Endogenous Carbohydrates, Organic Acids, and Pine Pollen Viability[1])

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An external source of carbohydrates is usually required for in-vitro germination of pollen (VISSER, 1955). Some pollens, such as that of pine, do not require glucose or sucrose to facilitate germination when freshly harvested. However, no attempt has been made to relate the cellular levels of these chemical components to the inherent germination capacity of pollen. This paper presents results of an analysis of viable and non-viable stored pollens to determine if internal carbohydrates and organic acids are related to in-vitro germination capacity.

When pollens which do not initially require external carbohydrates for in-vitro germination are stored under varying periods and conditions, they frequently exhibit a marked response to added sugars in the germinating media (DOROSHENKO, 1928). In pollen of *Pinus sylvestris* from different provenances, rate of tube formation was related to pollen starch content measured by iodine staining (MARCET, 1951). Starch content was related to germination ability of Nymphaa *alba* and *Pinus laricio* pollen (KÜHLWEIN, 1938). VISSER (1955), related the physiological activities in pollen to the method of collecting and handling. A relation between pollen handling procedures and viability is well documented by plant breeders who have demonstrated that pollen stored at low temperatures and at specific controlled relative humidities generally maintain maximum viability (HOLMAN and BRUBAKER, 1926; DUFFIELD, 1954).

External substrates of sugars and acids have been shown by KÜHLWEIN (1939) and HELLMERS and MACHLIS (1956) to affect the formation of storage carbohydrates of germinating pine pollen. Studies of TANADA (1955) indicated that when pine pollen was germinated in glucose media more branching occurred than when sugar was absent. Seed studies also indicate that the concentrations of nutritive substrates in the seeds at the end of a period of storage can be related to the capacity of the seed to germinate (CROCKER and BARTON, 19'58). Other substrates, such as amino acids and carbohydrates, have been studied in pollen of pine and other plants (SEKINE and LI, 1942; HATANO, 1957: NILSSON, 1958). These constituents have usually been related to the nutritional quality or ecological distribution of the pollen (TODD and BRETHERICK, 1940; CALVINO, 1952).

Analytical procedures and the nature of the pollen used in this study, permit a comparison of cell chemical constituents to in-vitro germination ability of pine pollen stored under different relative humidities.

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Methods

Pollen samples. Pine pollen was collected and extracted at the Institute of Forest Genetics, Placerville, California, in 1945 by the method of CUMMING and RIGHTER (1948). Dr. N. T. MIROV started the experiment by storing samples in 20 ml. glass vials with cotton stoppers. He then placed the vials in a one-pint Mason jar. An open 50 ml. vial of sulfuric acid, with the density adjusted to maintain 10 to 25 percent relative humidity, was placed in the jar with the pollen vials. The jars were screwed tightly shut and placed in a refrigerator at 5° C.

Viability was tested on duplicate samples in 1960. Approximately 20 mg of pollen were sprinkled on each of two drops of 0.002 M calcium phosphate buffer, pH 5.9, on a microscope slide. The slides were placed in a moist Petri plate and incubated at 29° C. After 48 hours 100 grains were counted at random in each replicate drop and the mean of two replicates determined.

Carbohydrate analysis. Sugars soluble in ethanol were determined by extracting 200-mg samples of pollen for one hour with 70 ml. of 70 percent ethanol in a 250 ml. Soxhlet apparatus. The rate of refluxing was adjusted at 40 drops per minute. After 1 hour the alcohol was replaced with a fresh 40 ml. volume and refluxing continued for a second hour. At the end of two hours the extracted pollen was washed with 20 ml. of fresh 70 percent ethanol. The ethanol from extractions and washings was concentrated on a steam bath to 50 ml. Five ml. of the concentrated extract was heated to boiling with 5 ml., 1ON H_2SO_4 for 45 minutes. This treatment hydrolysed oligosaccharides to monosaccharides.

Starch and other polysaccharides in the residual pollen were also hydrolysed and assayed. Pollen residue remaining after ethanol extraction was air dried and boiled with 10 ml. of 1ON H_2SO_4 for two hours in a wide-mouth 100 ml. flask with "U" tube condensor. After cooling the solution was neutralized with N NaOH (SUMNER and SOMERS, 1949).

Glucose content of the concentrated ethanol extract, its hpdrolysate, and the hydrolysate of the pollen residue was determined by the SMOGYI-SHAFFER-HARTMAN test (SUMNER and SOMERS, 1949). TWO 5 ml. replicate aliquots of each solution were assayed. Values between replicates usually agreed within 3 percent. Two standard and two blank solutions were analyzed at the start and end of each series of assays. All solutions were prepared from reagent grade compounds.

