine whether, as a rule, only one or both are capable of fertilizing.

The spermatozoids described in the following were not fully developed but "abortions" 1—2 weeks before maturity. Older specimens which could be thoroughly examined were not found, they were always enclosed in membranes and more or less obscured *(fig. 14)*. Their size and perhaps also other characteristics described below may therefore not be fully exact.

With this reservation the spermatozoid of *P. menziesii (fig. 12)* could, at the stage of development in question, be described as an oviform, unicellular, multiciliated organism with a powerful locomotor apparatus. The latter trait was clearly demonstrated by the speed of the „rotating" spermatozoids *(fig. 10, 10 a)* and by the alacrity with which the spermatozoids slipped out of the body cell and pollen grain and swam about in the neighbourhood *(fig. 11, 11 a)*. The nucleus is enclosed in a densely granulated cytoplasmatic envelope, only the cap of the nucleus is seen. The size and shape of the cytoplasmatic granules are the same as described on p. 98 ("cytoplasm"). The anterior part is surrounded by a groove which may be the "head" mentioned p. 98. This "head" is covered by cilia, and cilia are also seen along the sides of the spermatozoids, except the posterior part. It is possible that also other parts carry cilia, but this could not be determined with cer-

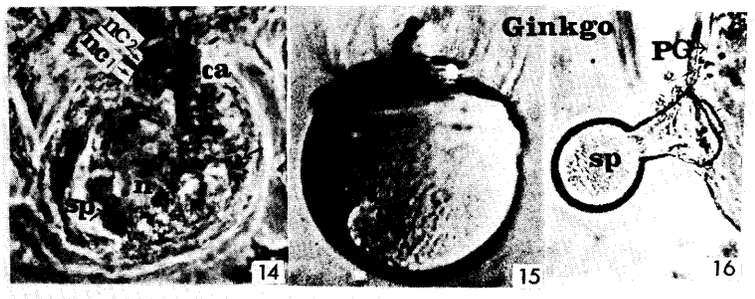
tainty. The length of a cilium is about 3—4 μ. The exact structure could not be determined, but the impression is, that each cilium consists of a tuft of fibres kept together by a "muff" about the middle (see inset *fig. 12,* arrow). The size of the spermatozoids at the stage of development in question is about 34 × 28 μ; the exact size at maturity is so far unknown, but the length of the spermatozoid in the capsule *fig. 14* seems to be about 45 μ, and they are probably considerably smaller than those of *Ginkgo* and of most *Cycads.* Also the cilial equipment seems to differ *(fig. 12, 15).*

"Rotating" spermatozoids were, as mentioned above, observed in a body cell of *P. menziesii* just before the spermatozoids left the cell. Similar occurrences have been observed in *Ginkgo* by SHIMAMURA (8) and in a *Cycas* by SWAMY (10). Obviously the "rotation" is caused by movements of the cilia of the spermatozoids, but it is not clear if the latter actually rotate. *Fig. 10* shows the "rotating" interior of a cell in which the outlines of two bodies, no doubt spermatozoids, are faintly seen. *Fig. 10 a* shows the same cell "rotating" at higher speed. On the left (arrow) this cell has a cap or knob, probably a connection between the spermatozoids and the cytoplasm of the pollen grain. It is interesting to note that a body cell of *Ginkgo* shown by M. FAVRE-DUCHARTRE (3), pl. III, fig. 5, reproduced in present *fig. 13,* containing two spermatids, also shows a cap like the one observed in *P. menziesii.*

The Pollen Tube of P. menziesii

The elongated part of the pollen grain is sometimes termed "pollen tube", but in view of the fact, that it does not grow into a style or apex of nucellus, but sprouts a special tube, through which the male cells are discharged, it seems doubtful if this term is justified. It may also be questionable whether the special, short tube ([2], fig. 35 and present *fig. 9, 9a)* is a pollen tube in the usual sense of the word; it is not clear if it is always a grown tube, or a tube made by mechanical means. However, in the following the term "pollen tube" is attached to the special short tube.

