5) Da auch dünne Sekundärstecklinge wie Primärstecklinge ein Jahr nach dem Steckzeitpunkt und sehr zahlreich blühen und ihre Bewurzelungsfähigkeit vom Primärsteckling zum Sekundärsteckling steigt, wird bei *Bombacopsis* auf Unabhängigkeit der Blühfähigkeit vom physiologischen Alter geschlossen.

Schlagworte: Bombacopsis quinata, Bewurzelungsfähigkeit, Primärstecklinge, Sekundärstecklinge, Provenienzunterschiede, Klonunterschiede, Einfluß des Durchmessers, Blühfähigkeit.

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On the Development of Pollen and the Fertilization Mechanisms of Larix and Pseudotsuga menziesii

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Introduction

Since GELEZNOV (8) and (5) in 1849 demonstrated the unique pollen-catching device in Larix, and STRASBURGER (18) and BELJAEFF (2) in 1884—93 discussed the character of the generative pollen cell in the species, the different stages of the development of the pollen and the pollination and fertilization mechanisms of Larix have been the subject of several investigations, but, nevertheless, irnportant details in these processes are still more or less in the dark.

This may seem strange, but it is a natural consequence of the absence of sufficiently detailed, continuous investigations of complicated processes like the pollination and fertilization mechanisms in conifers. The main causes are: undependable flowering, adverse climatic conditions, attacks by insects, great variations in structure even in closely related species or within the same genus (cf. FERGUSON (7), p. 217, point 14) and technical difficulties.

The knowledge in this field is therefore based on many descriptions of limited details (STERLING (17)) and sometimes, as it would seem, on homologies and analogies not too well founded.

STERLING ((17)p. 188) states:

"Among the gymnosperms the greatest amount of variation in sexual development occurs in the Coniferales and Taxales. It would appear that a closer investigation of gametophyte development in this group could provide further clues to their phylogenetic development and their interrelationships. Nevertheless there are many gaps in the knowledge of the different genera".

It also appears from STERLING'S review that the interpretations of different investigators are by no means always unanimous. He says f. inst., (p. 168):

"However, resemblances among lower and higher plants in the organization of the male garnetophyte (and its gametangium) are less readily perceived. Here only two homologues have been universally admitted: the first cell of the gametophyte (the spore) and the last one (the male gamete or sperm)".

and in the next paragraph:

"Because of the reduced structure of the gymnospermous male gametophyte, homologies of its cells have not been unambiguously interpreted. Differences in concepts of morphology have been reflected in differences of terminology."

It is evident that in the circumstances the study of the history of the pollen development in gymnosperms is extremely difficult although STERLING'S excellent review of the situation, particularly as regards the Coniferales and Taxales, is a great help. Particularly as regards the pollen cells, different interpretations and varying results of different investigations have, of course, numerous causes, most of them understandable. Remarkable are the uncertainties connected with the determination of the exact number of prothallial cells in several groups. When, f. inst. in respect of the Araucariaceae ((17)p. 192), it is documented that 13 to 40 secondary prothallial cells are produced, the suspicion involuntarily arises, that such — and perhaps other similar, although less spectacular — variations may be due to the recently discovered ability of pollen cells to divide (and produce haploid plants) when subjected to abnormal growth conditions. If this is possible, it involves a new complication of an already complicated situation, and new investigations may be required to find out the actual state of affairs.

The present investigation aims to fill in and elucidate some of the above mentioned gaps, which are of taxonomic as well as of practical significance. The subjects of the investigation are particularly the events occurring during the development of the pollen from the tetrad stage till the male gametes arrive at the archegones.

According to STERLING ((17)p. 189) the male gametophyte in Larix, Picea, Abies and Pseudotsuga before pollination consists of a five-celled row i. e.: 2 prothallial cells, 1 stalk (or sterile) cell, 1 spermatogenous cell and 1 tube cell. We found in Larix as a maximum: 2 prothallial cells, a dubious stalk cell, a spermatogenous cell, but no ordinary tube cell and no generative cell.

According to CHAMBERLAIN ((3), p. 227) Larix is taxonomically classified under the Abietaceae, the pollen of which produce a pollen tube and readily germinate on artificial substrate. According to our findings pollen of Larix (and Pseudotsuga) produce no ordinary pollen tube, and it has hitherto not been possible with pollen of the two species to make it discharge male cells on artificial substrate (4c).

The unusual interphase nucleus (fig. *10),* the unique internal structure of the mature pollen grain (figs. 15—18), the absence of a pollen tube and the impossibility of artificially producing discharge of the male cells seem to indicate that Larix, as regards the male gametophyte, must be placed somewhere between the Ginkgoales and pollen-tube-producing Coniferales.

It is suggested that in *Larix* (and *Pseudotsuga)* the mode of transport of the male cells from pollen grain to egg-cell is a transitional stage between the more primitive transport by spermatozoids (as f. inst. in *Cycadales* and *Ginkgoales),* and the more advanced transport by pollen tube (as in the *Angiosperms* and, according to the existing records, f. inst. in *Pinus* and *Abies).*

Material and Methods

Inflorescences of *Larix decidua* MILL. and *Larix leptolepis* (SIEB. & ZUCC.) GORD. were used. The flowering of *Larix* being rather undependable and liable to be damaged by frost, promising investigations have often been interrupted and postponed. Therefore, the results described in the following are derived from investigations made in the course of several years, and from different materials. This is of course not the ideal way to make observations of series of events, but it has at least the advantage of offering the opportunity repeatedly to scrutinize the same events. Trees for examination were placed at our disposal by the Director of the Tree Improvement Station of the Danish State Forestry, Mr. H. BARNER, who also arranged isolation and pollination for the necessary experiments; experience has shown that artificial pollination greatly increases the number of well pollinated ovules as compared with free pollination, which of course facilitates examination. The investigation of the sequence of the stages of development of the pollen grain and pollen cells was made partly in nature with intervals of 1—2 days, partly on inflorescences of twigs (rather large) forced in the laboratory at high humidity and room temperature. The latter method has the great advantage that the investigation can be started already in January and repeated by taking in new twigs as often as desirable for 2—3 months. Besides, the higher and constant temperature in the laboratory makes it much easier to find mitoses in the pollen cells. The forcing usually works satisfactorily during 8—10 days, up to the stages about dehiscence; thereafter the divisions often stop or become unreliable. It may be mentioned that already STRASBURGER used forced inflorescences for investigation.

