POLLEN VIABILITY TESTING STORAGE AND RELATED PHYSIOLOGY

REVIEW OF THE LITERATURE WITH EMPHASIS ON GYMNOSPERM POLLEN

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Abstract

This review of literature is concerned with the physiology and techniques involved in viability testing and storage of pollen, especially conifer pollen. Techniques involving pollen germination and staining are considered. Methods of pollen storage outlined include refrigeration, controlled humidity, freeze drying and vacuum storage. Related physiological topics discussed involve the identification and quantitative determination of carbohydrates, proteins, amino acids, enzymes and isozymes, as well as the measurement of respiration rates of pollen. Suggestions involving the use of physiological parameters to determine conifer pollen viability have been made by the authors. The authors have also considered improvements in current methods of conifer pollen storage which might be investigated.

Résumé

Les auteurs, dans une revue de la littérature sur le sujet, traitent de la physiologie et des techniques concernant les tests de viabilité et l'entreposage du pollen, spécialement le pollen des résineux. Ils traitent des techniques de germination et de teinture du pollen. Sur l'entreposage du pollen ils écrivent sur la réfrigération, l'humidité contrôlée, le séchage à froid et l'entreposage à vide. Ils discutent des sujets physiologiques incluant l'identification et la détermination quantitative des hydrates de carbone, des protéines, des acides aminés, enzymes, isozymes et le mesurage des taux de respiration du pollen. Ils font des suggestions relatives à l'usage de paramètres physiologiques pour déterminer la viabilité du pollen de résineux. Enfin ils suggèrent que l'on essaye de trouver des méthodes améliorées d'entreposage du pollen de résineux.

INTRODUCTION

With considerable attention now being focused on forest genetics and tree breeding, there is a need to maintain reserve supplies of viable pollen.

Douglas-fir, Pseudotsuga menziesii (Mirb.) Franco, is the most extensively harvested forest tree in the Pacific Northwest (Owens, 1973), and continuous reforestation has become necessary. Unfortunately, a good cone crop generally occurs about every 5 years, but this may vary between 2 and 7 years, depending on elevation of the tree (Owens 1973). Thus, in addition to eliminating the time barrier in breeding programs, successful storage of viable pollen can save time, effort and money expended each year in procuring supplies of pollen to produce seed. Successful viable pollen storage is also indispensable to studies on pollen physiology.

A rapid, reliable indication of pollen viability is essential, since the success of any storage program depends on the breeder's ability to distinguish between living and dead pollen.

The present literature review brings together the major studies on viability and pollen storage, as well as relevant pollen physiology.

PART I

TESTS FOR POLLEN VIABILITY

a) GENERAL

As a rule, studies on pollen viability and storage are inseparable. Historically, the papers of Pfundt (1910), Knowlton (1922), and Holman and Brubaker (1926) were the authoritative references on pollen viability. The work of these men encompassed a total of 231 species, representing 175 genera and 23 families. However, germinability on artificial media was their only measure of viability.

In Visser's review of 1955, pollen viability was established by fruit set and by germination tests. Since that time it has been shown that these tests do not necessarily give the same results (Johri and Vasil, 1961). In 1964, Jensen, working with 10 different genera, measured pollen viability in two ways: germinability defined as "the ability to produce pollen tubes under optimal growing conditions in vitro" and fertility defined as "the ability to produce normal seed set after controlled pollinations". He stated, "Germination is to be regarded as a measure of the state of vitality represented here by quantitative measurements of the rate in decrease of vitality on storage. Fertility, on the other hand, is regarded as a measure of survival of normal function of sperm nuclei." He further proved that pollen, which produced a positive fertility test, invariably showed a positive germinability test, but the reverse did not always occur.

Knowlton (1922), working with Antirrhinum sp. pollen, showed that pollen stored in the presence of stigmatic tissue was able to germinate after 670 days, whereas, without stigmatic tissue, the same pollen did not germinate after 474 days. Although the 670-day-old pollen was incapable of fertilization, some suggestion was evident that fertilizing ability might outlast germinability under artificial conditions. Streptocarpus sp. pollen, incapable of germinating in sugar solutions, was capable of fertilizing ovules (Roemer, 1915; Fetisov and Kryukova, 1961). Dempsey (1962), by means of fluorescence, observed that tomato pollen tubes were capable of growing in the stylet regardless of age and the seed or fruit-setting capacity. Hence, in vitro studies do not seem to be a reliable indicator of in vivo fertilization by pollen.

Recognition and concern for the problem of correlating germinability and fertility was also expressed by other authors, such as Orr-Ewing (1954), Stanley (1962), and was best summarized by Duffield (1954). Duffield stated, "The relationships between the pollen grain and the intended material tissue are so little known (especially in the gymnosperms) that artificial germination tests cannot be relied upon to give positive information as to the fertilizing potential of a sample of pollen. However, it seems reasonable to assume provisionally that pollen which cannot be made to germinate by means or which, on germination, shows unusually poor tube growth is likely to be ineffective in causing fertilization. Herein lies the present practical value of pollen testing as a routine procedure in breeding operations."

b) GERMINATION

Germinability *in vitro* has been used to indicate viability (Johnson, 1943; Santamour and Nienstaedt, 1956; Schoenike and Stewart, 1963). Pollen grain germination has been defined differently by many researchers; thus, a comparison of results is difficult. For example, some researchers have stated that a pollen grain was germinated when the length of the pollen tube equalled or exceeded the diameter of the pollen grain (Echols and Mergen, 1956; Dillon and Zobel, 1956; Brown, 1958; Ching, 1964). In other work, it is not clear whether burst pollen grains are included in the germination count (Oberle and Watson, 1953; Norton, 1966). In some of Pfeiffer's earlier work (1936) "apparently functional tubes" were considered germinated, while in later work (1955) the pollen tube length had to be three times the diameter of the grain. Perhaps these differences in definition were necessary because of pollen size differences in the genera studied. Christiansen (1969) criticized germination counts based on pollen tube measurements. He argued that this was only the first stage of development and did not indicate the capacity for fertilization. He therefore suggested that the immigration of the pollen cells into the tubes or mitosis in the pollen cells may be a more realistic and precise definition of pollen viability.

On the other hand, Kuhlwein and Anhaeusser (1952) found that for gymnosperms, pollen tube growth decreased with increasing age. This supported Duffield's (1954) and Dempsey's (1962) suggestion that the rate of pollen tube growth may be employed as a measure of fertilizing potential.

In vitro germination tests have been the most popular method of testing pollen viability (Holman and Brubaker, 1926; Bequerel, 1929; Pfeiffer, 1936, 1938, 1955; Duffield and Snow, 1941; Johnson, 1943; Bredemann *et al*, 1948; Santamour and Nienstaedt, 1956; Barber and Stewart, 1957; Fechner, 1957; Dobrinov, 1959; Worseley, 1959; Egorova, 1969). Often fertilizing potential (tested by controlled pollinations) accompanied the germination tests (Newcomer, 1939; Orr-Ewing, 1954; Ehrenberg, 1960; Stanley, 1962; Jensen, 1964; Livingston and Ching, 1967). In addition, Livingston, Ching and Ching (1962) used a combination of pollen germination percentage, seed set ability, seed viability and seedling growth as a measure of pollen viability. Techniques for germination tests have included: the vapor method (von Walderdorff, 1924), the hanging drop method (Righter, 1939) and the agar gel method (Duffield and Snow, 1941). Modifications of these and other methods appear in the literature (Duffield and Snow, 1954; Echols and Mergen, 1956; Dillon and Zobel, 1957).

