VITALITY AND METABOLIC PROPERTIES OF BINUCLEATE AND TRINUCLEATE POLLEN SPECIES UPON DEHISCENCE

CENTRALE LANDBOUWCATALOGUS
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Vitality and Metabolic Properties of Binucleate and Trinucleate Pollen Species upon Dehiscence

Proefschrift
ter verkrijging van de graad van
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STELLINGEN

I

In de evolutie van de Angiospermen treedt polyfyletisch de trend op naar snelle ontwikkeling van de stuifmeelbuis.

Dit proefschrift

II

Een gevorderde metabolische ontwikkeling van de manlijke gametofyt is een voorwaarde voor de voltrekking van de tweede mitose.

Dit proefschrift

III

De geringe vitaliteit van rijp, metabolisch actief stuifmeel behoeft het voortbestaan van de soort niet in gevaar te brengen.

Dit proefschrift

IV

De betrekkelijke ongevoeligheid van snelkiemende pollensoorten voor de aanwezigheid van cycloheximide in het kiemmedium berust op het reeds gevormd zijn van de voor kieming benodigde enzymen tijdens de ontwikkeling in de anthere.

Dit proefschrift

V

De door Ching c.s. aangeprezen snelle schatting van de pollenvitaliteit via bepaling van het ATP-gehalte, heeft slechts een beperkte toepasbaarheid.

Ching, T.M., Ranzoni, M.W., Ching, K.K. (1975): Plant Sci. Lett. *4*, 331-333;
Dit proefschrift

VI

Het drie-fasen patroon in de zuurstofopname van een kiemend pollenmonster vindt zijn oorsprong in de aanwezigheid van een niet homogeen over het monster verdeelde, kiemremmende factor.

Dickinson, D.B. (1965): Science *150*, 1818-1819;
Southworth, D. (1975): Nature *258*, 600-602

VII

De bewaring van pollen voor veredelingsdoeleinden in apolaire, organische oplosmiddelen kan alleen met succes worden toegepast indien eventuele lipofiele, exine-gebonden componenten geen essentiële rol spelen bij de aanhechting aan, en/of herkenning door de stempel.

Iwanami, Y. (1973): Plant Physiol. *52*, 508-509

VIII

Elektronenmicroscopische studies van in essentie droge plantenorganen die gefixeerd werden in een waterig medium, geven een onjuist beeld van de ultrastructuur *in situ*.

Thomson, W.W. (1979): New Phytol. *82*, 207-212

IX

Het is onjuist de participatie van de cyanide-ongevoelige, alternatieve oxidase alléén af te leiden uit de ademhalingsremming door hydroxamaten.

Lambers, H., Smakman, G. (1978): Physiol. Plant. *42*, 163-166;

Theologis, A., Laties, G.G. (1978): Plant Physiol. *62*, 243-248;

Parrish, D.J., Leopold, A.C. (1978): Plant Physiol. *62*, 470-472

X

De ADP/O-verhouding van mitochondriën die geïsoleerd zijn uit plantaardig materiaal, berekend volgens Chance en Williams, geeft geen uitsluitsel over hun kwaliteit, noch over de efficiency van de ademhaling *in vivo*.

Chance, B., Williams, G.R. (1956): Adv. Enzym. *17*, 65-134;

Rychter, A., Janes, H.W., Frenkel, Ch. (1979): Plant Physiol. *63*, 149-151

XI

Zolang hun bestaan niet is weerlegd, kunnen kabouters een functie vervullen bij de bestuiving van hogere planten.

Poortvliet, R., Huygen, W. (1976): Leven en werken van de kabouter, ISBN 90 269 4958 8

XII

De artikelen betreffende preventie en genezing van kanker, zoals die door sommige weekbladen in toenemende mate worden gepubliceerd, vormen een gezwel in ons geestelijk welzijn.

XIII

De plezierjacht is in ethisch opzicht de jacht op het plezier van de argeloze natuurliefhebber.

F.A. Hoekstra,

Vitality and metabolic properties of binucleate and trinucleate pollen species upon dehiscence.

26 oktober 1979.

Aan diegenen die lijden aan hooikoorts

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Frans L. Andela is er in geslaagd stuifmeel een eigen gezicht te geven, zoals de omslag duidelijk demonstreert.