Investigation of the pollen development during the 6—8 weeks on the stigmatic flap, in the micropylar canal and on the apex of the nucellus was mainly made by cutting off and dissecting, under a binocular dissecting microscope, the top of the integument or/and the apex of nucellus with the grains deposited on them. Part of the material was taken directly from the trees, part from pollinated female inflorescences forced for a few days in the laboratory.

During the periods January — June 1968 and 1969 a total of 539 fixations were made. Of these, 213 were inflorescences taken directly from the trees, 326 were inflorescences from twigs forced in the laboratory for different periods. Before fixation the inflorescences were cut through with a sharp blade and fixed in 45% acetic acid or CARNOY's fluid and later transferred to 70% alcohol.

For examination of structure etc. a phase contrast microscope is indispensable; the numerous membranes, often densely ornamented, totally obscure each other and the rest of the contents of the pollen grain if they are stained for examination by an ordinary microscope. An exception is FEULGEN staining for examination of intranuclear divisions, f. inst. in the embryonal cell; FEULGEN stain affects mainly parts containing chromatine, and it clarifies the cytoplasm; the FEULGEN hydrolysis may dissolve at least part of the granules, which often totally obscure the view. High temperature during forcing seems gradually to make the granules disappear. It must, however, be remembered that if the granules are dissolved and the cytoplasm cleared, by FEULGEN hydrolysis or by other means, intracellular tube junctions, "caps" etc. may be dissolved or bleached at the same time. Preparations of the large pollen grains of *Larix* cannot, in practice, be made permanent, neither by freezing nor by other known methods, without the risk of shrinkage and distortion. In the present investigation all plates of interest have therefore, when possible, been photographed and recorded, and the preparations made semi-permanent by using acetic orcein or 45% acetic acid plus 10—30% glycerine. Many of such preparations keep for months or longer, if evaporated fluid by and by is replaced with 10—30% glycerine in 45% acetic acid. The cytological technique used has been described in (1), (1a) and (4b).

The germination of pollen on the apex of the nucellus, the traces of the male cells through the nucellus and their contact with the egg cell has been studied on cut off nucellus tops and on microtome sections.

Terminology

In respect of seeds the term *Germination* means to sprout. When used in respect of conifer pollen it usually means that the pollen has produced a tube either on the apex of the nucellus or on artificial substrate.

The stage of development reached by the pollen, when a tube is produced, varies. In *Pinus,* f. inst., a pollen tube seems to be produced within a month after pollination, but the male gametes are formed the following summer. In *Abies* the male gametes may be formed before the tube is produced ((17), p. 189). By germination on artificial substrate the stage of development of the pollen cells is usually ignored.

In *Larix* and *Pseudotsuga* pollen, by which no pollen tube is produced, the elongation of the pollen has been termed germination. However, in view of the fact that also dead pollen of these species may elongate, the criterion is inadequate.

The time when the pollen of *Larix* begins to grow is difficult to determine, but it seems out of question that the term "pollen germination" should cover also pollen development before dehiscence. — Since the formation of the prothallial cells, which is relatively easily observable, takes place before dehiscence, and because practical purposes make it desirable that the stage termed germination is as close as possible to the stage of fertilization, *we have in the following used the term germination for the stage when the male cells are discharged from the pollen grain. — Generally the terminology of* STERLING *(17) is used.*

General Information

The pollen catching device in *Larix* and *Pseudotsuga,* which has been described in detail by DOYLE (5, 6), BARNER and CHRISTIANSEN (1, 1a), and others, differs from that of *Pinus* and *Abies* by the pollen remaining on the stigmatic flap for 6—8 weeks and thereafter being transported to the apex of the nucellus by fuid exuded and retracted through the latter. This exudation and retraction of fluid evidently coincides with the readiness of the egg cell for fertilization. In contrast to this the pollen of *Pinus* and *Abies* caught on the stigmatic arms of the integument, are within a few hours transferred to the apex of the nucellus (6a). The pollen catching devices of *Larix* and *Pseudotsuga* are practically identical. The structure and development of their pollen grains follow essentially the same lines, but

there is one conspicuous difference viz. while both are wingless, spherical, of about the same size and with no visible differences before dehiscence, the pollen grain of *Pseudotsuga* soon after pollination starts to elongate, and at germination time it is 5—6 times longer than originally (1a). The pollen grain of *Larix*, on the contrary, elongates only slightly, and its body-cell complex being of about the same size as that of *Pseudotsuga*, it is more compacted and difficult to investigate. For this reason it has not been possible to give a detailed description of the development of the pollen grain of *Larix* from pollination to germination, and such a description will probably not be possible until the pollen can be germinated on artificial substrate (4c). It seems however clear, that the processes are essentially the same as in *Pseudotsuga* and rather different from those in *Pinus* and *Abies*.

Meiosis

Meiosis in *Larix* has been described by SAX and SAX (15), KNABEN (11) and has not been reexamined during the present investigation. However, the telophase II and the