The past literature also disagreed in several aspects with respect to the medium required for germination of the same pollen. For example, optimal germination with pollen of *Corylus avellana* has been obtained in water and 15-20%, 25% and 35-50% sugar solutions (Visser, 1955). The conditions required for maximum germination *in vitro* have been subjected to considerable research (Brink, 1924; Kühlwein and Anhaeusser, 1938; Johnson, 1943; Bishop, 1949; Faull, 1955; Visser, 1955; Echols and Mergen, 1955; Dillon and Zobel, 1957).

Generally, the pollen grain requires water for the formation of a pollen tube (Visser, 1955). Visser (1955) further mentioned that "pollen

germination depends on the rate at which water is released from the medium and taken up by the pollen." He calls this phenomenon the "diffusion rate of water." Apart from water, a search of literature indicates that excellent germination was obtained in sugar media with or without a lower percentage of agar or gelatin. Visser (1955), however, agreed that "the presence of sugars is essential in order to create a certain osmotic value only." He continued: "The growth of the tube of many different pollens whether cultivated in vivo or in vitro is independent of the presence of nutrients in the medium in the form of sugars."

Generally, the supporters of endogenous nutrition of pollen tubes believed that even in vivo, the pollen tubes do not get any nourishment from the style. On the other hand, O"Kelley (1955) reported that externally supplied sugars do serve as a source of nutrition for the germination of pollen. Perhaps both sides were correct to some extent and, in the presence of a rich external source of nutrients, the pollen grain used these rather than its natural reserves for germination.

Iwakawa and Watanabe (1965) studied the effects of different constitutions of artificial media, different temperatures, different light conditions and pre-treatment of pollen on germination of pine pollen, "with a view to arranging a practical method of testing pine pollen germination." They concluded:

> "1) The media containing 0.5 to 1.0% agar and 0.1 to 0.3 M sucrose, being adjusted to pH 4.5 to 6.5 seemed to be the best media tested. Vapor or double-distilled water without nutrients was also an excellent medium for pine pollen germination, although it tended to give a slightly lower germination percentage. Tap water gave no germination. Citric acid decidedly gave better results than hydrochloric acid in adjusting pH value of media.
>
> 2) Pollen germination percentage was best at temperatures of 28 to 37°C, while considerably lower at 41°C and none at 47°C. At a temperature of 23°C or at room temperature the pollen germination was delayed, though the final germination was not affected.
>
> 3) Results from studies on light and dark incubation of pollen showed no significant difference in germination percentage under moderate light conditions, but tended to indicate a depression of germination percentage by strong light.
>
> 4) Humidifying of pollen at a temperature of 5°C in 90% relative humidity for 24 hours before placing it on a wet medium gave beneficial effects on the germination of pollen stored at low humidity" (See also Dengler and Scamoni, 1939).

Previously, Chira (1964) has determined the best temperatures for germinating 21 species and varieties of Pinus pollen and had concluded that

in the majority of cases, fresh pollen germinated best at 30°C, whereas pollen stored for 1 year germinated best at 20°C. Razmologov (1964) gave the optimal medium, temperature and date for germinating several species of gymnosperm pollen. He concluded that there appeared to be a similarity between "behaviour" of stored pollen and pollen under "normal conditions" (see also Kaurov and Vakula, 1964).

Sucrose of between 10 and 30% was commonly used as a medium for germinating conifer pollen (Johnson, 1943; Fechner, 1957; Bogiatto and Andrada, 1970). Istratova (1961) reported the best medium for Picea sitchensis was 12-15% sucrose, for Pinus strobus 15% sucrose, and for Taxodium distichum 8% sucrose plus 0.05% H_3BO_3. Bishop (1949), working with Tradescantia paludosa, germinated this pollen in a lactose medium as successfully as in a sucrose medium. Dillon and Zobel (1956) showed an increased germination of Pinus elliotti pollen with decreasing sucrose concentrations; in fact, the highest germination occurred in distilled water.

According to Christiansen (1969), Pseudotsuga menziesii (Mirb.) Franco var. viridis Schiver, Laux decidua Mill. and L. leptolepis (Sieb. and Zucc.) Gord. do not produce "ordinary" pollen tubes and cannot be germinated on usual artificial media. However, in 1972, Ho and Sziklai reported successful germination of Pseudotsuga menziesii pollen in a solution of boric acid, calcium nitrate, magnesium sulfate and potassium nitrate.

Boron may be important in pollen germination. In Nymphea pollen, there was very little germination in 1% glucose. However, when secretions of the stigma were added, this produced very good germination. The stigma contained approximately 10 ppm B_2O_3 (Schmucker, 1932). Fourteen of 40 species tested showed considerable increases in both germination and pollen tube growth in the presence of boron (Schmucker, 1935). Since those tests were made, hundreds of pollens belonging to the angiosperms have been found to germinate better with the addition of boric acid or other boron compounds. The optimal concentration of boron for germination in most plants was about 10 ppm and, in some cases, 50 to 100 ppm were necessary (Visser, 1955). Few species required less than 10 ppm, but none could tolerate concentrations up to 1,200 ppm (0.12%) of boron (Johri and Vasil, 1961). The effect of boric acid is not due to its acidity because borax works as well (Visser, 1955).

c) STAINING TECHNIQUES AS A METHOD OF TESTING VIABILITY

To a botanical technician, there is little difference in the terms "stain", "dye" or "bleach;" thus, the term "stain" will be used here to infer all three types of reaction. The great advantage of staining in determining pollen viability is simplicity and time. A number of tests have been developed specifically for the purpose of testing pollen viability. Some researchers have used both germination and staining techniques as viability tests (Worseley, 1959; Cook and Stanley, 1960; Allen and Sziklai, 1962). While comparisons of these two methods have indicated more reliable results with staining techniques (Maurin, 1956; Vazhnitskaya, 1961), Cook and Stanley (1960) and Giordano and Bonechi (1956) found that for pine, in many cases it was the germination method of indicating viability, not the staining method, which produced more reliable and consistent results. On the other hand, using pollen of rutabagas and turnips (Brassica spp.), Vazhnitskaya (1961)

found staining with acetocarmine gave a higher index of viability than did the germination tests.

Ostapenko (1956) tested the viability of apple, pear, apricot, peach, plum, damson and almond pollen after 10-15 days and 12 months' storage by a) germination on 10% sugar solution; b) staining with acetocarmine, and c) peroxidase determination. Germination on sugar solution gave a reasonable indication of viability, while the other two techniques were unsuitable for old pollen.

Maurin (1956), using pollen of Larix Kurilenses, Pinus sylvestris, Pseudotsuga menziesii and several genera of angiosperm tree pollen, had more reliable results with the simple peroxidase reaction than with the more complex germination in vitro using a sucrose medium (Maurin, 1962).

King (1960) was critical of both staining tests and germination tests. In his words:

> "The erratic responses of pollens by germination to an artificial environment necessarily make the germination test unreliable; while the technique now used for staining pollen grains (such as with acetocarmine or iodine-potassium iodide) gave results which cannot be associated with viability. They may, at best, indicate only degrees of pollen maturity."

King (1960) tested pollen viability based on the oxidation of benzidine by peroxidase in the presence of hydrogen peroxide. The reaction was "accompanied by emission of oxygen gas, which is freed from hydrogen peroxide by catalase." The viable grains enlarged, became colorless and ovoid; non-viable grains stained blue and did not enlarge, and aborted grains were distorted with a decreased internal content.

In concluding, King stated: "The test reaction has been specific for each kind of pollen, although pollens of closely related species seem to give a similar test reaction (as do the pollens of Solanum tuberosum and Lycopersicon esculentum)." Contrary to the results of Ostapenko (1956), the catalase reaction of King (1960) gave consistent viability comparisons among dated pollens.