The always empty pollen grains on the apex of the nucellus ([2], fig. 34) and analysis of the contents of pollen tubes and capsules ([2], fig. 35, "n" and present *fig. 9 a* and others) seem to show, that as a rule the whole body cell complex, including spermatozoids, remnants of the body cell and its tube connections, debris and dead cells pass into the pollen tube enclosed in the membranes surrounding the complex in the pollen grain. During the passage through the pollen tube the contents of the membrane are packed closely together *(fig. 9 a),* and on leaving the tube the whole load has the shape of a capsule, which proceeds to the vincinity of the egg cell ([2], fig. 40—41 a). That this is what happens is fairly well substantiated, but the intriguing question is, how it happens (see diagram *fig. 17).* One thing seems perfectly clear, a considerable amount of motive power is required to manoeuvre a container with a load like the above into the pollen tube and out of it, and it seems equally clear that this motive power must be supplied by the spermatozoids. That the spermatozoids of *Ginkgo* have the power required to break through the wall of a pollen grain, thereby pushing out a tube of inner intine, is reported by SHIMAMURA ([8], pl. 14, fig 10, reproduced in present *fig. 16),* who states that besides by this method the spermatozoids may leave the pollen grain by



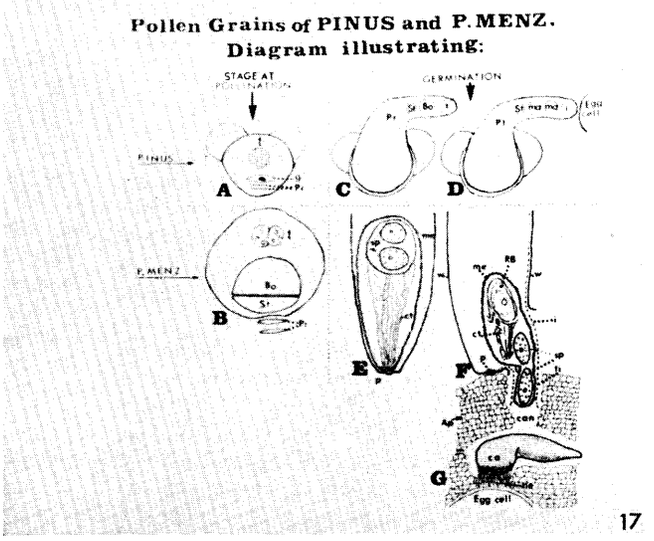


Fig. 14: Spermatozoid of *P. menziesii* in top of capsule at neck-cells. Piece of membrane and part of cytoplasm of sp removed by knife. Note nuclear cap (nc. 1) of sp and corresponding organ (nc. 2) above in membrane. Organelles at arrow. ×550. —

Fig. 15: Mature swimming spermatozoid of *Ginkgo biloba* (after SHIMAMURA [8]); average size about 95μ × 67μ; only anterior part has cilia. —

Fig. 16: Spermatozoid of *Ginkgo biloba* has broken through wall of PG enclosed in bulge of inner intine, (after SHIMAMURA [8] Pl. 14, *fig. 10).* —

Fig. 17: A: PG of *Pinus* ready for pollination, contains according to textbooks tube cell, generative cell and 2 prothallium cells. — *B:* PG of *P. menziesii* ready for pollination; contains "tube cell", body cell, "stalk cell", 2 prothallium cells ([2], fig. 24). — *C and D:* Germinating PG of *Pinus* shortly before fertilization; *C:* tube cell, body cell, stalk cell have passed into the pollen tube. *D:* body cell has divided into 2 male cells immediately before fertilization. Note! D takes place about an year after C. (Development according to textbooks). — *E and F:* Pore-end of germinating PG of *P. menziesii; E:* body cell has divided into 2 spermatozoids (corresponds to [2], fig. 38); *F:* spermatozoids (sp) and remnants of tube system and body cell (RB) enclosed by membrane of body cell complex (me) passing from PG through wall, forming short tube of intine (ti), into apex of nucellus (Ap), cf. p. 102, and on to egg cell (cf. G) (corresponds to stage in present *fig. 9).* — *G:* During passage through apex the enclosing membrane (me) assumes shape of capsule (ca) (corresponds to [2] fig. 40—41) containing spermatozoids, debris etc.; passage seems facilitated by exudation of enzymes. — *Abbreviations:* Ap = apex of nucellus; Bo = body cell; ca = capsule containing sp etc.; can = canal made by capsule; ct = connecting tubes; g = generative cell; i = intine; ma = male cells; me = membrane enclosing body cell complex; n = nucleus; nc. 1 = nuclear cap of sp; nc. 2 = organ corresp. to nc. 1; ne = neck cells; p = pore; PG = pollen grain; Pr = prothallium cell; Pt = pollen tube; RB = remnants of body cell; sp = spermatozoids; St = stalk cell; t = tube cell; ti = short intine tube; w = wall of PG.