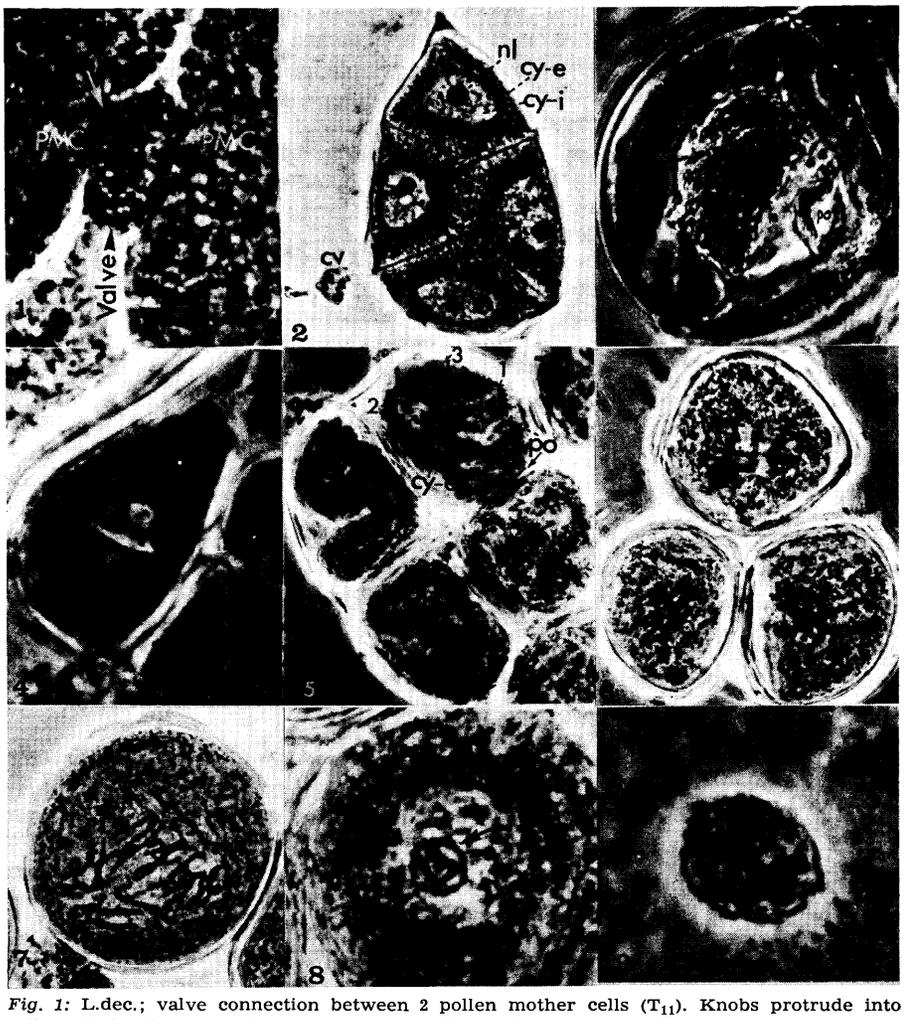

Fig. 1: L.dec.; valve connection between 2 pollen mother cells (T_{11}). Knobs protrude into cells; only left knob visible (arrow). ×3300. — *Fig. 2:* L.lept; tetrad, 4 cells with external (cy-e) and internal (cy-i) cytoplasm; one large nucleolus visible in all nuclei, in bottom cell also trace of second nucleolus and chromosomes of interphase nucleus, which in the other cells is hidden by cytoplasm. Left torn off valve (cv). ×725. — *Fig. 3:* Decaying tetrad cell showing valve connections. External cytoplasm and most of internal dissolved. CV_1: side valves between nucleus and external cytoplasm; CV_2: valve connection between nucleus and probably intercellular valves *(fig. 1)*. ×1780. — *Fig. 4:* Decaying tetrad cell. Cytoplasm dissolved. The mushroomlike organ (arrow) seen is probably membrane containing interphase nucleus *(fig. 10)*. ×610. — *Fig. 5:* Tetrad cells undergoing mitosis. Upper cell: cy-e split open revealing part of sack-like membrane with pore-orifice torn loose from pore and lifted up (dotted arrow). 1—2: side valves; 3: cap of outer top-valve; 4: inner top-valve corresponding to 3. Regards cells see text (cf. also *fig. 4*). ×1000. — *Fig. 6:* Tetrad cells with mitotic divisions. Top: Telophase of first pollen division (either formation of first prothallial cell or, possibly, vegetative cell *(fig. 29)*, see text). Bottom: metaphases. ×1000. — *Fig. 7:* Early anaphase of first pollen division, spindle figure shows traces of peculiar interphase nucleus, see text. ×300. — *Fig. 8:* Tetrad cell nucleolus (arrow) without cap (cap probably as *fig. 9*). The black dots are probably ends of chromosomes of interphase nucleus like *fig. 10* and may be cilial band initials (the 2 black circles). ×1700. — *Fig. 9:* L.dec.; nucleolar cap in mononuclear pollen, enlarged early stage. ×4000.

Abbreviations: cv = connecting valve; cy-e = external cytoplasm; cy-i = internal cytoplasm; L.dec = *Larix decidua;* L.lept = *Larix leptolepis;* n = nucleus; nl = nucleolus; PMC = pollen mother cell; po = pore.

tetrad stage were included in the investigation, inter alia because of mitotic divisions being found at the latter stage *(fig. 6)*.

The Anatomy of the Pollen Grain of Larix

According to WODEHOUSE (19): "The pollen grain of Larix is spheroidal, various in size, ranging in the different species from 62.5—102 μ in diameter, closely resembling those of *Pseudotsuga mucronata* (Douglas fir). Exine thin generally rupturing and frequently cast off completely when the grains are moistened; texture smooth, with no trace of the flecks. Intine thick and hyaline, but less thick than in the grains of *Juniperus*. Furrow, pores and bladders entirely absent". — According to MÜLLER-STOLL ((13), p. 629) the smooth exine of *Larix* and *Pseudotsuga* is relatively much thicker than in *Taxus* and similar pollen grains and has no texture. He compared the intine of *Pinus* and *Abies* with