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Algemene Inleiding

Studies betreffende vitaliteit en houdbaarheid van stuifmeel hebben een praktische kant enerzijds, waar het de plantenveredeling aangaat, anderzijds kunnen ze het inzicht verruimen omtrent bestuivingsfysiologische processen in samenhang met oecologische factoren.

Vitaliteit van pollen kon eerst worden geschat nadat pioniers op het gebied van de pollenfysiologie, waaronder van Tieghem (1869), Mangin (1886), Molisch (1893) en Lidfors (1896), er in waren geslaagd op kunstmatige wijze kieming en pollenbuisgroei tot stand te brengen. In later onderzoek werden de kunstmatige media verder geoptimaliseerd door toevoeging van suiker (zie de reviews van Visser, 1955, en Johri en Vasil, 1961), calcium (Brewbaker en Kwack, 1963) en borium, dat van nature in stempelsekreet bleek voor te komen (Schmucker, 1932).

De drijfveer van het zoeken naar omstandigheden en methoden die kunnen leiden tot verlenging van houdbaarheid van stuifmeel was van praktische aard. Immers, behoud van vitaliteit zou verzending mogelijk maken van pollen ten behoeve van hybridisering. Bovendien zouden kruisingen kunnen worden gemaakt tussen planten die op uiteenlopende tijdstippen in bloei komen. De relatieve luchtvochtigheid (RV) als factor van belang voor behoud van vitaliteit, werd grondig onderzocht door Pfundt (1910) bij een groot aantal pollensoorten. De keuze van deze factor lag voor de hand, omdat pollen van nature aan de invloed van de sterk variabele luchtvochtigheid is overgeleverd, zodra het de beschermende anthere heeft verlaten. Pfundt kwam tot de conclusie dat in veel gevallen blootstelling aan de lage RV van 30% een betere overleving van pollen mogelijk maakt dan blootstelling aan de hoge RV van 90%. Sommige pollensoorten trokken de aandacht vanwege hun extreem lange of korte houdbaarheid. Echter, Pfundt's pogingen om hierin ordening aan te brengen naar taxonomische verwantschap, hadden geen succes. Holman en Brubaker (1926) presenteerden een aanzienlijke aanvulling op Pfundt's lijst, met daarbij aangegeven de beste overlevings-RV. Ze kwamen met de classificatie niet veel verder, behalve in het geval van Gramineae pollen dat opviel door zijn lage bestendigheid tegen reeds geringe uitdroging, hetgeen later door Goss (1968) werd bevestigd. Afgezien van deze irreversibele daling in vitaliteit bij Gramineae, werd bij ander pollen soms een lage kiembaarheid waargenomen als gevolg van de zeer sterke uitdroging

boven geconcentreerd zwavelzuur. Echter, in het licht van meer recent onderzoek (Lichte, 1957; Jensen, 1970; Gilissen, 1977 en 1978) kan de oorzaak van dit schijnbare vitaliteitsverlies worden toegeschreven aan onvoldoende rehydratatie in vochtige lucht, voorafgaand aan kieming *in vitro*.

Bij angiospermen komt het pollen vrij in een twee- of driecellig stadium. Het tweecellige, ook wel binucleaat type genoemd, bezit één vegetatieve en één generatieve cel, welke laatste tijdens pollenbuisgroei nogmaals een mitose ondergaat. Het driecellige type (trinucleaat type) heeft één vegetatieve en twee generatieve cellen. De tweede mitose vindt reeds plaats tijdens de ontwikkeling van de pollenkorrel in de anthere. Zeventig percent van de plantenfamilies bezit stuifmeel dat vrijkomt in het binucleate stadium. Van de overige plantenfamilies, waaronder de landbouwkundig belangrijke Gramineae, Chenopodiaceae, Compositae en Cruciferae, leveren enkele of alle genera trinucleaat stuifmeel. Voornoemde verdeling in binucleate en trinucleate families werd opgesteld aan de hand van cytologisch onderzoek aan ongeveer 2000 soorten behorende tot 265 families (Brewbaker, 1967). Brewbaker (1957) en Pandey (1960) claimen voorts, dat trinucleate typen uitsluitend worden aangetroffen in fylogenetisch recente plantenfamilies.