sudden egress: "the membrane of the mother cell containing the two spermatozoids is suddenly thrust out of the pollen tube" (pollen tube means here the elongated pollen grain). This is interesting, because if the point of egress in *fig. 16* were near the apex of the nucellus, it would look very much like a germinating pollen grain of *P. menziesii* with the short tube. Another point of interest is that also the spermatozoids of *Ginkgo* sometimes leave the pollen grain enclosed in a membrane.

Although this has as yet not been observed in practice, there seems, in view of the above, not much doubt, that in *P. menziesii* the released spermatozoids, by moving the cilia and pressing against the enclosing membrane at the point where they want to get out, propel themselves and their cargo either through a grown pollen tube (cf. below) or right through the wall of the pollen grain. In the latter case the wall of the pollen grain breaks by this rough passage and a bulge of inner intine (which is evidently more elastic) is drawn out like in *fig. 16* (see *fig. 17)* till it bursts forming a short "pollen tube". Keeping up the pressure against the membrane. which has now assumed the form of a capsule, the spermatozoids under both alternatives continue to propel themselves and their cargo to the egg cell or to a point near it. The spermatozoid in *fig. 14* lying with the cap against the wall of the broad end of a capsule like the one shown in (2), fig. 41, seems to suit the propulsion theory well enough, although it is not quite clear how the propelling force is effected inside a seemingly closed capsule. The explanation is probably that there is circulation of fluid between the interior of the capsule and the surrounding fluid. The traces left by the capsule during its passage through the rather solid tissues of the apex of the nucellus suggest, that exudation of enzymes has facilitated the passage. — It was hinted above, that a *grown* pollen tube sometimes might be present. This was based on the fact, that the two elongated pollen grains shown in (2), fig. 37, actually seem in the process of sending out pollen tubes at the ends of the pollen grains opposite the pore, although the spermatozoids still seem to be in the cells. It is, of course, impossible to be sure that the cells are not empty, but the possibility cannot be excluded, that when the end of the pollen grain opposite the pore is in contact with the apex of the nucellus, the elongated pollen grain may be able to *grow* a pollen tube, through which the capsule leaves the pollen grain, while, in the case when the pore end is in contact, the capsule has to break through the wall of the pollen grain.