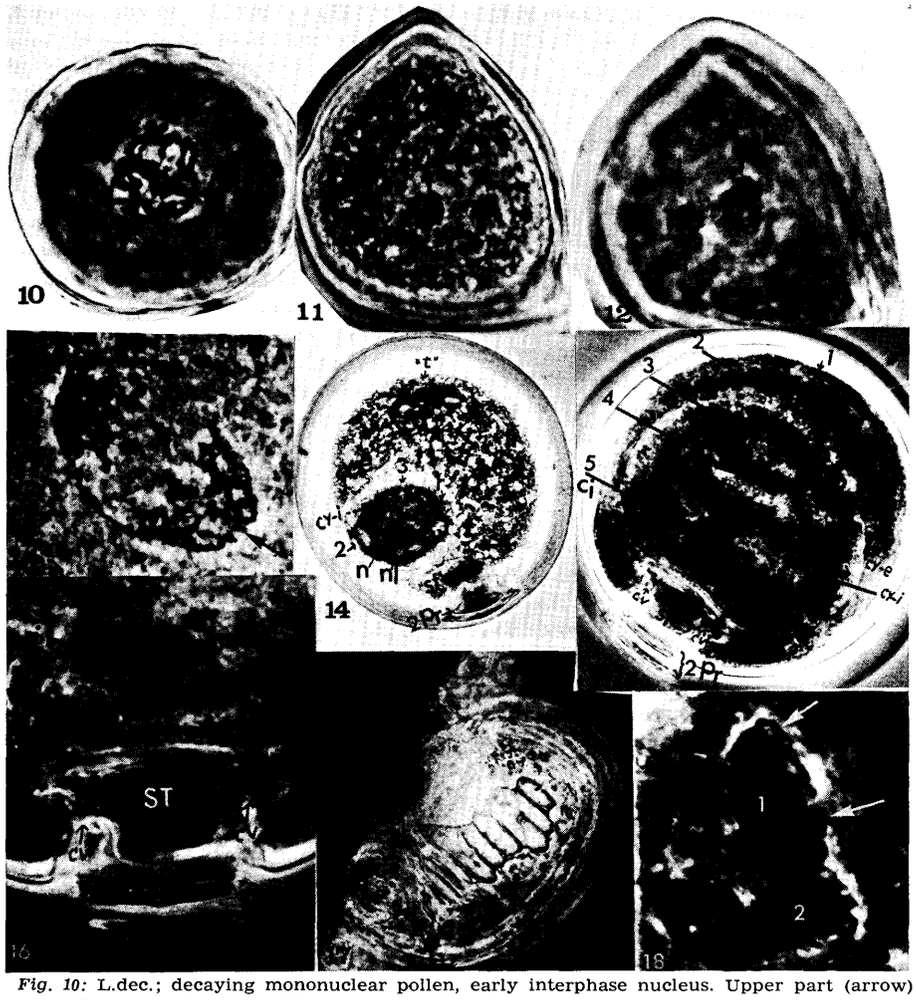

Fig. 10: L.dec.; decaying mononuclear pollen, early interphase nucleus. Upper part (arrow) normally covered by cap *(fig. 9)* and nucleus hidden under internal cytoplasm. ×380. — *Fig. 11 and 12:* L.lept.; mononuclear pollen, developping interphase, near beginning of prophase, same pollen, different focus. *Fig. 11:* 2 nucleoli with caps (arrows), rest of interphase nucleus hidden under internal cytoplasm. *Fig. 12:* same cell as 11, lower focus, interphase nucleus with unexplained typical submedian constriction and chromosomes used as structural units to maintain peculiar shape. The 2 nucleolar caps (arrows) are still visible, they seemingly cover chromocenters, see text. ×380. — *Fig. 13:* L.dec; mononuclear pollen, decaying interphase nucleus, nucleolar cap dissolved; probably chromocenters at arrows. ×380. — *Fig. 14:* L.lept.; embryonal cell after second division, nucleus pushed out of position, shows valves connecting nucleus with external cytoplasm (cf earlier stage *fig. 3*). 1—2: side valves; 3: top valve; "t": supposed „tube nucleus". ×380. — *Fig. 15:* L.dec.; spermatogenous cell probably about a week before fertilization. Presumably 2 male gametes above each other; "stalk cell" with 2 distinct valve connections (see also *figs. 16 and 17*), spiral band (probably cilial (ci)), caps 1—5. 1—3: top valves; 4—5: nucleolar caps of small and large male gametes respectively (5: cap in center of spiral) ×500. — *Fig. 16:* Enlargement of pore-region of *fig. 15* showing cilial spiral of large gamete and "stalk cell" with valve connections (cv) to external cytoplasm, 2 prothallial cells (without connections to tube system). ×1400. — *Fig. 17:* L.dec.; dead and decayed pollen from apex of nucellus. Structure consisting of 8—10 ringformed sections reaching from pore to top. Upper 6 sections probably carry the 2 male gametes *(fig. 15)*. Lower part seems to comprise "stalk cell" and pore region (cf. text). ×400. — *Fig. 18:* L.dec.; decaying spermatogenous cell about week before fertilization; containing one male gamete more or less intact (1), one damaged (2). 1: probably interphase nucleus including chromocenters (arrows); unexplained row of "fins" on left side. ×950.

Abbreviations: ci = cilial spiral band; cv = connecting valve; cy-e = external cytoplasm; cy-i = internal cytoplasm; L.dec. = *Larix decidua;* L.lept. = *Larix leptolepis;* n = nucleus; nl = nucleolus; po = pore; pr = prothallial cell; st = stalk cell; t = "tube cell".

that of *Larix* and found that in the two first-mentioned species the intine consists of one seemingly rather homogeneous layer, while in *Larix* and *Pseudotsuga* it is differentiated into an outer, relatively easily swelling layer, and an inner, very thin layer (p. 637).

In the main the above is in accordance with our own observations; we can, however, supply some supplementary information: The pollen grain of *Larix* has a pore like that in *Pseudotsuga;* the pollen grains shown in (1a), *figs. 16—19* may serve as illustration. It has also, at least at younger stages, beginning with the tetrad cells, an aperture at the distal end where dry pollen is often caved in. The interiors of pollen mother cells, and often of young pollen lying close together, are connected by a sort of valve shown in *fig. 1.* These valves are seldom found in situ, but often lying about; they are sitting very loosely.

Already at the tetrad stage *the pollen of Larix* has two layers of cytoplasm: an external and an internal, *(fig. 2)* the latter enclosing the nucleus, the chromosomes of which, to judge from phase contrast observations, are embedded in a somewhat different substance (nucleoplasm, *fig. 10).* The two layers of cytoplasm are separated by a sack-shaped (prothallial) membrane, the orifice of which is the pore, and through which the internal tube connections between the nucleus and the external cytoplasm are conducted by means of complicated valves *(figs. 3, 14, 16).* The internal cytoplasm is also, at least at certain stages, covered by a heavily ornamented membrane, seemingly like that found in *Pseudotsuga* ((4b), *fig. 2).* In all observable respects the pollen grains of *Larix* and *Pseudotsuga* continue to appear identical until after pollination, when, during the 6—8 weeks stay on the collapsed stigmatic flap, the grain of *Pseudotsuga,* as mentioned above, elongates to a length corresponding to 5—6 pollen diameters, while the grain of *Larix* remains more or less spheroidal or becomes slightly ovate. The pollen grains of both species are closely packed with granules which sometimes completely obscure the interior. The granules seem to dissolve gradually, particularly at higher temperatures, and they probably serve as nutrition during the development of the grain.