Other staining tests are available. A technique using sodium-biselenite indicated degrees of viability of stone and pome fruit pollen (Jacopini, 1954). Although no species were mentioned, a technique for indicating pollen grain viability using methyl green and phloxine in glycerol jelly was described by Owczarazk (1952). This method has been used for gymnosperm pollen (Worseley, 1959). When Heslop-Harrison and Heslop-Harrison (1970) immersed pollen from Impatiens neltanii, Cucurbita pepo, Phleum pratense, Tagetes patula and Naegelia zebrina in a solution of fluorescein diacetate and sucrose, viable pollen grains rapidly accumulated free fluorescein (see also Kozubov, 1967).

Tetrazolium salts have been widely used as a test for pollen viability (Vietzev, 1952; Oberle and Watson, 1952; Cook and Stanley, 1960; Diakonu, 1962; Chira, 1963; Hauser and Morrison, 1964; Sarcella, 1964; Norton, 1966; Novak and Betlach, 1967). According to Smith (1951), the

2, 3, 5-triphenyltetrazolium chloride (TTC) test involved reduction of the colorless dye by dehydrogenase enzymes to an insoluble red formazan complex in living cells. Cook and Stanley (1960) compared the TTC test with the germination in agar of Pinus sp. pollen. They treated pollen samples with TTC at 55°C for 90 minutes, then examined the pollen at X100 magnification, using Munsell's book of color for stain intensity. Statistical analysis indicated fair agreement between germination and stain intensity when large samples were used, although this staining technique may have slightly underestimated germinability. The results were not correlated with fertility. Using a modification of Diakonu's (1962) triphenyltetrazol method, Chira (1963) distinguished between "good, dying and dead" pollen of some species of Pinus through the use of color using triphenyltetrazols of chloride and suggested that this method be used to test pollen stored for long periods.

Hauser and Morrison (1964) compared a Nitro Blue Tetrazolium staining (Nitro-BT) with aniline-blue-lactophenol stained samples using thirty species of angiosperms. Nitro-BT discriminated between pollen grains that had a capacity for oxidative metabolism and those that did not. Tetrazolium red gave more consistent results than solium biselenite with the pollen of Pinus species.

d) OTHER VIABILITY TESTS

Stanley and Poostchi (1962) analysed viable and non-viable stored pollen, relating carbohydrates and organic acid content to in vitro germination capacity. Low molecular weight sugars and organic acids were higher in viable than in non-viable grains, and thus retention of viability may be related to intracellular respiration during the storage period. Hence, an indication of pollen germinative capacity of Pinus sp. might be accomplished by a chemical assay. From the amino acid content of apple pollen, Tupy (1932) concluded that the proline-histidine content was an important indication of pollen fertility.

Two observations that may be related to viability include different manners of floating between pollen stored in favorable and unfavorable conditions, and the more rapid uptake of water by pollen in unfavorable storage (Duffield, 1954). Jovancevic (1962) suggested estimating germinability of tree pollen by microscope observation after soaking in a 12% Kl solution. Pollen grains of "abnormal size, form or color are likely to be sterile."

PART II

POLLEN STORAGE

a) INTRODUCTION

The need to store viable pollen has been recognized for some considerable time. Visser (1955) cited a paper by Zirkle (1935) which revealed that transporting pollen of the date palm took place as early as 2400-2000 B.C. Systematic investigations on pollen storage began in the last century.

Pollen storage is necessary in most plant breeding programs as it is often impossible to make the controlled crosses at the time of natural pollen maturation and dehiscence; for example, the selected parents may flower at different times or in widely separated regions. In gymnosperm tree-breeding, long-term storage of pollen is required because controlled pollinations may not be possible for 1 or more years following pollen collection. Also, pollen production may vary with individual trees and particular years.

Information about pollen storage is included in several literature reviews and general works on pollen physiology (Visser, 1955; Brewbaker, 1959; Walden, 1959; Johri and Vasil, 1961; Linskens, 1969). A review of commercial pollen storing, shipping and research was compiled by Antles (1951).

Holman and Brubaker (1926) showed a "tendency toward uniformity in pollen longevity within many genera, although wide variations do occur within some genera." (See also Pfundt, 1910; Duffield, 1954). The longevity extremes noted in Holman and Brubaker's (1926) work were 1-2 days for Zea mays pollen and 336 days for pollen of Typha latifolia.

Clearly, there is no general statement concerning pollen storage.

b) VARIATIONS OF TEMPERATURE ABOVE 0°C AND RELATIVE HUMIDITY

Research in gymnosperm pollen storage began in the latter part of the 19th century, with work by Mangin (1886), Rittinghaus (1886) and Molisch (1893). Pollen of 80 species was stored under dry air conditions at room temperature with little or no success.

In the early part of this century, workers such as Goff (1901), Sandsten (1908) and Roemer (1915) became aware that low temperatures prolonged pollen viability. Working with Antirrhinum sp., Knowlton (1922) concluded that as storage temperatures decreased, viability of pollen increased.

Pfundt (1910) was the first to study the effects of relative humidities of 0, 30, 60 and 90% at approximately room temperature (17-22°C); maximum longevity occurred at R.H. 0-30%. Holman and Brubaker (1926) confirmed Pfundt's results with 52 additional species.

Pfeiffer (1936, 1939 and 1944) studied storage conditions of pollen from Lilium spp., hybrid Amaryllis (Hippeastrum vittatum), Cinchona spp.

and Gladiolus spp. She concluded that favorable storage environments for these pollens were 10°C, 35-50% R.H. and darkness.

Newcomer, in 1939, working with Ginkgo biloba pollen, produced normal fruits after 16 months' storage at 7°C over $CaCl_2$, although no germination occurred on a variety of media and stimulatory substances.

In a pollen longevity study of Pinus strobus and P. resinosa, Duffield and Snow (1941) obtained 80% germination with both species after 1 year's storage at 50% R.H. and 0-4°C. Reduced germination occurred at 0-10% R.H. regardless of temperature, and at room temperatures up to 30°C regardless of humidity. However, germination could not be restored by 75% R.H. at 4°C for 12 hours (see also Nebel and Ruttle, 1937; Pfeiffer, 1944 and 1955); thus, it may be necessary to rehydrate pollen stored under dry conditions. This may also explain some of the poor results of earlier workers.

Johnson (1943) stored pollen of several forest tree species under a series of different R.H.'s, temperature and light. After 1 year's storage, pollen longevity was favored by low temperature and darkness. Pollen from Pinus spp. gave 96-99% germination at 50-75% R.H., 2°C and dark storage, while pollen from Picea spp. gave only 58-63% germination even at a wider R.H. range (10-75%), 2°C and dark storage. An interesting observation from this study is that in all species successfully stored pollen germination was higher after 12 months' storage than after 8 months' storage. This improvement of pollen germination with storage has been reported by other researchers (Fechner, 1957; Dobrinov, 1959; Wright, 1959; Ehrenberg, 1960). In addition, Bullock and Snyder (1946) observed that viability was quickly lost if R.H. was allowed to fluctuate during storage.