Organic acid assay. The ion-exchange procedure detailed by RONSON (1955) was used to assay the non-volatile acids contained in the 70-percent ethanol extract. An

anion-exchange column was prepared from amberlite IRA-400, 20-50 mesh. Five gms. of dry resin were placed in 500 ml. N Na_2CO_3 and allowed to stand for three hours with occasional stirring, then packed in a 1.5 cm. diameter glass column. The resin was then backwashed three times with N HCl and N Na_2CO_3, and rinsed between each reagent with deionized water free of CO_2. This anion column was placed in an acid absorbing condition by washing through the carbonate cycle. The cation-exchange column was prepared from Dowex 50, 50-100 mesh. Five gms. of resin were soaked in 0.2 N formic acid for two hours, then packed in a 0.9 cm. diameter column, and washed with distilled water until free of acid. In actual use a constant blank was obtained, as titrated with standardized NaOH.

Five ml. aliquots of the pollen extract were placed on the anion-exchange column, and washed with water to remove the non-acidic substances. Acids were recovered by eluting with about 200 ml. N Na_2CO_3; the acids then regenerated by passing through the cation exchange resin column. Acid quantity in the extract was determined by titration with standardized NaOH to a phenopthalein end-point. Solutions of organic acids of known concentration were run through the procedure for comparison of recovery efficiency.

Results and Discussion

Pollen stored 15 years at 10 percent relative humidity had a higher content of ethanol-extractable free glucose and of low molecular-weight oligosaccharides than pollen stored at 25 percent relative humidity *(Table 1)*. The total hydrolysable carbohydrates in the pollen samples varied between approximately 28 percent and 69 percent of the fresh weight of the pollen. Bound carbohydrates and hydrolysable polysaccharides remaining in the ethanol-extracted pollen changed less during storage than the extractable carbohydrates. A large number of branched tubes were present in the more actively germinating pollen. These branched tubes were similar to those observed by TANADA (1955) when pine pollen was germinated on a glucose media.

Table 1. — Relation of carbohydrates to in-vitro germination of pine pollen stored 15 years at 5° C. and 2 relative humidities.

Pine species	Relative humidity	Germination	I[1] Free glucose + oligosacch	II[1] Hydrolysable polysacch.	$\frac{I}{I+II} \times 100$
	Percent		*mg./100 mg.*		
Ponderosa	10	58	25.65	17.10	60.0
	25	0	21.80	18.10	52.5
Echinata	10	34	16.47	15.20	52.0
	25	0	14.55	14.20	50.6
Lambertiana	10	30	31.15	37.10	45.6
	25	0	26.00	35.70	42.0
Radiata	10	6	24.05	34.60	40.8
	25	0	14.25	35.10	28.9

[1]) Note: I = 70 percent ethanol extract of pollen.
II = Hydrolysed pollen residue.

Breakdown of complex carbohydrates would probably occur slowly in stored pollen. However, it is also possible that the stability of the polysaccharides may indicate slight accumulation of an insoluble oligosaccharide during storage. Enzymes for β-1, 3-glucosan, which give rise to callose in pollen, are well known (KESSLER *et al.*, 1960) and could be expected to function at a low level of activity even during storage. Thus, products accumulating during storage and germination will depend upon the kinds of enzymes present and their equilibrium activities.

These results indicate that storage conditions which maintain pollen viability restrict the activity of enzymes which degrade simple carbohydrates in stored pollen. Unfortunately, these pollen samples were not analyzed at the time of storage for chemical constituents. Thus, the overall changes during storage cannot be evaluated. However, chemical analyses of pine pollen by other workers, have generally reported carbohydrate content in the range of 50 to 70 percent (NILSSON, 1956; KRESLING, 1891). If carbohydrates are metabolized, as *Table 1* suggests, then it might be of interest to determine if intermediate products such as organic acids accumulate or if the sugars are totally oxidized.

The organic acid content was consistently higher in viable pollen than in non-viable pollen *(Table 2)*. This does not preclude carbohydrate degradation by the usual oxidative metabolic pathways such as the Krebs Cycle. Rather, we can interpret these results as indicating that acid-degrading enzymes are more active in pollen stored under moist conditions favoring rapid loss of viability and inactivation of pollen than in pollen stored in a less moist condition. Alternatively, organic acid synthesis may be higher in viable pollen, or, the mere presence of higher amounts of monosaccharides, direct precursors of organic acids, might be expected to result in higher yields of organic acids. Humidity during extraction may play an important role in maintaining pollen viability (DUFFIELD, 1954; VISSER, 1955). Environmental humidity and temperature during storage, will even more drastically affect enzyme activities and substrate utilization.