The puzzling question how the enormous spermatogenous cell and the other pollen cells are held in position and nourished seems to some extent to be answered by *figs. 15 and 17. Fig. 17* shows a dead pollen grain found at germination time on or near the apex of the nucellus. It is decaying and the pollen cells have disappeared thereby disclosing a peculiar tube-like structure, consisting of 8—10 ring-formed sections and reaching from the pore to the distal end of the grain. It seems evident that the pollen cells seen in *fig. 15* are arranged, one above the other, on such a structure, which is no doubt also serving nutritious purposes. The cells in *fig. 17* were probably the two male gametes (cf. *figs. 15, 16)* which, having accomplished the development, failed for some unknown reason before the last barrier. Further interpretation of this interesting and, as far as known, unique, structure has not been possible.

The internal tube system of the pollen grain of *Larix* is much the same as in *Pseudotsuga* ((4b), *figs. 3, 3a)* and the present *figs. 3, 14.* There is a big tube connecting the pore with the distal pole; it is interrupted by valves where it traverses the prothallial membrane enclosing the embryonal/spermatogenous and the prothallial cells. There is another, transverse, tube connection through the first mentioned cell to the external cytoplasm; also this has valve connections where it traverses the prothallial membrane.

Fig. 5 shows 4 tetrad cells about the first division of the embryonal cell. The upper cell to the right has burst open showing part of the prothallial membrane (with the pore) containing the embryonal cell. The dark masses in the cell are evidently external cytoplasm and membranes, which are not dissolved during the intranuclear divisions. The strange patterns they form do not seem to be accidental; they are frequently seen, and they may be caused by the reduced space left for spindel formation, f. inst. when the temperature is near the threshold of chromosome movement and the pliability of the cytoplasm reduced (4a). Pollen mitoses at the tetrad stage have been reported in tetraploid *Larix* by CHRISTIANSEN (4), and they seem to occur rather frequently in inflorescences also of diploid *Larix* forced at room temperature.

The Interphase Nucleus in Larix

The chromosomes are evidently not despiralized during interphase; they have a certain resemblance to somatic metaphase chromosomes and seem bent and shaped so as to support the shape of the nucleus, cf. *figs. 10—13.* The same situation is found in *Pseudotsuga* ((4b), *figs. 7, 7a). Fig. 4* shows the interphase nucleus of a decaying tetrad cell, enclosed in a membrane. At full interphase the nucleus is strongly condensed *(fig. 10).* When preparing for division the membrane of the nucleus and the inner cytoplasm disappear and the nucleus gradually expands filling out the inner space, the chromosomes take on a more normal shape and the divisions, including the prophase, seem normal. It is, however, interesting to note that although the metaphase stage often is fully normal, divisions like that shown in *fig. 7* with chromosomes outside the spindle, and seemingly striving to maintain the peculiar shape of the nucleus, are not infrequent. It is also interesting that while interphase nuclei of the *Larix-Pseudotsuga* type described above do not seem to be common in plants, they seem to exist in animals, f. inst. in the Syrian hamster (12).

Nucleoli of interphase nuclei in *Larix,* particularly before meiotic prophase and before mitotic prophase in young pollen, have been examined. They show great variation in size and appearance: They are usually two, not always at the same level, and they are often very conspicuous. At late meiotic telophase II more than 12 faint nucleoli, probably " prenucleolar bodies" (cf. (14), p. 316), could sometimes be counted. They soon fuse, however, to larger units, and one gets the impression that the main part of the nucleoplasm consists of fused prenucleolar bodies. After late T. II two large nucleoli, one at each end of the nucleus, are usually seen. Actually they seem to be chromocenters consisting of parts of several chromosomes *(fig. 10)* brought together inside a "cap" *(fig. 9).* These caps, protruding through the internal cytoplasm, is what is usually seen in pollen at interphase, while the nucleus itself is hidden under the cytoplasm. *Fig. 11* shows a young pollen with 2 nucleoli, *fig. 12* shows the same pollen at lower focus and the interphase nucleus underneath. *Fig. 2* shows a tetrad with 4 cells, each with external and internal cytoplasm and interphase nucleus of which only one nucleolus is seen; the other nucleolus and the interphase nucleus are hidden under the internal cytoplasm. The caps and nucleoli disappear during divisions, but before that they enlarge considerably.

The Cells of the Pollen of Larix

According to STERLING ((17), p. 187) the embryonal cell of the male gametophyte in *Ginkgo* (or *Pinus),* to the latter