Duffield (1954) published the results of a number of studies, including pollen storage of seven species of Pinus. Pollen was stored at four levels of temperature and R.H. (0, 5, 10, 22°C and 10, 25, 50, 75% R.H.). It was concluded for all species tested that best storage conditions were at 0-5°C and 10-25% R.H. and low germination occurred at 25°C and 50% R.H. and at 22°C and 75% R.H. at all temperatures. One species (Pinus radiata) is sensitive to a combination of low temperature and humidity. Pinus lambertiana may also be sensitive to such storage conditions. Visser (1955) suggested a negative correlation of pollen longevity with relative humidity for optimal storage. However, grass pollen requires a humidity for germination but remains viable for only short periods of time. Using Pinus pinea and Pinus hamiltonii, Magini (1956) found that after 1 year's storage at 3-5°C and 30% R.H. pollen gave 70% germination.

The first studies of pollen storage of Douglas-fir (Pseudotsuga menziesii) were published by Orr-Ewing (1956). Pollen was stored at 4°C in lightly stoppered flasks with no humidity control. Germination declined during 2 years of storage, while controlled crosses indicated an extreme reduction in the fertilizing potential of the same pollen.

Pollen of Tsuga canadensis stored for 1 year at 1, 7, 16 and 20°C and at 10% and 50% R.H. resulted in only slight differences for the temperature range of 1-16°C, but much lower germination at the lower humidity (Santamour and Nienstaedt, 1956).

Fechner (1958) reported that pollen of Picea pungens, Pinus ponderosa, P. contorta and P. flexilis gave favorable germination after storage for 1 year at 0-4°C and 25-50% R.H. Worseley (1959) reported that Pinus sylvestris was stored successfully for 3 years and Cedrus atlantica pollen for 2½ years. Worseley did not indicate length of storage for all species, but concluded that pollen of Abies amabilis, A. concolor, var loweana, Larix loptolepis, Picea breweriana, Picea smithiana, Pinus banksiana, Pinus contorta, Pinus mugo, Pinus nigra, Pinus penke, Pinus ponderosa, Pinus strobus and Pinus sylvestris may be stored at 0°C and 10% or 25% R.H. successfully. Pollen of Pseudotsuga menziesii was most successfully stored at lower temperatures (0-5°C) and at 25% R.H., but a reduction of up to 50% in the germination potential occurred for pollen stored at 10% relative humidity.

More recently, several authors have suggested that moisture content of pollen itself, rather than moisture level of the storage atmosphere, is the most critical factor in pollen storage. Snyder (1961) published a practical paper, including a brief literature review of storage methods for gymnosperm pollen. Working with southern pine pollen, he stressed that pollen must be dry for successful storage and described methods for determining and adjusting pollen moisture content. He further recommended that pollen be stored in 2-0z or smaller containers, half filled, and plugged loosely with cotton. Storage temperature should be 0°C or slightly above and R.H. 22%, maintained by a saturate solution of potassium acetate. Lanner (1962) measured the moisture level of pollen by placing pollen samples in a series of constant R.H. chambers until equilibrium moisture content was reached. From this, a curve was derived from which a predetermined moisture content of pollen could be selected. Lanner calculated the moisture content of stored pollen of nine species of conifers, including Pinus, Abies, Pseudotsuga and Cedrus. (Lanner was studying the influence of moisture content on viability of deep frozen pollen, but we felt that this could be adapted to pollen stored above 0°C to find the most successful storage moisture with reference to viability.)

Data on pollen stored for 5 to 15 years have been published by Stanley (1962), Stanley et al. (1960), Stanley (1962), Fechner and Funsch (1966) and Callahan and Stainhoff (1966). Stanley et al. (1960, 1962) found that Pinus ponderosa pollen, after 15 years' storage at 10% R.H. and 0° or 5°C, gave 77% germination but produced only hollow seeds under controlled pollinations. After 11 years' storage, Fechner and Funsch (1966) found highest germination (no actual values were given) for Pinus ponderosa pollen stored at 25-50% R.H. and 0-4°C rather than pollen stored at higher temperatures and R.H. In the same research study, Pinus contorta and Pinus flexilis pollen failed to germinate after 11 years at 0-4°C and 0,25, and 50% R.H. Picea pungens pollen gave the highest germination at 50% R.H. and 0-4°C.

Storing pollen over $CaCl_2$ (0% R.H.) has resulted in conflicting observations. Newcomber (1939) obtained fruit set with Ginkgo biloba pollen after 16 months' storage over $CaCl_2$; Manzhos (1958) had favorable results for 1 year with Pinus sylvestris, Pinus contorta and Pinus banksiana; Egorova (1969) had favorable results from pollen stored for more than 2 years with certain species of Cupressaceae; Dobrinov (1959) also had favorable results for up to 1 year with Pinus sylvestris. On the other hand, Santamour and Nienstaedt (1956) observed a marked decrease in pollen germination of

Tsuga canadensis when stored over $CaCl_2$ (0% R.H.) for 1 month, and after 1 year a complete failure to germinate. In 1954, Scepotjiv and Pobegailo suggested that Inglans nigra pollen should not be stored in a desiccator over $CaCl_2$ because of the poor germination obtained.

King (1970) studied the effects of different drying agents on Cunninghamia lanceolata and C. konishii pollen. After 3 years' storage, germination was 26% over $CaCl_2$, 20% over silica gel and 15% over H_2SO_4 for C. lanceolata, and 19%, 18% and 12%, respectively, for C. konishii. The R.H. over all three drying agents was 67%. When no R.H. is measured, the R.H. may be 67% or less, possibly approaching 0% if the R.H. of the atmosphere is already low. King (1970) suggested that because of the similarity of germination rates with the three drying agents and R.H. values, the drying agents had no direct effect on pollen viability but the humidity within the desiccator was important.

In summary, early work suggested that low humidity was the answer (Pfundt, 1910; Holman and Brubaker, 1926). Newer references such as Duffield and Snow (1941) and Johnson (1943), who investigated different species or varieties and a range of humidities, demonstrated that a relative humidity near zero had an unfavorable effect on nearly all pollens tested. However, the great contrasts between older and newer data concern different pollens and it is most likely that differences in storage or germination techniques may be responsible for the large discrepancies found. For example, Pfundt (1910) found a maximal longevity of 70 days with apple pollen when stored at room temperature over concentrated H_2SO_4, while Visser (1955) reported a complete loss of germination within 3 days. Nonetheless, the overwhelming evidence of literature indicates that longevity of pollen increases with decreasing relative humidity. It is believed that high humidities allow greater physiological activity, resulting in shortened lifetimes. This should not be considered a rule, since data from many pollens indicate that water can only be lost to a certain level before loss of viability becomes evident. Thus, in these latter cases, it would appear that there is a definite optimal relative humidity below which physiological damage results.

c) RELATIVE HUMIDITY EFFECTS BELOW 0°C AND DEEP FREEZING STUDIES

Grape pollen stored at -12°C retained its viability longer at 28% R.H. than at a higher R.H. (58%) (Olmo, 1942). However, other investigators had favorable results without controlling humidity during storage (Knowlton, 1922; Viser, 1955). For example, Griggs, Vensell and Iwakiri (1953) reported that pollen can be stored in a home freezer (0°F) in cotton-plugged vials with no humidity control. Wright (1959) undertook comparative trials for seed set with fresh white pine (Pinus monticola) pollen kept at 40°F and 25-40% R.H. and pollen stored at 0°F and undetermined R.H. for 1 year. He reported a higher percentage of filled seeds with fresh pollen, but in total seed set per cone, stored pollen sometimes gave higher percentages of filled seeds. According to Visser (1955), relative humidity became less important as temperatures decreased. He gave no reason for this statement, but physiological activity would be greatly reduced.

Uncontrolled R.H. levels may give favorable results at temperatures of 0°C or below only if the pollen itself has been sufficiently predried before subjecting it to lower temperatures. Damage may result through water

condensation (Newcomer, 1939) or the high moisture content of the pollen may actually lead to physical freezing damage (Visser, 1955).