Table 2. — Non-volatile organic acids in pine pollen stored 15 years at 5° C. and 2 relative humidities.

Species	Relative humidity	Germination	Mg. equiv. acid in 70% EtOH extract of 100 mg. pollen
	Percent		
P. ponderosa	10	58	33.89
	25	0	27.55
P. echinata	10	34	32.77
	25	0	28.61
P. lambertiana	10	30	31.90
	25	0	27.50
P. radiata	10	6	33.19
	25	0	28.87

The experimental data reported here suggests that viability of pollen during storage is related to levels of metabolic substrates, such as endogenous carbohydrates, available to the pollen when it is placed in an environment suitable for germination. Pollen is probably much like seeds with respect to the effect of tissue water content during storage on enzyme activities (STANLEY, 1958). Maintenance of a low water content in pine pollen during extraction and extended periods of storage would limit enzyme degradation of endogenous substrates essential to support initial growth of the pollen tube.

Knowledge of the mechanism by which pollen survives extended periods of storage may possibly aid in selection of the best pollen for breeding purposes. A simple boiling ethanol extraction and an assay of the soluble acids might provide a chemical index of viability. Although most pol-

lens germinate in-vitro in two to three hours, pine pollen germination tests require two or three days. A rapid chemical assay may be of value in selecting the most viable among samples of pine or other slow germinating pollen.

Summary

The carbohydrate and organic acid content of pine pollen were compared after 15 years storage at 5° C. Pollen stored at 10 percent relative humidity germinated; pollen stored at 25 percent relative humidity did not germinate. Low-molecular-weight sugars and organic acids were higher in viable pollen than in non-viable pollen. The results suggest that the mechanism by which pollen cells retain their viability is related to intracellular rates of respiration during the storage period. The possibility exists for determining germinative capacity of pine pollen samples indirectly by chemical assay.

Zusammenfassung

Titel der Arbeit: *Endogene Kohlehydrate, organische Säuren und die Lebensfähigkeit von Kiefernpollen.*

Der Gehalt an Kohlehydraten und organischen Säuren wird bei Kiefernpollen nach 15jähriger Aufbewahrung bei 5° C vergleichend untersucht. Pollen, der bei 10% relativer Feuchtigkeit aufbewahrt worden war, keimte noch; Pollen, der bei 25% relativer Feuchtigkeit lagerte, keimte nicht mehr. Einfache Zucker (mit niedrigem Molekulargewicht) und organische Säuren waren bei lebenden Pollen in höherem Maße vorhanden als bei nicht mehr lebenden Pollen. Die Ergebnisse deuten darauf hin, daß der Mechanismus, durch den die Pollenzellen ihre Lebensfähigkeit erhalten, mit der Höhe der intrazellulären Atmung während der Aufbewahrungsperiode in Beziehung steht. Es ergibt sich somit die Möglichkeit, die Keimfähigkeit von Kiefernpollen-Proben indirekt durch eine chemische Prüfung zu ermitteln.

Résumé

Titre de l'article: *Hydrates de carbone endogènes, acides organiques, et viabilité du pollen de pin.*

La teneur du pollen de pin en hydrates de carbone et en acide organique a été étudiée après 15 années de conservation à 5° C. Le pollen conservé à 10% d'humidité relative a germé; le pollen conservé à 25% d'humidité relative n'a pas germé. Les sucres à faible poids moléculaire et les acides organiques existaient en plus forte proportion dans le pollen viable que dans le pollen non viable. Ces résultats suggèrent que le mécanisme par lequel les cellules polliniques conservent leur viabilité est lié à la respiration intracellulaire au cours de la période de conservation. Il paraît possible de déterminer indirectement la faculté germinative du pollen de pin par analyse chimique.