Artificial germination of pollen of P. menziesii has, as far as known, never been successfully carried out. The reason for this may have been, that formation of a pollen tube was expected, and that the spermatozoids were overlooked, but more often the pollen grains probably degenerated before they reached the germination stage. In an effort to overcome this latter difficulty the pollen grains were left in the micropylar canal till about the time for natural germination viz. first half part of June, and then transferred to hanging drops consisting of sugar solutions of different percentages or of fluid pressed out of the nucellus, sometimes diluted with boiled tap water. It is regretted that a detailed description of the procedure cannot be given; as a matter of fact the finding of the spermatozoids was as much an accident as the result of systematic research. In view, however, of the importance of the matter, a detailed description of the sequence of events is given in the following. The germination attempt took place during the period June 8—28, 1964, and on account of lacking flowering and of the time consuming work, the attempt has not been repeated. The germination of the elongated pollen grain, in which rotation and spermatozoids were observed, took place in a hanging drop consisting of fluid pressed from several nucelli diluted with about ¼ of boiled tap water; it was kept at room temperature, about 23° C, and inspected several times daily. The drop contained about a dozen other elongated pollen grains. In order not to risk detrimental influence on the germination, no antibiotica was used, but it would indeed be very desirable if some means could be found of preventing the development of the multitude of microorganisms always invading such cultures. The forcing was started on June 8th. On June 9th it was observed that in one of the pollen grains the body cell was swollen and more translucent than in others, and the culture in question was therefore singled out for more frequent observation. In the morning of June 10 th the situation seemed unaltered, but in order to facilitate inspection and to avoid shaking the hanging drop, the culture was left in a microscope. Inspection was carried out about once an hour, and about 16 o'clock a member of the staff, lic. agro. Ib Linde-Laursen, looking in the microscope, observed movement in the body cell of a pollen grain near the one under observation. The movements gradually changed into rotation *(fig. 10, 10 a),* the velocity of the rotation increased, and after a few minutes a spermatozoid rushed out with amazing speed, immediately followed by another. Both swam about inside the pollen grain a few moments, whereafter they left through a gap in the wall of the grain, where a piece had been broken off. Continuing to swim about with considerable speed *(fig. 11, 11 a)* they kept all the time near their "home", until they after about half an hour grew tired and lay still. While keeping the two spermatozoids under constant observation we noticed two more coming out of the pollen grain. As there were no other cells in the pollen grain they must have been passing from another one in the neighbourhood. Movement was also observed in a couple of other pollen grains, but in a hanging drop the pollen grains are frequently changing their position, and it could not be determined, whether the one primarily under observation was among them. During the observations a series of exposures was made with a camera mounted on the microscope, but microphotography of objects in a hanging drop being rather tricky, many exposures were unsuccessful, and furthermore only rather low magnification could be used. It was therefore considered important to try to make the preparation permanent i. a. in order to investigate the cilia, which were only faintly seen by low magnification. The attempt proved successful. The cover glass with the hanging drop was placed on a slide in a drop consisting of 3 parts acetic orcein plus 1 part of glycerine, and by good fortune the 2 spermatozoids we had under observation were found in the place where they had stopped. 3 other spermatozoids were found elsewhere in the preparation. The observation of the cultures continued till June 28. Motion was seen in two cells but they stopped again, and no spermatozoids were seen. As we could not, however, inspect the cultures all 24 hours, movement may have been overlooked.

Although the above particulars are not as adequate as desirable, they should make it possible to work out a method for germination of pollen grains of *Pseudotsuga* (and perhaps of *Larix),* especially if the material is taken from the micropylar canal about the time when natural germination is taking place. The first step must no doubt be to find the most suitable substrate and the best tem-

perature for germination, and that may be troublesome enough, because on account of the short life of the spermatozoids inspection may be necessary during all the 24 hours.

Discussion

It might of course be tempting and interesting to try to make a more detailed comparison between the fertilization mechanisms in *P. menziesii, Ginkgo* and *Cycads.* There is, however, still several gaps in the knowledge of the development of the male cells and the fertilization mechanism of *P. menziesii,* and a detailed comparison is therefore better postponed, untill these gaps are filled.

Little is known about the structure of the spermatozoids of Phanerogams, and it may therefore be of interest, that according to KNUT NORSTOG (6), who has recently carried out an investigation of the fine structure of the spermatozoid of *Zamia integrifolia,* this spermatozoid has a fine structure resembling in some respects that of motile cells of algae, mosses and ferns with similarities in structure both of flagella and basal bodies. He also found that the spiral organization of the flagellar apparatus in *Zamia* is essentially like that of the fern spermatozoid.

Although the fertilization mechanism in *P. menziesii* is unusual, its individual phases show certain parallels with those of other plants. It is thus well known that the tip of an ordinary pollen tube carrying male cells may continue to work its way a considerable distance through the tissues after it has lost its connection with the rest of the tube and with the pollen grain. It is also well known that a pollen tube may contain other nuclei or cells than the male nuclei (cf. [1] and [1 a]) and that these may even be discharged into the egg cell. Elongated pollen grains occur also in *Ginkgo, Cycads, Sequoia sempervirens, Cryptomeria japonica* and perhaps in other species. The novelty in this case is that spermatozoids are occurring in *P. menziesii,* and occurring in connection with a kind of pollen tube, signifying, perhaps, a passing stage between fertilization by spermatozoids and fertilization by pollen tube.

