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

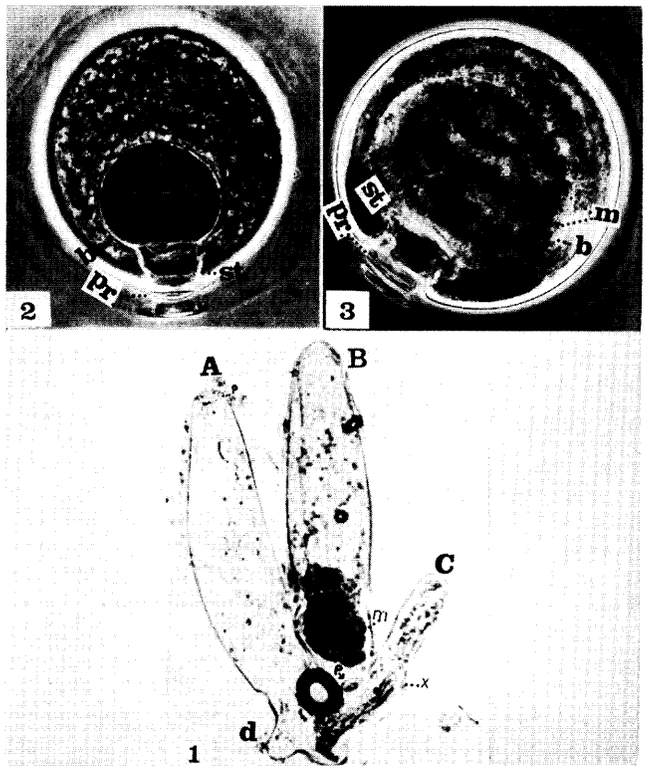

Fig. 1: A and B: elongated pollen grains of *Pseudotsuga menziesii*. C: short intine tube presumably made by male cells breaking through pollen grain wall at distal end of A (cf. text and [3] fig. 9, 9 a. A and C are empty; in A pore at top. In B pore near bottom of pollen grain touching black air bubble; one male cell (m) is near pore, one male cell and debris may have slipped out through wall left or right just above remaining male cell. C: debris inside lower half part of tube, other contents and male cells may have slipped out through break at x. d: bulge may be attempted but abandoned small escape tube. (Original photo by G. S. ALLEN, reproduced with consent of Canadian Forest Research Laboratory, cf. p. 106). — *Fig. 2:* Pollen grain of *Larix decidua* fixed May 9. 1968, about a week after pollination, shows 2 proth. cells, "stalk cell' and body cell, no tube cell visible. — *Fig. 3:* Pollen grain of *L. dec.* fixed May 25, 68, about 3 weeks after pollination, shows 2 proth. cells, "stalk cell" and a much enlarged and complicated body cell, perhaps just after division. Neither "stalk cell" nor body cell can, as yet, be interpreted with certainty, but the former shows no sign of emanating from a pollen cell division. Tentatively the body cell in *fig. 3* is estimated to contain two male cells, one (with the black "knob") above the "stalk cell" and, above it, a smaller one, each of them has a black, somewhat oblique, grove suggesting ciliabands like in the cycads. The male cells are seen more or less in side view. *However, although the interpretation is uncertain, it is quite clear that a pollen grain at the stage in fig. 3 is a much better criterion for fertilization capacity than the one in fig. 2 or earlier.*
Abbreviations: b = body cell; d = attempted but abandoned tube; m = male cell; p = pore; pr = prothallium cell; st = stalk cell; x, see text to *fig. 1.*

Magnifications: *Fig. 1.* ×100. *Figs. 2 and 3.* ×400.

overlooked. This danger is of course particularly imminent if the investigator is looking for pollen tubes. In fact the possibility cannot be excluded that some of the previous germination attempts with *Larix* and *Pseudotsuga* pollen, which were discarded as abortive, were actually successful, only that the motile cells escaped notice. This possibility should of course be checked; if it is confirmed it may save much research work.

Viability Tests

The importance of pollen germination on artificial substrate being mainly due to its use for estimation of the fertilizing capacity of the pollen tested, a short outline of the viability tests generally applied to pollen of coniferous forest trees is necessary: —

Viability Tests of Tube-producing Pollen

Among the coniferous forest tree species the male cells of the pollen of f. inst. *Pinus, Picea* and *Abies* are, as far as known, carried from the pollen grain to the egg cell by means of a regular pollen tube. When such pollen grains are subjected to a "viability" test, it is usually done by forcing them on artificial substrates, and rating such pollen grains as viable, which have produced a pollen tube of a length of about 3—5 pollen diameters and of sound appearance. Obviously the object of a test like this is to find a basis for estimation of the capacity of the pollen for *fertilization.* Actually however the test involves only the first stage of the development of the tube, and in spite of even a sound appearance of the pollen tube nobody can tell if it is not already at the very last stage of exhaustion. Furthermore unsuitable substrates may cause production of false tubes and other irregularities which are not always easy to distinguish from genuine tubes. The reliability of the results is therefore frequently not high, and probably the less so the lower the quality of the pollen is.