With success at 0°C or slightly lower, parallel research was undertaken at extremely low temperatures. In 1948, Bredemann et al. quick froze Lupinus polyphyllus pollen in liquid N_2 (-196°C) and stored it at 0°C. Immediately after freezing, almost 80% of the pollen germinated but pollen germination decreased rapidly with increasing length of storage at 0°C.

Duffield and Callahan (1959) outlined a method of deep-freezing pine pollen. After desiccation over $CaCl_2$ for 1 week, pollen was transferred to 5°C storage. In their words, "crude estimates" indicated no appreciable difference between the ability of quick-frozen pollen and fresh pollen to fertilize, although more round seeds were set in cones pollinated with fresh pollen.

Pinus sylvestris L. and Pinus contorta var. Mungana Engelm. pollen was stored at room temperature, 4°C and -18°C (Ehrenberg, 1960). Fertilization capacity was tested with 3-year-old pollen. Storage at -18°C gave more favorable results than the 4°C storage. After 1 year, pollen stored at room temperature had lost its viability. At -18°C, pollen retained its viability after 7 years and germinated better than pollen stored at 4°C for the same length of time.

Callahan and Steinhoff (1966) stored pollen of Pinus jeffreyi, Pinus monticola, Pinus contorta var. murrayana, Pinus ponderosa and Pinus sabiniana up to 5 years at -20°C. After 5 years, the total yield of seed was the same for frozen and fresh pollen. However, the pollen stored at -20°C for 5 years produced only 50% filled seeds compared to fresh pollen (see also Bingham and Wise, 1968).

Ichikawa and Shidei (1971) found -35 to -45°C to be the best temperatures for storage of pollen for Cryptomeria japonica, Larix leptolepsis and Cedrus deodora. As stated in this review, a procedure such as this must be accompanied by low water content in pollen and a rapid cooling rate (such as with liquid nitrogen) if injury is to be avoided. These workers also had a very successful result for 100 days with pollen of Cryptomeria japonica by pre-freezing at -30°C for 4-5 hours and then storing at -196°C in liquid nitrogen.

Some references suggest avoiding low temperatures for preservation of pollen (Stubbe, 1936; Hermann, 1969), but the majority indicate that low temperature favored pollen longevity (Goff, 1901; Sandsten, 1908; Roemer, 1915; Knowlton, 1922).

Removal of water seems to be required before subjecting pollen to storage at temperatures below 0°C; hence, those pollens able to maintain their viability at a low moisture level can be stored at freezing temperatures. This assumption is supported by the fact that pollens belonging to different genera and which can be kept viable at dry conditions were found to profit from storage at temperatures below 0°C; for example, Amaryllis, Antirrhinum, Lilium, Lupinus, Lycopersicon, Phoenix, Pistachia, Prunus and Vitis (Visser, 1955). This led Linskens (1963) to suggest that drought-resistance and temperature resistance are linked to one another so that study of one may

provide information concerning the other.

At a temperature as low as -190°C, all physiological activities of pollen should be reduced to near zero; therefore stored at such a temperature would retain its initial germination capacity for an indefinite period of time. Thus, pollen, provided it is resistant to low temperature, should be given "eternal life". However, temperatures as low as -180°C or -190°C obtained by using liquid oxygen or liquid nitrogen, respectively, although appearing to be ideal, are far too expensive and require costly and elaborate storage facilities. However, deep-freeze temperatures of -20°C may be a general and practical way of storing pollen from one season to the next.

d) FREEZE DRYING STUDIES

Freeze drying as a method of storing pollen has not been generally accepted, but has given good results for some genera. For example, Pfeiffer (1955) found that after a freeze-drying treatment and storage in evacuated tubes at 20°C, 0.5°C or 5°C, germination percentage was low. However, when stored Lilium pollen was exposed to 65% R.H. and 8°C for 5-7 days prior to germination, the percentage germination increased. King (1959 and 1961) extended the longevity of several genera using a freeze-drying method of storage, but Hesseltine and Snyder (1958) with Pinus palustris pollen did not.

Livingston et al. (1962), working with Pseudotsuga menziesii pollen, reported that viability was reduced after freeze-drying. Freeze-drying for 2 hours reduced the residual water content to 2% or less, with consequent reduction in viability. The magnitude of reduction in viability was related not only to the duration of treatment but also to conditions immediately following collection.

Freeze-drying may increase the permeability of the membrane and loss of viability results (Ching and Ching, 1964). Such damage may occur during vacuum-drying rather than the initial freezing step (Davies and Dickinson, 1971). Measuring O_2 uptake manometrically, they reported modified patterns of respiration of Lilium longiflorum pollen. Frozen undried pollen showed a similar O_2 uptake to unfrozen undried pollen, but with increasing length of vacuum-drying (15, 30 and 60 minutes), O_2 uptake significantly decreased. Thus, in addition to loss of viability, vacuum-drying may also be responsible for the apparent increased permeability observed by Ching and Ching in 1964.

The first promising results concerning storage of freeze-dried pollen were reported by Ching and Ching in 1964. Pretreating pollen of Pinus monticola by air-drying for 4 hours or refrigerating "slightly" air-dried pollen for 6 weeks and then freeze-drying for 30-60 minutes was an effective means of retaining high viability (see also Ching and Slabaugh, 1966).

Recent work dealing specifically with Pseudotsuga menziesii has been reported by Livingston and Ching (1967). They applied three pre-treatments to Douglas-fir pollen: Lot 1 - air-dried or prechilled with a moisture content of 16%; Lot 2 - air-dried for 16 hours, moisture content 6%; Lot 3 - air dried for 4 hours, moisture content 9% and prechilled at 0°C for 36 days. The pollen was prefrozen at -78°C, vacuum-dried and sealed in

lyophil tubes. Pollen was stored at 20°C, 3°C and -18°C. Germination percentage and moisture content were determined at each step, and samples of pollen from all treatments were used in controlled pollinations to check fertility. It was concluded that germinability of pollen was reduced by freeze-drying but that a pretreatment of air-drying and prechilling lessened this effect. The pretreatment also enhanced retention of fertility - which was noticeably higher in freeze-dried pollen than non-freeze-dried pollen. After 2 years' storage, only freeze-dried pollen stored at 3°C and -18°C was capable of producing viable seed.

Opinions on freeze-drying for pollen storage have differed widely. Jaranowski (1965) stated that freeze-drying was the most effective method of artificial storage for pollen but that it must be exposed to a predetermined temperature and R.H. prior to pollination. However, Hermann (1969) suggested that low temperature freeze-drying was undesirable due to the risk of "mutative modifications" from such treatment. Although not serious, this together with the respiratory changes and membrane effects described earlier leave some questions concerning this method of storage.

e) VACUUM DRYING AND STORAGE IN VACUO

The early work on storage in reduced air pressure produced conflicting results (Visser, 1955). As early as 1929, Bequerel stored pollen of Antirrhinum majus and Nicotiana tabacum in vacuum for 5 months and concluded that metabolic activity was suspended in vacuo. Pfeiffer (1938), however, reported complete loss of viability with two species of Lilium stored in vacuum at 20°C.

The loss of pollen viability may be greatly reduced by vacuum-drying at higher temperatures than those used in freeze-drying treatments. Layne and Hagedorn (1963) compared the effects of vacuum-drying, freeze-drying and storage environment on the viability of pea pollen. Between -25°C and 8°C there was no difference between freeze-drying and vacuum-drying if the vacuum period was less than 120 minutes. Because the vacuum-drying seems responsible for loss of viability of the pollen (Davies and Dickinson, 1971), if vacuum is employed it should not be too severe.