Summing up the evidence it seems probable that the spermatozoids of both *P. menziesii* and *Ginkgo* swim from pollen grain to egg cell, but while those of *Ginkgo* swim freely, the spermatozoids of *P. menziesii* swim enclosed in a membrane *(fig. 17).* The reason for this difference is not known, but it might perhaps be imagined that an "armoured" spermatozoid penetrating hard tissues in less exposed to accidents than is a naked one. While the fertilization in both cases is effected by spermatozoids, the mechanism in *P. menziesii* seems to be a step in the direction of the formation of a pollen tube.

It seems probable already now, that the fertilization mechanism in *Pseudotsuga menziesii* is more like that of *Ginkgo* than like that generally accepted as regards the *Pinaceae (fig. 17),* but it is perhaps in this connection worth recalling that IKENO and HIRAZE (4) (Ann. of Botany. vol. 11, p. 345, 1897) said: "HOFFMEISTER long ago stated his supposition that in the pollen tube of conifers spermatozoids would be found to be produced". Be that as it may, but at all events, it is inter alia still unexplained how a non motile male nucleus works its way from the membrane of the egg cell to its nucleus. This latter problem also exists in the Angiosperms, where, according to SHARP ([7], p. 298), the male nuclei of species of *Lilium, Tulipa* and *Fritillaria,* in the opinion of several investigators, have the power of independent movement. In *Sequoia sempervirens* the fertilization mechanism has, according to LAWSON's (5 a) description, considerable resemblance to that of *P. menziesii;* the pollen grains in the micropylar canal elongate, and when the archegonia are ready for fertilization the two male cells, to quote LAWSON, "move toward the wall of the pollen tube and take up positions immediately opposite the necks of two neighbouring archegonia. The wall of the male cell and of the tube in the region opposite the neck-cells evidently become dissolved, for the nucleus of the male cell with a very small amount of cytoplasm surrounding it, squeezes through the narrow canal between the neck-cells and immediately advances toward the egg-nucleus". The male cells are in LAWSON's opinion not spermatozoids, but he does not suggest any explanation as to how they move the relatively long distance out of the pollen grain, between the neck-cells and through the egg cytoplasm. In *Cryptomeria japonica* the situation is, according to LAWSON (5 b), much the same as in *Sequoia sempervirens,* and the distance the male cells have to move seems also approximately the same. — The resemblance between the ovules and the pollination mechanism of *P. menziesii* and *Larix* and the fact, that pollen of *Larix,* like pollen of *P. menziesii,* cannot be germinated in vitro, seem to justify a suspicion that a more exhaustive investigation of the fertilization mechanism of *Larix* might be worth while.

A more detailed investigation of the fertilization mechanism of these — and perhaps other — species might be of considerable interest, and not only for taxonomic and other theoretical reasons. It seems probable that motile spermatozoids are more sensitive to climatic conditions than male cells enclosed in a pollen tube, and it is, where spermatozoids are involved, an open question whether frost during meiosis in early spring, or low temperature, although above zero, in June, is most detrimental to the seed setting. It is, of course, also possible that more exact knowledge of the pollination — and fertilization — mechanisms would enable us to produce hybrids, and perhaps valuable hybrids, between species, which it now seems impossible to cross.

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Summary

1. It appears that three mitotic divisions take place in the embryonal nucleus of the pollen grain of *Pseudotsuga menziesii* forming 2 prothallium cells and 2 male cells. No generative cell was observed, and the assumption is advanced, that the tube cell and the stalk cell are both units in the nutritive system of the body cell and of the pollen grain, and that they are not daughter cells of the embryonal cell.

2. Two motile, oviform, multiciliar spermatozoids were observed emerging from the seemingly rotating interior of a body cell and swimming in the neighbourhood.