Het is de grote verdienste van Brewbaker (1957, 1959) dat hij wees op het verband tussen enige pollenfysiologische eigenschappen, waaronder houdbaarheid, en het aantal cellen. Binucleaat pollen blijft gedurende een lange periode levensvatbaar, levert een hoog percentage kieming *in vitro*, en de taxa worden gekarakteriseerd door het gametofytische type van incompatibiliteit. Trinucleaat pollen daarentegen heeft slechts een beperkte vitaliteit, kiemt moeilijk *in vitro*, en de taxa worden over het algemeen gekarakteriseerd door het sporofytische type van incompatibiliteit. Men nam aan dat de beperkte vitaliteit van trinucleaat pollen het gevolg was van gebrek aan metabolieten, veroorzaakt door de tweede mitotische deling. Vergelijkende bepalingen van gehalten aan suikers en andere metabolieten (Stanley en Linskens, 1974), ondersteunen deze opinie echter niet.

Ondanks deze belangrijke correlaties is er nauwelijks vergelijkend onderzoek gedaan aan de fysiologie van twee- en driecellig pollen, terwijl juist veel informatie beschikbaar is met betrekking tot de kieming van tweecellig pollen. In het onderhavige proefschrift wordt uitgebreid aandacht besteed aan de fysiologische en metabolische eigenschappen die leiden tot het unieke karakter van trinucleate pollensoorten. In de discussies van de aparte hoofdstukken worden deze eigenschappen besproken in relatie tot het evolutionair geavanceer-

de karakter van trinucleaat pollen.

Voor het goede verloop van dit onderzoek moest eerst een aantal trinucleate pollensoorten met succes kunnen worden gekiemd *in vitro*. De weinige voorbeelden daarvan uit de literatuur geven aan dat de wijze van waterdosering een precaire kwestie is. Kubo (1955 en 1956) loste dit probleem op door pollen van Compositae en *Triticum* te laten kiemen op zeer dunne laagjes suikerhoudend medium, dat een zeer hoog gehalte aan gelatine bevatte (60%). Goede kieming van Cruciferae pollen geschiedt in media waaraan het osmotisch actieve polyethyleenglycol is toegevoegd (Ferrari en Wallace, 1975). Recente studies geven aan dat de cruciale factor voor het mislukken van kieming bij trinucleate typen de te hoge snelheid is waarmee water wordt opgenomen (Bar-Shalom en Mattsson, 1977). Opmerkelijk zijn in dit verband de resultaten van het vergelijkend onderzoek naar het voorkomen van droge of natte stempels (Heslop-Harrison en Shivanna, 1977). Planten met trinucleaat stuifmeel zijn voornamelijk in het bezit van droge stempels, zonder overvloedig exudaat.

In Hoofdstuk I (Hoekstra en Bruinsma, 1975 a) worden media geformuleerd voor optimale pollenkieming *in vitro* van vertegenwoordigers van een aantal genera der trinucleate Compositae. Bijzondere aandacht is besteed aan de calcium- en boriumbehoefte. Het effect van voorafgaande equilibratie in vochtige lucht op het percentage gerealiseerde kiembuizen is bestudeerd, en het weglaten van deze behandeling wordt bediscussieerd in relatie tot eerdere literatuurgegevens.

De relatieve luchtvochtigheid en temperatuur tijdens vrijkomen van stuifmeel zijn onderzocht op hun betekenis voor de dagelijkse variatie in kiemkracht. Omstandigheden worden aangegeven voor de meest gunstige bewaring van dit trinucleate Compositae stuifmeel.

In Hoofdstuk II (Hoekstra en Bruinsma, 1975 b) wordt een vergelijkend onderzoek beschreven naar de ademhalingsactiviteit in vochtige lucht (RV=97%) en de daarmee gepaard gaande vitaliteitsdaling van trinucleate pollensoorten ten opzichte van enkele binucleate typen. Deze studies zijn gedaan omdat de indruk was verkregen dat speciaal trinucleaat stuifmeel in een toestand van hoge metabolische activiteit verkeert (zie Hoofdstuk I). De gaswisseling is gemeten met behulp van gaschromatografie. De ademhaling van heterogene pollenmonsters is gecorrigeerd voor slecht functionerende individuen via een speciale kleurmethode. Voorts is de ademhaling van verschillende pollensoorten bij lagere RV bestudeerd in relatie tot hun overleving.

Ook de specifieke gevoeligheid van trinucleaat Gramineae stuifmeel voor uitdroging is door middel van ademhalingsmetingen nader geanalyseerd.