Viability Tests of Pollen of Larix and Pseudotsuga

In *Larix* and *Pseudotsuga* no regular pollen tube is produced, and in the latter species motile male cells have been observed (3). The male cells of *Pseudotsuga* leave the pollen grain through a short tube and propel themselves through the tissues of the nucellus top to the egg cell. The investigation of *Larix* is as yet not quite finished but its fertilization mechanism is as mentioned above very much like the one of *Pseudotsuga.*

In these two species the applied criteria for viability are usually the throwing off of exine and elongation of the pollen grain. Similar to the test in the pollen tube producing species, this covers only the first stage in the series of events in the pollen grain leading to fertilization. What is worse is that long dead pollen grains (if not injured, f. inst. by overdrying or too high humidity) may keep their swelling capacity, throw off their exine and elongate and, thus, be rated as viable, cf. ([9], p. 630, [2], p. 93—94).

Improvement of Viability Tests. New Criteria for Viability

That viability tests like the above mentioned, in spite of their obvious shortcomings, nevertheless are widely used is due to the fact, that the only dependable fertility test available, viz: the *seed setting* after controlled pollination with the pollen under test, and comparison with the seed setting after pollination with pollen of known capacity of fertilization, is, for several reasons, only practicable in special cases.

The only satisfactory solution of this problem seems to be to try to improve the existing viability test methods, as for example: —

(a) to work out for each species the best storage method and treatment of the pollen before (and after) the test, the best temperature and humidity degrees to be used during the test and, on this basis, to compose a substrate, if necessary separately for each species, which secures optimal, regular growth of the pollens *under sterile conditions* (maintaining, of course, strictly the stipulated regime). If a standard scheme of "germination" could be found, it would of course be still better, but this seems unlikely. —
(b) to try to find a criterion for viability nearer fertili-

zation stage than the present, f. inst., as regards pollen tube producing pollens, the stage when the pollen cells wander into the tube, or when divisions show that the pollen grain still has sufficient energy. Little is however known about when and how such events take place, and how the pollen cells behave on substrates. Thus from COULTER and CHAMBERLAIN (4) it appears that in nature no pollen cells of *Pinus laricio* move into the tube during the year of pollination, while DENGLER and SCAMONI (5), in pollen of *Pinus montana* after 3 days of growth in distilled water, observed germinated pollen grains with tubes containing immigrated pollen cells. The behaviour of the pollen cells and tubes of coniferous forest trees by germination on artificial substrates needs therefore investigation. The situation in *Pseudotsuga* (and probably in *Larix),* is more clear. Here we know that at germination the motile male cells are released from the pollen grain and for practical reasons not very suitable as criterion for viability testing (cf. below). However, the earlier stage, when the body cell moves away from the pore toward the distant pole (cf. [2], fig. 32), ought to be sufficient criterion for viability. This also applies to *Larix,* but on account of the smaller elongation of the pollen grain, the huge size of the body cell rather than its distance from the pore is more likely to be the better criterion, or perhaps both these characteristics may be used together (see *fig .3).*

Summing up: For the improvement of the reliability of the viability tests it is necessary: (1) to make sure that the substrates and regimes applied secure optimal and normal growth of tubes of faultless pollen under test and (2) that criteria for viability are found that better than the tubes alone reflect the future fertilizing capacity of the pollen. As regards *tube producing species* it is suggested above that the immigration of the pollen cells into the tubes (or possibly mitoses in the pollen cells) is used. As regards *Larix and Pseudotsuga* the movement of the bodycell, perhaps together with its size, toward the middle of the elongated pollen grain is suggested as criterion. If a suitable substrate can be found perhaps also the small tube emerging at germination may be used.

It is true that the time needed for germination may be longer, and that a microscope will be needed for inspection of the cultures, but it will hardly be more difficult or time consuming than the chromosome control of polyploid plants on agricultural experimental stations, if phase contrast is used.

I had through several years the privilege to be in close contact and compare notes with the late Dr. G. S. ALLEN of the *Canadian Department of Forestry, Forest Research Laboratory, Victoria, B. C.* on matters concerning the fertilization mechanism of *Pseudotsuga,* and, particularly, on the germination on artificial substrate of its pollen grain. ALLEN was, as well known, a pioneer in this field, and it is of interest to know, that he was rather optimistic as regards the possibility of finding a suitable substrate for *germination of Pseudotsuga pollen.* A couple of months before his much regretted death, in September last, he informed me that he had succeeded in producing in vitro the stage when the body cell moves away from the pore, i. e. the stage which may perhaps be used as criterion for viability, and also in the production of the small tube from elongated pollen grains cf. present *fig. 1* corresponding to ([3], fig. 9—9 a). He used as a medium a Sugar-Vitamin-Boron-Calcium mixture, but his time ran out before he could send further details.