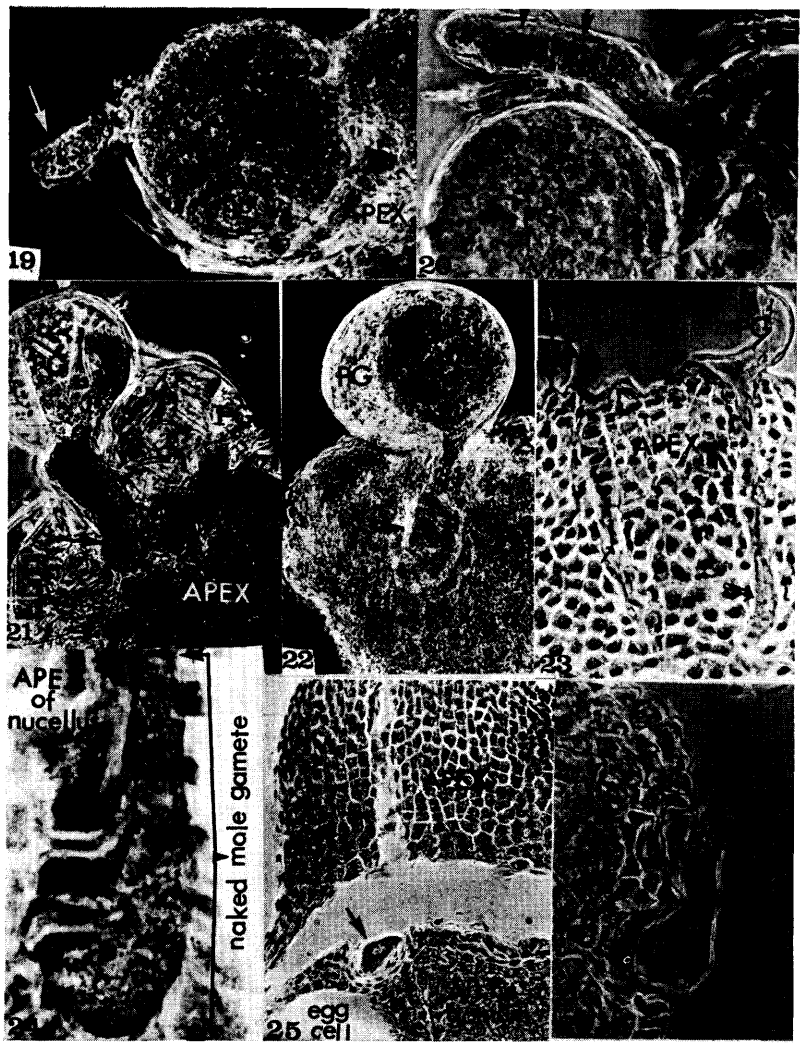

Fig. 19: L.dec.; germinating pollen on apex of nucellus. Male gamete in bulge of intine discharging itself (arrow). Remaining pollen cells unidentifiable. ×300. — *Fig. 20:* L.dec.; segments of two (perhaps 3) PG on apex. Top: 2 male gametes (arrows) and debris in bulge of intine from right segment trying to bypass left PG, resembling ((4b), *fig. 9a)* and *fig. 26.* ×300. — *Fig. 21:* L.dec.; 3 decaying PG (like *fig. 17)* on apex. In centre bulge of intine from torn off and lost PG, containing cells and debris, arrow. ×250. — *Fig. 22:* L.dec.; PG on apex discharging at least one male cell (arrow) in bulge of intine. ×250. — *Fig. 23:* L.dec.; microtome section of apex of nucellus with remnants of two PG and traces of passage of male gametes through apex. Left track: short tube seemingly terminating in piece of tube of dead male gamete (arrow). Right track: only short piece of intine bulge, thereafter pieces of membrane followed by a piece of the gamete (double arrow) shown in *fig. 24* from next microtome section. ×200. — *Fig. 24:* (see also *fig. 23):* male gamete naked, strongly elongated, nucleus (touched by knife) partly covered by pieces of apex cell walls traveling through apex of nucellus (see text). ×590. — *Fig. 25:* Upper part: trace in apex after passage of gamete (dotted arrow); below: male gamete at archegonium, dissolving tissue (arrow). ×175. — *Fig. 26:* Two gametes like those in *fig. 20* in thin membrane resembling end of pollen tube (arrows), evidently trying to penetrate into different egg cells (see text). ×200.

Abbreviations: L.dec. = *Larix decidua;* PG = pollen grain; po = pore.

of which *Larix* is taxonomically referred, divides twice and cuts off two prothallial cells, divides again forming an inner generative cell and a peripheral tube cell. The generative cell divides into an inner sterile cell and an outer spermatogenous cell. Eventually the latter divides to form two male gametes, which makes a total of five divisions (cf. *fig. 27).* According to our investigations, however, the development of the male gametophyte in *Larix* differs considerably from the above schedule. Actually seen were only 3 divisions viz. 2 divisions giving rise to the two prothallial cells and 1 division forming the male gametes *(fig. 28).* As in *Pseudotsuga* (4b) no tube cell, stalk cell or generative cell were found. As mentioned above it was, on account of technical and climatic difficulties, not possible to carry out a continuous investigation of the development of the male gametophyte from tetrad cell to germination of the pollen, and new details may be disclosed, when it becomes possible to germinate *Larix* pollen on artificial substrate. In the following the evidence available is presented.

The Prothallial Cells

2 prothallial cells are found in *Larix*, which is in accordance with STRASBURGER (18). Each cell is separated from the external cytoplasm by a layer of intine and they deteriorate quickly. It is often said that the prothallial cells are "cut off", and so it might seem when the cells are flattened. The mitotic divisions, by which the cells are produced, are however quite normal and so are the prothallial cells immediately after divisions. The reason why they deteriorate and flatten is no doubt starvation; it seems that when the embryonal cell divides, one of the daughter cells, the central, remains connected to the external cytoplasm and survives, the sister cell has no such connections and must die (cf. *fig. 16*).

The generative Cell

The history of this cell is difficult to trace because the term "generative" has not at all times been used in the same sense; besides, descriptions of the formation and division of the generative cell are very few. Convincing documentation could not be found showing that a generative cell (an a tube cell), a body cell and a sterile cell are formed in *Larix*.

Judging from the material available, the embryonal cell, after the production of the two prothallial cells, functions as spermatogenous cell and produces two male gametes by the third division (cf. diagram *fig. 28*).

As regards the history of the tube, generative and sterile (= stalk) cells, the reader is referred to STERLING ((17), p. 182 ff.). A point of interest is, that in view of the complicated construction of the embryonal cell (including valves and enclosing membranes), it is difficult to imagine how a generative cell (and tube cell) could be produced; it would involve the "cutting off" of the tube cell to the side opposite to the prothallial cells, where the important top-valve is situated and removal of the "cut off" cell to the external cytoplasm, where a tube-junction (see tube cell) already exists *(fig. 14)*.

The Pollen Tube Cell (or Nucleus)

Generally there has always been some doubt as to the role of the pollen tube cell in the development of the pollen tube. STERLING (17) says, (p. 168): "The male gametophyte of modern gymnosperms is oligocellular. It seems to have no arrangement of cells which resembles, even slightly, the antheridium of a moss or a fern. Moreover it is distinguished by a special outgrowth of dubious relationship, the pollen tube". He says further, p. 175, as regards the Cycads: .... "(The general cytoplasm of the pollen grain is the cytoplasm of the tube cell). Development of a pollen tube is common to all the Cycads (Text — *fig. 3, B—E*). However, this tube appears to serve a function which is more haustorial than siphonogamous. In all those Cycads which have been carefully investigated, motile spermatozoids have been found" .... and, p. 176: "Although the pollen tube of all the Coniferales and Taxales has definitely a siphonogamous role, to a great extent its functional activity must be nutritive, as witness the cell growth which occurs within the male gametophyte". It is of interest in this connection, that recently HERICH (9) found that in *Tulipa* the vegetative nucleus does not participate directly in the regulation of growth of the pollen tubes, but that the vegetative nucleus and its nucleolus take part in the division of the generative nucleus. He also found that the vegetative nucleus disappears after formation of spermatic nuclei. Although *Larix* and *Pseudotsuga* are intermediate between fertilization by spermatozoids and by pollen tubes, it is clear that their situation as regards tube cell and pollen tube is closer to that in *Cycads* and *Ginkgo* than to that f. inst. in *Pinus*, but a comparison with the cell-complexes of these species is not possible without a more thorough investigation. It is however evident that, on account of the absence of pollen tubes, the "tube cells" of *Larix* and *Pseudotsuga* have no siphonogamous role, and no doubt their functional activity must predominantly be nutritive as stated above by STERLING.