Hoofdstuk III (Hoekstra, 1979) behandelt een uitvoerig onderzoek betreffende de activiteit en de mate van ontwikkeling van mitochondriën in ongekiemd pollen, en veranderingen daarin tijdens kieming *in vitro*. Vergeleken worden 3 binucleate pollensoorten en 1 typisch trinucleate soort. Deze soorten zijn gekozen op grond van hun zeer uiteenlopende ademhalingsactiviteiten in vochtige lucht. Dit onderzoek is opgezet omdat verschillen in ontwikkeling van mitochondriën mogelijkerwijze de basis zouden kunnen vormen voor de waargenomen verschillen in ademhalingsactiviteit tussen bi- en trinucleaat pollen. Methoden zijn uitgewerkt ter isolering van actieve mitochondriën uit diverse typen pollen. Het vermogen tot oxydatieve fosforylering *in vitro* is geregistreerd met behulp van polarografische zuurstofmeting.

Mitochondriën uit de diverse typen pollen worden gekarakteriseerd op grond van de snelheid waarmee de maximale capaciteit van elektronentransport wordt bereikt bij kieming *in vitro*. Deze snelheid wordt gecorreleerd met die waarmee de kiembuizen verschijnen. Voornoemde gegevens uit isoleringsexperimenten worden voorts ondersteund door de bepaling van de ademhalingssnelheid van intacte korrels en door directe extractie en meting van adenylaatfosfaten. Met behulp van het laatste wordt de 'energy charge' (EC) berekend, die een aanwijzing geeft voor het al dan niet kunnen voldoen door het ademhalingssysteem aan de vraag naar ATP door anabolische stofwisselingswegen. Mitochondriële ontwikkelingsverschillen tussen bi- en trinucleaat pollen en tussen verschillende binucleate typen worden bediscussieerd.

In het geval van een relatief lage mitochondriële ontwikkelingsgraad is ook getoetst of *de novo* synthese van eiwit een onmisbaar element is voor opbouw van het elektronentransportsysteem en of veranderingen optreden in de concentraties van de verschillende cytochromen. Mede naar aanleiding van deze gegevens worden parallellen besproken met de ontwikkeling van mitochondriën in kiemende zaden.

In Hoofdstuk IV (Hoekstra en Bruinsma, 1979 a) is getracht de efficiency aan te geven waarmee ademhaling in vochtige lucht, voorafgaande aan kieming *in vitro*, plaats vindt. Eerder onderzoek (Hoofdstuk II) wees uit dat onder vochtige condities de houdbaarheid van bi- en trinucleaat pollen veel meer uiteenloopt dan de ademhalingssnelheid. Een onderzoek is ingesteld naar een mogelijk lage efficiency van de fosforylatie in de cytochroomroute en

naar de betrokkenheid van de minder efficiënte, alternatieve, CN^--ongevoelige route bij incubatie in vochtige lucht. De participatie van de verschillende routes van elektronentransport wordt vastgesteld na infiltratie van het pollen met specifieke metabolische remmers. De aanpak via infiltratie was noodzakelijk omdat in de meeste gevallen directe metingen aan mitochondriën, geïsoleerd uit ongekiemd stuifmeel, wegens isoleringsproblemen niet juist verliepen (zie Hoofdstuk III). Specifieke effecten van ontkoppelaars worden bediscussieerd, en waar mogelijk wordt de ATP-turnover geschat. Speciale aandacht is gericht op het van nature voorkomen van een geremd ademhalingssyteem.

In Hoofdstuk V (Hoekstra en Bruinsma, 1979 b) wordt bestudeerd in hoeverre een geavanceerd mitochondrieel systeem in pollen samengaat met een actief eiwitsynthetiserend apparaat. Daartoe wordt tritium-gelabeld leucine geïnfiltreerd in pollen,en de snelheden van incorporatie zijn bepaald tijdens ademhaling in vochtige lucht en kieming *in vitro*, al dan niet in aanwezigheid van de remstof cycloheximide. Ter ondersteuning van de incorporatiegegevens is de aanwezigheid van polyribosomen nagegaan met behulp van gradiëntcentrifugering. De waarnemingen worden geëvalueerd in relatie tot de lengte van de te bestuiven stijlen en bediscussieerd in het licht van de evolutionaire trend tot versnelling van de bestuivingsfase.