3. It is suggested that at germination the spermatozoids, remaining inside the membrane surrounding the body cell complex, are propelling themselves and the contents of the membrane through a short (pollen) tube into the apex of the nucellus and on to the vincinity of the egg cell.

4. Certain resemblances to the fertilization mechanism in *Ginkgo* were found, and it appears that both in *P. menziesii* and in *Ginkgo* the fertilization is effected by spermatozoids, but while in *Ginkgo* the spermatozoids are swimming freely from pollen grain to egg cell, in *P. menziesii* they swim inside a membrane from the pollen grain through a canal in the apex of the nucellus and the neck cells (formed by exudation of enzyme) to the egg cell.

5. It is suggested that the fertilization mechanism in *P. menziesii* may be a passing stage from fertilization by spermatozoids to fertilization by pollen tube.

6. The question of the sensitiveness of motile spermatozoids to low temperature is touched, and it is thought desirable that the fertilization mechanism of f. inst. *Sequoia sempervirens, Cryptomeria japonica* and *Larix* are subjected to a more detailed investigation, with a view, inter alia, to the possibility of making crosses which cannot now be effected.

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On the Germination of Pollen of Larix and Pseudotsuga on Artificial Substrate, and on Viability Tests of Pollen of Coniferous Forest Trees

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Introduction

As reported by Kühlwein (8), Müller-Stoll (9), Illies (7), Barner and Christiansen (1), Eriksson (6) and probably experienced in practice by many other workers, all attempts to germinate pollen of *Larix leptolepis* (Sieb. & Zucc.) Gord. and *Larix decidua* Mill. on artificial substrate have hitherto proved unsuccessful; this peculiarity in *Larix* also applies to the pollen of *Pseudotsuga menziesii* (Mirbel) Franco var. *viridis* Schwer.

By "germination" is here understood the production, on artificial substrate, of a pollentube, which in nature is destined to carry the male cells (or nuclei) from the pollen grain to the egg cell.

As is well known "germination", or "viability", tests are widely used in other species as a basis for estimation of the viability of pollen destined for controlled pollination, pollenstorage tests etc.

The pollen grains of *Larix* and *Pseudotsuga*, however, produce no ordinary pollen tube, and the usual viability tests cannot be applied, which is a serious shortcoming. Eriksson (6) says about this: "The working out of a convenient method for estimation of pollen sterility in *Larix* must be regarded as of utmost importance for the future research concerning seed setting in *Larix*". — The working out of a method of this kind, and particularly in respect of a cytologically difficult species like *Larix*, is, however, no easy matter, but some new information given in the following concerning the fertilization mechanism of *Larix* and *Pseudotsuga*, may be of use in this connection.

The Fertilization Mechanism of Larix and Pseudotsuga

The reason why the pollens of *Larix* and *Pseudotsuga* do not respond to the usual pollen germination methods is probably, as mentioned above, that no ordinary pollen tube is produced, and that the fertilization mechanisms of the two species in several respects differ considerably from those of coniferous forest trees producing regular pollen tubes. The fertilization mechanisms of *Larix* and *Pseudotsuga* are in many respects more like that of *Ginkgo* than of f. inst. *Pinus*, and the structure of the pollen grains are much more complicated than in the latter species cf. (3) and the present *figs. 2 and 3.*

Besides the long resting period on the collapsed stigmatic flap (6—8 weeks), which may also suggest a prolonged forcing period on artificial substrate with increased risk of contamination, the main difficulties are probably caused by the motile male cells (Actually motile male cells have as yet not been observed in *Larix* but the fertilization mechanism is essentially the same as in *Pseudotsuga).* Their life (in a hanging drop) seems short, about 1 hour, and they probably need special nutrition if they are to live longer. The movement and apparent rotation of the male cells observed in the bodycell of *Pseudotsuga* at germination time (actually only the cilia are moving) are relatively easy to see, but as soon as they break out of the body cell they aim straight at the pollen grain wall, which they penetrate, or they slip out through an existing crack in the wall, and disappear, much like the spermatozoids of *Ginkgo*. The cultures must therefore be kept under strict observation, otherwise the emergence of the male cells may easily be