References

Calvino, M.: Le sostanze di riserva dei pollini e il loro significato, filogenetico, ecologico embriologico. N. Gior. Bot. Ital. n. s. **51**: 1—25 (1952). — Crocker, W., and Barton, L. V.: Physiology of seeds. Chronica Botanica; Waltham, Mass., 267 pp. (1953). — Cumming, W. C., and Righter, F. I.: Methods used to control pollination of pines in the Sierra Nevada of California. U. S. Dept. of Agr. Cir. 792, 18 pp., illus. (1948). — Doroshenko, A. V.: Fiziologia Phltsy. Trudy po Prikladnoi Bot. **18**: 217—344 (1928). — Duffield, J. W.: Studies of extraction, storage and testing of pine pollen. Z. Forstgenetik **3**: 39—45 (1954). — Hatano, K.: Amino acids in the pollen and female flower of *Pinus thunbergii*. Bul. Tokyo. U. For. **48**: 149—151 (1955). — Hellmers, H., and Machlis, L.: Exogenous substrate utilization and fermentation by the pollen of *Pinus ponderosa*. Pl. Physiol. **31**: 284—289 (1956). — Holman, R. M., and Brubaker, F.: On the longevity of pollen. Univ. Calif. Pub. in Botany. **13** (10): 179—204 (1926). — Kessler, G., Feingold, D. S., and Hassid, W. Z.: Utilization of exogenous sugars for biosynthesis of carbohydrates in germinating pollen. Pl. Physiol. **35**: 505—509 (1960). — Kresling, K.: Beiträge zur Chemie des Blütenstaubes von *Pinus silvestris*. Arch. d. Pharm. **229**: 389—425 (1891). — Kühlwein, H. von: Pollenphysiologische Studien zum Stärkeproblem. Bot. Arch. **39**: 245—262 (1939). — Marcet, E.: Pollenuntersuchungen an Föhren (*Pinus silvestris* L.) verschiedener Provenienz. Mitt. Schweiz. Anst. Forstl. Versuchs. **27**: 348—405 (1951). — Nilsson, M.: Constituents of pollen I. Low molecular carbohydrates in pollen from *Pinus montana*. Acta. Chem. Scand. **10**: 413—415 (1956). — Ranson, S. L.: Non-volatile mono-, di-, and tricarboxylic acids. Modern methods of plant analysis II. Ed. Paech and Tracey. Berlin-Göttingen-Heidelberg, 1955, 539—582 pp. — Sekine, H., and Li, T.: Composition and vitamins of pine pollen. Rept. Jap. Assoc. Adv. Sci., Tokyo. **16**: 563—566 (1942). — Stanley, R. G.: Gross respiratory and water uptake patterns in germinating sugar pine seeds. Physiol. Pl. **11**: 503—515 (1958). — Stanley, R. G., Petersen, J., and Mirov, N. T.: Viability of pine pollen stored 15 years. Pac. Southwest Forest and Range Expt. Sta. Res. Note 173. 5 pp. (1960). — Sumner, J. B., and Somers, G. F.: Laboratory experiments in biological chemistry. Academic Press, N. Y., 1949, 169 pp. — Tanada, K.: The pollen germination and pollen tube development in *Pinus densiflora* Sieb. et Succ. I. The effects of storage, temperature and sugars. Tôhoku U. Sci. Rpt. Ser. 4. **21**: 185—198 (1955). — Todd, F. E., and Bretherick, O.: The composition of pollens. J. Econ. Entomology **35**: 312—317 (1940). — Vissser, T.: Germination and storage of pollen. Med. Landbouw. **55** (1): 1—69 (1955).

Ergibt die Kreuzung Populus tremula x Populus alba (und reziprok) luxurierende Bastarde?

(Ein Beitrag zum Heterosisproblem bei Waldbäumen)

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1. Einleitung

W. v. Wettstein fand bei Züchtungsexperimenten, daß Kreuzungen zwischen der Silberpappel *(Populus alba)* und der Aspe *(Populus tremula)* luxurierende Bastarde ergeben (17, 21, 22, 23, 24). Diese Befunde wurden von ihm selbst und anderen Autoren wiederholt zitiert. Bastardwüchsigkeit, W. v. Wettstein spricht von „Heterosis", sollte demnach bei erwähnten Hybriden eine Erscheinung sein, mit der der Züchter regelmäßig rechnen darf.

Bereits früher habe ich berichtet, daß die Jugendwuchsleistung von Silberpappel/Aspen-Bastarden in unseren Versuchen nicht den Erwartungen entspricht (14); das Material war damals jedoch noch nicht umfangreich genug, um Sicheres aussagen zu können. Unterdessen liegen Aufnahmeergebnisse von insgesamt 13 Versuchsflächen vor, die eine eindeutigere Stellungnahme zur Frage der „Heterosis" bei erwähnten Bastarden gestatten.