Dit proefschrift wordt besloten met een samenvatting van de resultaten waarna een slotbeschouwing volgt betreffende de evolutie van pollen in de richting van een beperking van het aantal zelfstandig door de manlijke gametofyt uit te voeren processen, de essentie van het trinucleate aspect daarin, en de implicaties voor bloei en bloem.

LITERATUUR

Bar-Shalom, D., Mattsson, O.: Mode of hydration, an important factor in the germination of trinucleate pollen grains. Bot. Tidsskrift *71*, 245-251 (1977)
Brewbaker, J.L.: Pollen cytology and self-incompatibility systems in plants. J. Hered. *48*, 271-277 (1957)
Brewbaker, J.L.: Biology of the angiosperm pollen grain. Ind. J. Gen. Pl. Breed. *19*, 121-133 (1959)
Brewbaker, J.L.: The distribution and significance of binucleate and trinucleate pollen grains in the angiosperms. Amer. J. Bot. *54*, 1069-1083 (1967)
Brewbaker, J.L., Kwack, B.H.: The essential role of calcium ion in pollen germination and tube growth. Amer. J. Bot. *50*, 859-865 (1963)

Ferrari, Th.E., Wallace, D.H.: Germination of *Brassica* pollen and expression of incompatibility in vitro. Euphytica *24*, 757-765 (1975)
Gilissen, L.J.W.: The influence of relative humidity on the swelling of pollen grains in vitro. Planta *137*, 299-301 (1977)
Gilissen, L.J.W.: Post-X-irradiation effects on *Petunia* pollen germinating in vitro and in vivo. Env. Exp. Bot. *18*, 81-86 (1978)
Goss, J.A.: Development, physiology, and biochemistry of corn and wheat pollen. Bot. Rev. *34*, 333-358 (1968)
Heslop-Harrison, Y., Shivanna, K.R.: The receptive surface of the angiosperm stigma. Ann. Bot. *41*, 1233-1258 (1977)
Hoekstra, F.A.: Mitochondrial development and activity of binucleate and trinucleate pollen during germination in vitro. Planta *145*, 25-36 (1979)
Hoekstra, F.A., Bruinsma, J.: Viability of Compositae pollen: Germination in vitro and influences of climatic conditions during dehiscence. Z. Pflanzenphysiol. *76*, 36-43 (1975 a)
Hoekstra, F.A., Bruinsma, J.: Respiration and vitality of binucleate and trinucleate pollen. Physiol. Plant. *34*, 221-225 (1975 b)
Hoekstra, F.A., Bruinsma, J.: Control of respiration of binucleate and trinucleate pollen under humid conditions. Offered for publication (1979 a)
Hoekstra, F.A., Bruinsma, J.: Protein synthesis of binucleate and trinucleate pollen and its relationship to tube emergence and growth. Planta in press (1979 b)
Holman, R.M., Brubaker, F.: On the longevity of pollen. Univ. Calif. Publ. Bot. *13*, 179-204 (1926)
Jensen, C.J.: Some factors influencing survival of pollen on storage procedures. FAO/IUFRO section 22, Working group meeting on the sexual reproduction of forest trees, Finland 1970
Johri, B.M., Vasil, I.K.: Physiology of pollen. Bot. Rev. *27*, 325-381 (1961)
Kubo, A.: Successful artificial method of germination of Compositae pollen. J. Sci. Hiroshima Univ. ser. B *7*, 23-43 (1955)
Kubo, A.: On the artificial pollen grain germination of Gramineae, I. *Triticum vulgare* Vill.. J. Sci. Hiroshima Univ., ser. B *7*, 103-118 (1956)
Lichte, H.F.: Über die Physiologie von Angiospermenpollen und ihre Bedeutung für die Pflanzenzüchtung. Angew. Bot. *31*, 1-28 (1957)
Lidfors, B.: Zur Biologie des Pollens. Jb. Wiss. Bot. *29*, 1-38 (1896)
Mangin, L.: Recherches sur le pollen. Soc. Bot. France Bull. *33*, 512-517 (1886)
Molisch, H.: Zur Physiologie des Pollens, mit besonderer Rücksicht auf die chemotropische Bewegungen der Pollenschläuche. Sitz. Ber. Ak. Wiss. Wien, Math. Naturw. Kl. 1, *102*, 423-449 (1893)
Pandey, K.K.: Evolution of gametophytic and sporophytic systems of self-incompatibility. Evolution *14*, 98-115 (1960)
Pfundt, M.: Der Einfluss der Luftfeuchtigkeit auf die Lebensdauer des Blütenstaubes. Jahrb. Wiss. Bot. *47*, 1-40 (1910)
Schmucker, Th.: Bor als physiologisch entscheidendes Element. Naturwiss. *20*, 839 (1932)
Stanley, R.G., Linskens, H.F.: Pollen. Berlin-Heidelberg-New York: Springer Verlag 1974
Van Tieghem, P.: Végétation libre du pollen et de l'ovule et sur la fécondation directe des plantes. Annls. Sci. Nat. Bot. *12*, 312-328 (1869)
Visser, T.: Germination and storage of pollen. Mededel. Landbouwhogeschool Wageningen *55*, 1-68 (1955)