Vacuum-stored pollen samples are usually superior in retention of viability to samples stored at the same temperature but without the vacuum; for example, species of pollen evacuated for 2 hours to a final pressure of -.015 mm Hg. and stored in ampoules at 5°C or room temperature. After 1 year of storage at 5°C in vacuo, pollen of tobacco (Nicotiana tabacum), potato (Solanum tuberosum), clover (Trifolium sp.), pea (Pisum sp.) and beet (Beta sp.) were capable of setting seed, and after 2 years, tobacco and pea pollen could effect seed set. Pine pollen (Pinus sp.) germinated after 2½ years at 5°C in vacuo. On the other hand, Barber and Stewart (1957) found 1 year of storage of jack pine (Pinus banksiana), white spruce (Pinus glauca) and Norway spruce (Pinus abies) pollen in a vacuum gave results similar to pollen stored in a refrigerator at atmospheric pressure. However, Muto (1962) did increase longevity of Picea pollen stored in vacuum at -8°C to 10°C.

Schoenike and Stewart (1963) found that pollen of Pinus banksiana, Picea glauca and Picea abies after 5 years' storage under 1 mm Hg vacuum at

4°C had a germination range from 44-90%. Vacuum storage gave greater viability than no vacuum, and vacuum treatment plus a chlorophylls or Demin additive gave greater viability than vacuum alone. However, vacuum treatment plus iron chelate resulted in a lower viability.

In 1959, the Institute of Forest Genetics and Forest Tree Breeding at Schmalenbeck reported a simple inexpensive method for preserving pollen in glass ampoules under vacuum. By using a vacuum of 190 ton, about 100 ampoules per hour were evacuated and sealed under vacuum. The closed ampoules were stored at -18°C. Pollen of Scots pine (Pinus sylvestris), Norway spruce (Picea abies) and European larch (Larix aecidua) germinated after 4 years under these conditions.

Because it is relatively inexpensive and has given promising results, vacuum storage should be investigated as a general method for pollen storage.

f) OTHER POLLEN PRESERVATION ATTEMPTS

High percentages of CO_2 in the storage atmosphere increased the longevity of Antirrhinum sp. pollen, whereas storage in pure oxygen proved less favorable (Knowlton, 1922).

Tulecke (1954) collected unopened male strobili of Ginkgo biloba, surface sterilized them and immediately stored them at 5°C over anhydrous $CaCl_2$. Dehisence took place in the cold; this pollen had 35-45% germination after 2 years' storage, compared to 50-60% germination of fresh pollen.

Worseley (1959) suggested pollen fractionation as a method of selecting viability of pollen samples. He fractionated "live" from "dead" pollen in a gently moving vertical column of air. Pseudotsuga menziesii and P. taxifolia pollen gave the best fractionation; samples retained in the column were 98% viable. Pollen for storage could be tested to insure that the maximum amount of viable pollen is actually stored.

Iwanami and Nakamura (1972) suggested storage in organic solvents. This study, however, dealt with very short-term storage.

PART III

PHYSIOLOGY OF POLLEN

a) INTRODUCTION

Rosen (1968) stated, "Pollen offers opportunities and challenges because it is unique among higher plant tissues. Yet this very uniqueness also adds to its value in research on fundamental and universal problems." Pollen is haploid (Rosen, 1968). It is a unicellular organism unconfined by other cells. This provides the cell physiologist with genetically "simple" material to work with. The major drawback is the difficulty in obtaining enough uniform samples of pollen grains to do experiments. Thus, major studies have been confined to plants producing large amounts of pollen, such as Zea mays or Pinus.

Generally, the physiology of pollen is an old subject in botany. The first detailed chemical analyses of pollen were published in 1829 by Braconnot. In 1963, it was reported that in the preceding 50 years some 8000 articles had been written on the subject. This review will attempt to cover the major topics.

b) CARBOHYDRATES

Johri and Vasil (1961) stated that at various stages of pollen development, pollen grains showed considerable quantities of starch but this starch disappeared during the time of pollen shedding. According to Iwanami (1959), starch is used while the new cell wall is being laid down and its disappearance just before anthers dehisce was though to be due to its conversion into sugars.

Linskens (1969) and others (Johri and Vasil, 1961; Rosen, 1968) claimed that carbohydrates in pollen have been frequently detected in protein fractions. Stanley (1971) stated that carbohydrates in pollen occur primarily in the cell walls and as cytoplasmic polysaccharides. Much earlier, Tischler (1917) and Luxemburgowa (1928), in embryological studies, reported the presence of starch and fat. Ribose and deoxyribose, associated with nucleoproteins, were also present. Starch accumulation was observed in pine pollen as exogenous sugar was absorbed on germination (Hellmers and Machlis, 1956; Iwanami, 1959). Quadrio (1928) and Mameli (1952) reported that generally wind-pollinated plants have starchy pollen, while pollen of insect-pollinated plants was rich in fat and sugar. Moreover, Mameli (1952) stated that a correlation between starchy pollen and aporogamy and fatty pollen and prorogamy exists. However, Linskens (1969) argued there are so many exceptions that this cannot be considered a rule.

Simple sugars are the substrates used by germinating pollen. According to Stanley (1971), the amount of sugar present depended somewhat on the number of nuclei. Most grass pollens, which do not retain viability when shed, are trinucleate. These include Zea mays which contains 36-40% dry weight as carbohydrates. On the other hand, pollens relatively stable in storage, such as the date palm (P. dactylifera) pollen, contain only 1-2% soluble carbohydrate.

The major component of free sugar in pollen varies with each species. In angiosperms, sucrose comprised 20-50% of the free sugars, whereas in pines, it could be more than 93% (Stanley, 1971). According to Stanley (1971), many soluble sugars were present in most pollens; of 15 conifer pollens examined, raffinose occurred in all, stachyose in 10; arabinose, xylose and galactose occurred frequently, and these three sugars could occur in hydrolysates of pectin and hemicelluloses. He reported that rare sugars such as turanose and nigerose may also be present, but stated these may only be fragments from degraded polysaccharides. Kuhn and Low (1949) reported lactose in pollen of Forsythia, and according to Johri and Vasil (1961), this is the only authentic record of lactose in the plant kingdom. Several unidentified sugars have also been reported in corn (Gunning and Pate, 1969) and cotton pollen (Jensen and Fisher, 1961).

Storage affects soluble sugars in pollen, as reported by Stanley and Poostchi (1962) and Zolotovich, Secenska and Deceva (1962). Stanley and Poostchi (1962) found that pine pollen, after 15 years of storage at 25% R.H. and 5°C did not germinate, and contained less glucose and sucrose than pollen stored at 10% R.H. and 5°C, capable of germinating. According to Stanley (1971), no one has pursued these leads or determined the significance of sugars in pollen growth.

c) PROTEINS AND AMINO ACIDS

Type and composition of proteins and amino acids present in pollen has been investigated to determine the active principle causing pollen-induced allergy (see Sarker et al, 1949; Nielsen et al., 1955; Takashima, 1954; Virtamen and Karl, 1955; Varma and Varma, 1956; Iwanami, 1959). The total amount of protein in pollen generally runs between 11 and 30% (Stanley, 1971). Pollens with slower growing tubes and those with rapid growing pollen tubes generally have lower and higher amounts of protein, respectively. Using radioisotopes, Mascarhenas and Bell (1969) showed that protein synthesis occurred during germination and when functioning polysomes were present.