As mentioned before no formation of a generative cell and tube cell could be found in *Larix*. However, already from the tetrad stage a (sometimes) nucleus-like tube-junction is seen in the external cytoplasm at the place, where a tube cell would be expected to appear after the production of the two prothallial cells, (in *fig. 3* above the "top valve", in *fig. 14* marked "t"). Origin and minor structural details of this organ could not be determined, but in view of the fact that it sometimes shows a certain resemblance to a decaying tube cell in angiosperms, and that mitotic divisions were observed at the tetrad stage, it might, perhaps, be suspected, that a "tube" cell is formed *before*, instead of after, the production of the prothallial cells *(fig. 29)*. From a nutritious point of view this would seem an advantage, and the process would be more like that existing in the angiosperms.

The question must, however, be left open; this spring all inflorescences of *Larix* were damaged by frost and useless for investigation of later stages of development.

The Stalk (= Sterile) Cell

The stalk (= sterile) cell is reported to be produced together with the spermatogenous cell by division of the generative cell. STERLING ((17), p. 181) says in this connection inter alia: .... "Suffice it to remark that there is no general

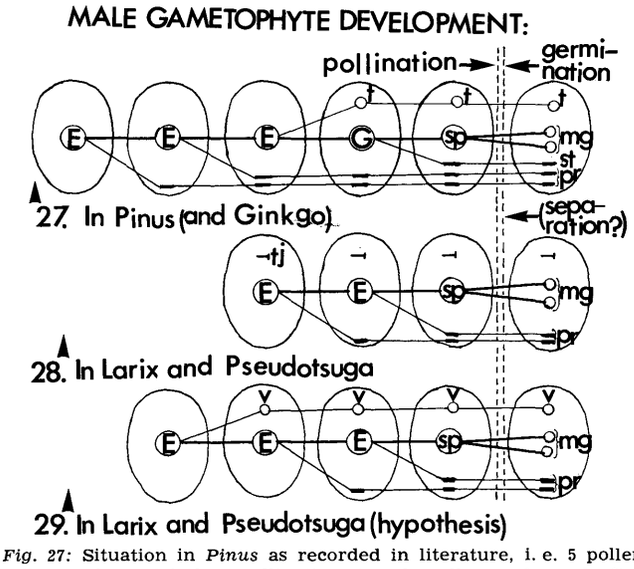

Fig. 27: Situation in *Pinus* as recorded in literature, i. e. 5 pollen cells are formed before pollination, 2 male cells are formed after pollination. — *Fig. 28.* Situation in *Larix* and *Pseudotsuga* according to own investigations. 3 pollen cells (and perhaps also the two male gametes) are formed before pollination. No generative cell, tube cell and stalk (sterile) cell as described in Pinus were found. A tube-junction (tj) was found at all stages including those where the tube cell should be. — *Fig. 29:* shows a possibility (mentioned in text) that the tube-junction (tj in *fig. 28*) is actually a vegetative cell formed *before* the two prothallial cells.

Abbreviations: E = embryonal cell; G = generative cell; mg = male gametes; pr = prothallial cell; sp = spermatogenous cell; t = tube cell; tj = tube junction; v = vegetative cell.

rule for all the gymnosperms (not even for the more primitive ones) regarding either the orientation of the division figure during mitosis of the generative cell or the relative positions of sterile cell and spermatogenous cell within the gametophyte".

In *Larix* no evidence could be found neither as regards formation by mitotic division of stalk (sterile) and spermatogenous cells, nor of any of the transitional stages, which such divisions must involve.

The impression is that the main structure of the embryonal/spermatogenous cell exists already at the tetrad stage *(fig. 4)*, and that this structure includes a ring-shaped, funnel-formed organ *(figs. 15, 16)*, through which the main vertical tube *(fig. 17)* leads, connecting pore with top. It probably also serves as support for the embryonal/spermatogenous cell. The said organ is connected with the external cytoplasm through at least two valve connections, clearly seen *figs. 15, 16* at right and left, and that is probably what is usually termed stalk cell or sterile cell. It has no resemblance to the two decaying prothallial cells seen below, and the possibility that it could have been produced by a recent mitotic division seems remote.

The Male Gametes

Two unequal male gametes were found in *Larix* by Strasburger (18a), and this seems to be in accordance with our observations *(figs. 15 and 26)*. It is not clear when exactly the spermatogenous cell divides, but it probably divides early (by the third division) soon after the formation of the prothallial cells. It seems, however, that the two gametes after the division remain very close together like those of *Ginkgo* ((16), text *fig. 1)* and *Pseudotsuga* (4b), *fig. 4*, until they separate at germination, before they leave the pollen.

Evidence as regards the anatomy of the male gametes is scarce, and it is mainly derived from decaying pollen; the gametes in sound pollen are so well protected and covered that only a piece of extraordinary luck may bring them to light. *Fig. 17* shows the internal structure of a mature decayed pollen on the apex of the nucellus. The structures correspond fairly well to the stage shown in *fig. 15*, which is a couple of weeks younger, and has retained most of its cytoplasm. *Fig. 15* is interesting on account of the various "caps" seen and of the spiral structures (see also *fig. 8)*; the latter have a certain resemblance to the cilial spirals seen in *Ginkgo* on Shimamuras text *fig. 1.* (cf. above). They may imply spermatozoid origin. *Fig. 18* shows the interior of a decaying spermatogenous cell, containing as it seems one more or less intact male gamete, and another damaged and pushed out of position. The intact gamete has the shape of an interphase nucleus including chromocenters and caps.

Germination of Pollen of Larix in Nature

Figs. 19—23 show "germinating" pollen on the apex of the nucellus. As in *Pseudotsuga* (4b) the male gametes seem to discharge themselves from the pollen into the apex of the nucellus through a pushed-out short bulge of intine *(figs. 19, 20)* and thereafter to continue on their own to the egg cells. As a rule they seem to leave the pollen enclosed in a membrane and, judging from the traces they leave in the apex, they dissolve the cells barring their way. *Fig. 25* shows a male gamete at an archegonium and trace of its passage through the apex of the nucellus. *Fig. 26* shows 2 male gametes in a common membrane seemingly trying to penetrate into different archegones. *Fig. 24* shows that naked gametes having left the membranes travelling through the apex, may occur. — Incidentally: the "end of pollen tube" shown in ((1), *fig. 37)* was no doubt actually an independent, but somewhat deformed, male gamete.