Although some experimenting may still be necessary, I think that with the present knowledge of the fertilization mechanisms of *Larix* and *Pseudotsuga* and the particulars given in the present paper, the solution of this pollen germination problem should be well within reach.

While we must leave the composition of substrates and regimes for pollen germination to specialists in pollen biology and chemistry, our experience as regards methods for study of the fertilization mechanism of *Larix* and *Pseudotsuga* and culture of in vivo material may be of interest.

On the study of the fertilization mechanism of Larix and Pseudotsuga: Examination by means of a phase contrast microscope of developing pollen grains, dissected out from the collapsed stigmatic flaps and either fixed in CARNOY'S fluid or transferred to a hanging drop culture for further observation, is one of the most important means for study of the pollen grain and its contents. If an ordinary microscope is used few details are seen on account of the thick membranes, and the necessary heavy staining additionally obscure the contents. Preparations may be made in acetic orcein or carmine mixed with about 10% glycerine, and dilluted with 45% acetic acid till the transparency is satisfactory; *the cover glass must not be pressed,* and if older pollen grains are processed, the cover glass should be supported by small splinters of cover glass, otherwise the cells will be flattened and thrown out of position. Such preparations, sealed with some suitable medium, will as a rule keep in a freezer for a few weeks.

The use of disinfectants in artificial cultures before knowing, how they may influence the motile cells, should be avoided; the latter may be killed or injured. It is therefore advisable to make the forcing on artificial substrate as short as possible, and it is recommended to dissect pollens for study out of the upper part of the micropylar canals, when they are about the desired stage of development and transfer them, under as sterile conditions as possible, to a hanging drop culture (tentatively: 1 part boiled water + 3 part sap from crushed ovules, at about 23° C., or possibly 10% cane sugar solution as used for *Ginkgo* spermatozoids ([10], p. 418) for further development and study.

For experiments with germination substrates for use in practice, living pollen at the desired stages of development may be dissected out and transferred to the substrate under test, as far as possible under sterile conditions, for observation of the further development (or non-development).

Developing pollen in a hanging drop culture may be used to test the influence of disinfectants against invading microorganisms.

Furthermore the development of living pollen in a hanging drop culture may be used as a control of the development of pollens under test on other substrates, if the pollens are started at about the same time and stage.

The best time to see "germination" is of course when in nature the first pollen grains of a given tree are transferred from the collapsed stigmatic flap to the tip of the nucellus. If fully developed pollen grains are dissected out of the cones of such a tree and transferred to a hanging drop, the emergence of the male cells may take place any time, wherefore inspection must take place at least hourly all round the clock. It is possible, and often more advantageous, to take twigs with female inflorescences, and pollen in the micropyles at too early a stage in for forcing in a jar with water, and place jar and twigs in a plastic bag. The inflorescences, and the pollens on the collapsed stigmatic flaps, will develop more quickly than in nature, and the

processing is easier. It will save tedious dissecting work if a suitable number of female inflorescences are bagged (after removal of the male inflorescences from the twigs to be bagged) and pollinated with pollen from other trees with intervals of one day during the receptive period. Thus self pollination is avoided, and the number of pollen grains per stigmatic flap will be much higher than after wind pollination. The time table of development of the pollen grains in the top of the micropylar canal may vary from tree to tree and probably also on account of climatic conditions. The stages of development of the pollen grains in the top of the micropylar canal of *Pseudotsuga menziesii* during the period May 13 — June 23 are shown in (2), figs. 30—33. In *Larix* the development is approximately the same, only a few days earlier, and the elongation of the pollen grains is much smaller. The stage which may perhaps be used as criterion for viability is, as mentioned above for *Pseudotsuga:* somewhere between the above mentioned figs. 31 and 32; for *Larix* about the stage shown in the present *fig. 3*, cf. also *fig. 2*.

Desirability of Review of Literatur and More Research

Visser (11) in his work "Germination and storage of pollen" mentions that no up to date and comprehensive review and study on the physiology of pollen germination and storage was known to him (1955). His ensuing work fills many gaps as regards fruit-trees and garden plants, but it would be very desirable if a similar work as regards germination and storage of pollen of coniferous and other forest trees, together with the necessary research, could be made.

Although pollen germination is a highly difficult field, and much insight is needed both into pollen physiology, chemistry etc. to compose a substrate and determine the regime, which secures optimal growth and development of the pollen tubes of a given species, much could no doubt be attained if the matter be given due attention.

Acknowledgements

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Summary

It is assumed that the reason why pollens of *Larix* and *Pseudotsuga* cannot be germinated on usual artificial substrates is the peculiar fertilization mechanisms of the two species. Ordinary pollen tubes are not produced, and the male cells propel themselves from near the tip of the nucellus to the egg cell; they resemble in several respects the spermatozoids of *Ginkgo*.

The efficiency of the usual viability tests of pollens of coniferous forest trees is discussed; improvements and new and more reliable criteria for viability are suggested.

Methods for further study of the development of the pollen grains and the male cells of *Pseudotsuga* and *Larix* are described.

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