Analysis has been done on protein fractions from pollens. Lunden (1954) found a considerable amount of the protein obtained was of low molecular weight and contained carbohydrates. Other proteins were free or bound to pigments and included artefolin, protensin and trifidin. Lunden (1954) reported the presence of nucleoproteins in birch pollen.

Varma and Varma (1956), Iwanami (1959) and others showed that most of the common amino acids occur in pollen grains and that these either occur in a free state or are bound to proteins. However, Johri and Vasil (1961) reported that phenylalanine, tryptophane, hydroxyproline, tyrosine and amino-butyric acid do not usually occur.

Payne and Fairbrothers (1973) found, in pollen of Betula populifolia, a considerable change in protein composition as pollen grains aged. Where proteins can be readily extracted or diffuse, they may prove useful in measuring viability.

d) GROWTH SUBSTANCES

1) Vitamins

Generally, pollen of gymnosperms is low in vitamin content, while angiosperms are very rich in "B" vitamins, but lacking in fat-soluble vitamins (Lunden, 1954). Johri and Vasil (1961) reported that biotin, folic acid, inositol, nicotinic acid. Pyridoxine, riboflavin, thiamin, ascorbic acid and vitamins A, D, E and K were present in pollen of maize, date plum and many other plants (see also Weygand and Hofmann, 1950; Nielsen et al., 1955). Stanley (1971) reported some vitamins occurred in low levels, while others, such as nicotinic acid, occurred in generally high levels.

According to Johri and Vasil (1961), pollen grains appeared rich in water soluble vitamins. Added Stanley (1971), "Pollen vitamins assayed as essential in insects and mammalian nutrition often behave chemically as enzyme co-factors."

Nielsen, 1956, found that vitamins of pollen of Pinus montana, Alnus glutinosa, A. incana and Zea mays hardly changed after 1 year's storage in a cool place. Exceptions were riboflavin and pantothenic acid. Offered Stanley (1971), "Such losses may cause reduction of growth of pollen tubes or low viability when older, stored pollens are germinated."

2) Promoters

According to Johri and Vasil (1961), Rosen (1968) and Stanley (1971), many oestrone and plant hormone-like substances have been found in pollen. Stanley stated many vitamin-like substances often occurred in mg/g dry weight levels. Sweet and Lewis (1941), with Pinus radiata pollen, reported thirteen active substances. These included five auxins, four gibberellins, and one compound which had some properties of both gibberellins and cytokinins and which may be involved in pollen tube growth.

As discussed before, sugars and boron may promote pollen tube growth. Other materials such as Ca^{2+} have effects in addition to growth promotion, although maize pollen cannot be germinated without Ca^{2+} (Cook and Walden, 1965). Kwach and Kim (1967) reported that Ca^{2+} protected pollen tubes against growth-inhibitors. Ca^{2+} became bound to the pectic regions of the wall, which decreased permeability and increased rigidity of the tube wall.

After the discovery of steroids in animals, plant biochemists searched for these substances in plants. They found cholesterol, sitosterol and other steroids (Standifer et al., 1968). Hoeberichts and Linskens (1968) reported that steroid components often occurred with fatty acids in pollen. According to Stanley (1971), the physiological role of steroids in pollen is not known.

Little is known about the role hormones play in pollen. Konar (1958), with Pinus roxburghii, found that pollen accumulated greater quantities of starch when supplied with kinetin and indole-3-acetic acid. He suggested that hormones might direct the mobilization of sugars between pollination and

fertilization.

3) Inhibitors

Pollen may contain growth inhibitors as well as promoters (Larsen and Tung, 1950; Tanaka, 1958). Tanaka (1958) found two growth inhibitors in the acid fraction from ether extracts of Pinus densiflora pollen. Sweet and Lewis (1971) reported three inhibitors in Pinus radiata pollen. The function of these inhibitors is not clearly understood.

e) ENZYMES AND ISOENZYMES

Pollen enzymes have been reviewed by Paton (1921) and, more recently, by Mäkinen and Macdonald (1968). Other references to pollen enzymes are in research on chemical composition or physiology (Lunden, 1956; Johri and Vasil, 1961; Linskens, 1969; Rosen, 1968; Brewbaker, 1971). Pollen enzymes were studied to determine growth requirements, metabolism and incompatibility reactions of pollen.

In 1894, Green observed that enzyme activity of some pollens was much greater in sugar solutions than in water. Stanley and Linskens (1964), using Petunia hybrida pollen, followed CO_2 release from germinating pollen which had been exposed to labelled sucrose. They found high levels of enzyme activity before tubes were formed, and this activity was related to the osmotic pressure of the culture medium. Enzyme activity was lowest at low osmotic pressures. Stanley (1971) reported that pollen contains many enzymes which increased during germination, including amylase, fructofuranosidase, phosphorylase and transaminase. However, he suggested that germination was not dependent on net enzyme synthesis.

Enzymes diffused rapidly from pollen when placed in a germinating medium (Brewbaker, 1971). Makinen and Brewbaker (1967) reported no correlation between germination and diffusion of enzymes because enzyme diffusion continued for hours in 1% NaCl, and no germination took place.

Isoenzymes of esterases, amino-peptidases, catalases, amylases and acid phosphatases were released from pollen of Oenothera organensis (Makinen and Brewbaker, 1967). This isoenzyme analysis was done through gel electrophoresis, which yielded 13 independent bands of esterases at the end of 19 hours of current flow. A series of papers have been published on isoenzyme polymorphism in flowering plant pollen (Makinen and Brewbaker, 1967; Makinen and Macdonald, 1968; Hamill and Brewbaker, 1969).

Further studies of pollen isoenzymes may show the causes of pollen viability loss during storage (Brewbaker, 1971). For instance, for peroxidase isoenzymes, pollen gave a "unique isoenzyme pattern" which differed from the pattern obtained after 1 hour's storage at room temperature. This may indicate that a loss of peroxidase activity was related to a loss of viability. Stanley (1971) reported that the loss of peroxidase is not a total loss of activity, but that zymograms of the peroxidase bands accompany the loss of viability. Heslop-Harrison (1971) reported that certain enzymatic activity was lost slowly during storage and that this alone cannot account for the rapid loss of viability in corn pollen.

Generally, pollens have similar enzyme activities to other plant structures. This indicates the complexity of form in the pollen grain. Isoenzyme analysis through gel electrophoresis may provide further evidence for the specialization of the pollen grain, and may provide answers to the cause of loss of viability during storage.

f) METABOLISM - A GENERAL DISCUSSION

Pollen is capable of metabolizing sugars not contained within it naturally; these include glucose, lactose and galactose (Stanley, 1971). However, glucose is the most favorable carbohydrate for pollen suspension when measuring the oxygen uptake of pollen from Lilium spp., Cammelia spp., Papaver spp., Tulipa spp., Thea spp. and Pinus spp. (Okunuki, 1937). Pine pollen was capable of germinating in distilled water in vitro (Righter, 1939; Echols and Mergen, 1956; Giordano and Bonechi, 1957; Brown, 1958), but exogenous sucrose resulted in doubling the oxygen uptake (Stanley, 1971). Starch accumulates in germinating pollen grains (Hellmers and Machlis, 1956). Dickinson (1968) related this starch build-up with increased respiration in germinating Lilium longiflorum pollen. During 2 hours' incubation in pentaerythritol, the respiratory quotient (R.Q.) was one. Endogenous sucrose decreased rapidly during incubation. These two factors suggested that an endogenous carbohydrate was the substrate for starch synthesis and respiration (Dickinson, 1968).