Germination of Pollen of Larix on Artificial Substrate

Ho and Rouse (10) have recently reported germination of pollen of *Larix sibirica* in vitro attaining a stage showing three cells with four nuclei. They found no production of a pollen tube, which is in accordance with our observations. However, for practical use, as a criterion for viability tests (cf. 4c), it would be very desirable if the authors could find their way to continue the experiments and find a method to grow *Larix* pollen on artificial substrate till discharge of the male gametes from the pollen. This stage should, if found, be relatively easy to determine, and it tells a lot more about the fertilization capacity of the pollen than earlier stages.

Discussion

On account of discrepancies observed through several years, between the development of the male gametophytes in *Larix* and *Pseudotsuga* and the existing reports as regards the said development in other conifers (plus a small number of own observations), it has been tried to elucidate, although on a small scale, the most important discrepancies and their causes. The main discrepancies concern the internal structure of the pollen, the formation of the pollen cells and their number and sequence, and the formation (respectively non-formation) of a pollen tube.

As touched upon above, the study of the development of the male gametophyte of the gymnosperms has always been a difficult field as witness the high number of reports and the disagreements as to their interpretation. The irregular occurrence of flowering years, adverse climatic influence, and a degree of variation higher than expected, are no doubt the main causes for this situation.

Although the present investigations of the male gametophyte of *Larix* and *Pseudotsuga* are not exhaustive and still leave several gaps unfilled, they nevertheless seem to show that Sterling's prediction (cf. p. 166) is well founded, and that important and interesting information is still to be found in studies on the gametophyte development of the gymnosperms.

As regards the male gametes of *Larix* and *Pseudotsuga* the latter, as described in (4b), produces some kind of spermatozoids which, enclosed in a membrane, propel themselves from the pollen to the archegones. — In *Larix* the general situation in this respect seems similar, but at the archegones only a thin membrane, if any, was found, and the shape of the two male gametes have often a certain resemblance to a terminal of a pollen tube *(fig. 26)*. This may of course be artefacts, but it might perhaps be, that *Larix* has reached a somewhat more advanced stage than *Pseudotsuga* in the direction of forming an ordinary pollen tube.

One of the main problems still requiring clarification is, which kind of motility the male gametes of *Larix* and *Pseudotsuga* exactly possess, and how they propel themselves from pollen to egg cell and further through the cytoplasm of the egg cell. Perhaps Molé-Bajer's excellent endosperm-culture technique combined with electronic flash photography and slow-motion film could supply the answers, particularly if and when discharge of the male gametes on artificial substrate becomes possible.

Another problem of considerable interest is presented by the "tube cell". If it turns out that a vegetative "tube cell" is formed *before* the production of the two prothallial cells, it would seem to alter the situation as regards the genera-

tive cell and tube cell in *Larix* (and probably also in *Pseudotsuga)* to something more like that existing in the angiosperms (cf. diagram *fig. 29).* — The solution of this problem ought to be possible when, somewhere, a normal flowering year occurs or, if and when, it becomes possible to grow *Larix* pollen at about the tetrad stage on artificial substrate.

As far as known the following information has not previously been reported in conifers: the internal tube and valve system of the pollen, the structural peculiarities in mature pollen, the interphase nucleus, the structure of the male gametes and their discharge at germination, and the occurrence of mitoses at the tetrad stage of pollen of diploid *Larix.*

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Summary

It is endeavoured to give a survey of the development of the male gametophyte and of the fertilization mechanisms in *Larix* (and *Pseudotsuga)* and, as far as possible, to compare the results with the corresponding processes in *Pinus.* As regards *Larix* and *Pseudotsuga* the survey is based on own investigations through several years and on existing records. As regards *Pinus* it is based on existing records only, but supplemental materials in respect of *Pinus, Abies* and *Picea* are in preparation.

The main differences between *Larix* (and *Pseudotsuga)* and *Pinus* seem to be:

Pollen grains of Larix and Pseudotsuga produce no ordinary pollen tubes, they germinate and fertilize in nature 6—8 weeks after pollination but cannot be germinated on artificial substrate. The fertilization mechanisms of the two species seem to represent transitional stages between fertilization by spermatozoids and fertilization by pollen tubes. Generative cell, tube cell and stalk cell, reported in other gymnosperms, could not be found in *Larix* and *Pseudotsuga,* but interphase nuclei with chromocenters and "caps", peculiar structures of pollen grains including cilial bands, suggesting, perhaps, a spermatozoid past, were observed. The possibility is mentioned that a "tube cell" (vegetative cell) may be formed *before* the production of the two prothallial cells.

Pollen grains of Pinus produce, according to existing records, ordinary pollen tubes; they germinate in nature shortly after pollination, but stop further development till next spring when fertilization takes place; pollen grains of *Pinus* germinate readily on artificial substrate. Generative cell, tube cell and stalk cell are recorded in *Pinus,* but interphase nuclei and internal structures as found in *Larix* and *Pseudotsuga* seem as yet not to have been observed.

It is suggested that *Larix* and *Pseudotsuga,* in respect of the development and structure of their male gametophytes and of their fertilization mechanisms, are closer to *Cycads* and *Ginkgo* than to pollen-tube producing conifers, as f. inst. *Pinus.* As regards the transitional spermatozoid/pollentube stage *Larix* may possibly be a trifle more advanced than *Pseudotsuga.*

Zusammenfassung

Im Gegensatz zu *Pinus* produzieren die Pollenkörner von *Larix* und *Pseudotsuga* keine üblichen Pollenschläuche. In der Natur keimen und befruchten sie 6—8 Wochen nach der Bestäubung. Auf künstlichem Substrat können sie aber nicht keimen. Die Befruchtungsmechanismen dieser beiden Gattungen können als Übergangsstadien zwischen einer Spermatozoiden- und einer Pollenschlauch-Befruchtung aufgefaßt werden. Sie stehen dabei *Cycas* und *Ginkgo* näher als *Pinus.*

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