O'Kelly (1955) measured oxygen uptake of pollen from Tecoma radicans (Trumpet vine) manometrically. He found external sucrose-C, fructose-C and glucose-C were all utilized in respiration. Stanley et al, (1958) demonstrated that cations modify the pathway by which Pinus ponderosa pollen metabolizes glucose. They observed the incorporation of carbon dioxide into the organic amino-acid and protein fractions of germinating pollen.

Oxygen uptake by Pinus mugo pollen, measured with the Warburg apparatus, showed considerable variation in respiration rate, depending on pH and temperature(Nyaard, 1969). Also, oxygen uptake was reduced with the addition of D-mannose or deoxyhexoses (Nygaard, 1971).

g) RESPIRATION

In 1933, Okunuki observed that pollen in the anther had a low respiration rate; upon shedding and germinating, respiration increased significantly. Since then Dickinson has studied the metabolic steps for pollen tube initiation and growth (Dickinson and Cochran, 1968; Davies and Dickinson, 1971; Hopper and Dickinson, 1972). Using Lilium longiflorum pollen, Dickinson (1965) measured the oxygen uptake during germination manometrically at 30°C. He observed an initial high rate of respiration before pollen tube growth; a low rate of respiration, and another high rate accompanying extensive pollen tube elongation. Dickinson (1966) suggested the three distinctive rates of respiration in pollen may be due to variations in the rate of adenosine triphosphate (ATP) synthesis.

Dickinson (1966) concluded that ATP may be regulating respiration in pollen. 2, 4-dinitrophenol (DNP) inhibits an intermediate in oxidative phosphoryation and is thought to stimulate ATP-ase, and the oxygen consumption

on intact mitochondria. DNP stimulated respiration to an equally high rate over all three phases. Further, oligomycin, which inhibits only phosphorylating electron transport, inhibited all three periods. DNP reversed this oligomycin induced inhibition.

Okunuki (1933) observed that KCN, methylene blue and phenylurethane affected the O_2 utilization of Thea sinensis pollen. Less O_2 uptake occurred in dried pollen than in fresh pollen and dried pollen was sensitive to cyanide (KCN) and carbon monoxide (CO).

During germination and pollen tube development of Pinus densiflora starch disappearance was accelerated by sodium azide and DNP, whereas tube growth and pollen germination were inhibited (Tanaka, 1957). Azide inhibited growth resumption and starch disappearance, but malonic acid promoted it. Dickinson and Cochran (1968) found, in Lilium, starch was accumulated in pollen in the presence of enough dimethyl sulphoxide (DMSO) to prevent tube initiation. At the same time, DMSO inhibited the high rate of respiration associated with Lilium pollen tube growth. Although DMSO is not a general inhibitor of pollen metabolism, Dickinson and Cochran (1968) suggested that this inhibitor might reversibly inhibit reactions necessary for tube initiation and tube growth. Because of the high concentration necessary (5%), it is more likely that DMSO had an osmotic effect rather than a direct enzyme inhibitory one.

Livingston (1971) measured O_2 consumption of irradiated and non-irradiated Pseudotsuga menziesii (Douglas-fir) pollen. He observed higher respiration rates with radiation doses up to 64 Kr and an inhibition of respiration at higher radiation levels. Livingston stated that it was not clear whether the decrease in oxygen consumption at the highest radiation level was due to a lower germination percentage or to a real decrease in the respiratory rate. However, at 256 Kr, he got lower amounts of O_2 consumed than at lower dose rates, and germination was only 1% less than the control or 32 Kr.

To discover whether radiation could alter the metabolic pathway of pollen, Livingston measured the R.Q. values under various doses of radiation. He concluded that the metabolic pathway was not altered because he got R.Q. values consistently greater than unity. He suggested that aerobic fermentation or an organic acid substrate could be responsible.

At radiation doses up to 64 Kr, the greater growth rate was due to an accelerated metabolism. The decrease in respiration at higher Kr doses, while germination was not affected, may be due to changes in the cell wall or membrane structure. Osmotic disturbances may alter the cell wall permeability and elasticity to such an extent that physical factors become more important than metabolism in cell elongation (Livingston, 1971).

O_2 uptake was measured on Tecoma radicans pollen germinating in the presence of various concentrations of boron (B). The B effect on pollen tube elongation was not closely related to respiration (O'Kelly, 1957).

Walden (1959) cited a series of papers by Okunuki and one by Nishikawa on pollen respiration. These workers had found that the ability of pollen of Thea spp., Camellia spp. and Lilium spp. to assimilate oxygen

was extended for 6 months when stored over $CaCl_2$ (no temperature was given). However, germinability ceased prior to cessation of O_2 uptake in all cases. Walden (1959), therefore, suggested that respiration might continue after loss of viability although respiration played a leading role in retention of viability.

The highest respiration of corn pollen was in 30% sucrose at 45°C. The pollen was stored at a series of humidities ranging from 80% R.H. to 0% R.H.; viability was reduced at low humidites (Nishikawa, 1957 cited by Walden, 1959). Walden (1959) measured the O_2 uptake of corn pollen. The rate of oxygen consumption for pollen no older than 4 days was more dependent on the percentage moisture than on the age of the pollen. He suggested that a change of moisture content in pollen has a direct effect on respiration and is irreversible.

Nygaard (1973) indicated that in pollen of Pinus mugo, a pool of adenosine triphosphate can be detected which may be exhausted by the onset of germination but restored by oxygen uptake.

Respiration plays an important part in the metabolic function of the pollen grain. If the longevity of pollen is to take place in storage, this respiration must be curtailed.

PART IV

SUMMARY AND RECOMMENDATIONS

Despite the volume of often conflicting work carried out on pollen, some general points can be made.

1) No satisfactory laboratory test has been found to determine pollen viability, the most widely employed method at present being in vitro germination. However, germination of a pollen grain does not mean that it still can fertilize the egg. This is particularly applicable to pollens in which the interval between pollination and fertilization is 6 weeks or more. Further, the failure of the pollen to germinate may be the fault of the medium used; the pollen may still be viable. Germination can be a good tool to indicate whether pollen is dead or alive, but it is not a reliable indication of viability.

According to unpublished results, respiration seems to be a promising test of viability (Binder, 1974). By measuring respiration with an oxygen electrode, it is possible to predict viability state.

2) In order to obtain an effective method of pollen storage, the respiration of the pollen must be reduced to conserve its food reserves. The different storage methods used are either expensive or are very limited in positive results. Deep freezing with liquid 0_2 or nitrogen may be promising with its "eternal pollen life" concept, but this method is expensive and requires delicate operations, such as the proper thawing rate. Also, there is some concern regarding the mutagenic effects of deep freezing on pollen. Methods of vacuum storage and near zero temperatures look promising and are less expensive and less drastic than deep freezing.

Temperature and humidity of stored samples should be monitored because these two variables seem to be of prime importance to longevity of pollen in storage.

3) The pollen grain is a very complex, self-contained organism. Metabolic pathways in pollen are common to most non-green tissues. Pollen contains sugars, starch, proteins, amino acids, vitamins and enzymes common to most cells. Decreased pollen viability after dehiscence seems related to metabolism of endogenous substrates in the pollen. Further, the enzymes themselves change in dead pollen compared to live pollen.

The final solution to the problems of testing viability and storing pollen lie in as complete a knowledge of the physiology and chemistry of the pollen grain as possible. Some important problems that still need satisfactory answers are the following:

1) How are respiratory parameters chemically correlated with pollen viability?

2) Why do some pollen grains grow at 0.05 mm/h and others at 6.50 mm/h or above?

3) What role do pigments, sterols, nucleotides and isoenzymes play in pollen physiology?

If the problem regarding storage of economically important plants such as the Douglas-fir tree is to be solved, this is at least one direction that research could and should take.

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