Z. Pflanzenphysiol. Bd. 76. S. 36–43. 1975. Received January 22, 1975

Hoofdstuk I

Viability of *Compositae* Pollen: Germination *in vitro* and Influences of Climatic Conditions during Dehiscence

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Summary

Effects of various components upon germination *in vitro* were studied in order to develop an optimal germination medium for *Compositae* pollen. Equilibration of pollen in humid air, preceding germination, improved the reliability of results considerably.

Irregular germination ability of pollen samples, originating from different collections, was studied by exposing flowering plants to different climatic conditions. High relative humidity and temperature at dehiscence cause a rapid decrease in pollen vitality. Data for an optimal germination medium and for acquisition of good pollen quality are presented.

Introduction

Media for the *in vitro* germination of pollen have been developed for a large number of plant species (Johri and Vasil, 1961; Visser, 1955). Sugars are used as osmotic agent and a source of energy (O'Kelley, 1955; Stanley and Linskens, 1964). For optimum germination and tube growth sucrose concentrations from 5 to 40 % are preferred by different pollen species (Visser, 1955). Agar or gelatin are added to solidify the medium.

The importance of boron during germination and tube growth was discovered by Schmucker (1932). Up to 100 μg/ml H_3BO_3 greatly enhances germination and tube growth *in vitro* (Visser, 1955). Lack of Ca causes an increase in membrane permeability, resulting in leakage of metabolites (Brewbaker and Kwack, 1963; Dickinson, 1967). A much neglected aspect of pollen germination *in vitro* is reconditioning in humid air (Visser, 1955; Lichte, 1957). Upon storage at low temperatures, low relative humidities, or freeze drying, high germination percentages could not be obtained without equilibration under humid conditions (Jensen, 1970). Ignorance of the importance of reconditioning may be the cause of repeatedly observed irregular germination percentages (Holman and Brubaker, 1926; Nebel and Ruttle, 1936).

Germination *in vitro* of *Compositae* pollen is difficult, requiring high sugar percentages (30–40 %) and special water-restricting conditions. Other trinucleate pollen

species behave similarly, their longevity being very restricted (BREWBAKER, 1959). When *Compositae* pollen was successfully germinated, short pollen tubes have frequently been noticed (PFUNDT, 1910; PODDUBNAJA-ARNOLDI, 1936; MATSUBARA and TSUKAMOTO, 1968). In the present study the optimum conditions for the *in vitro* germination of *Compositae* pollen are established and the effects of climatic conditions during dehiscence on the pollen viability studied.

Material and Methods

Different species of *Compositae* were grown in soil in plastic pots in growth chambers, at 30,000 lux fluorescent light (Philips TL 33 R) at flower height during 14 hrs per day at 24 °C, night temperature 17 °C, relative humidity (RH), day 65 % and night 90 %.

In order to eliminate the influence of differences in desiccation, pollen was equilibrated on a slide in an atmosphere saturated with water vapour at 30 °C prior to germination *in vitro*. In each germination test, 0.5 mg pollen was incubated on a slide in 150 μl medium at 30 °C, allowing for optimum oxygen supply. Germinated grains were stained with an aqueous, decolorized aniline blue solution and the germination percentage was determined under an ultraviolet microscope. For the observation of tube growth *in vivo*, styles were macerated in 1 N NaOH prior to staining in aniline blue and squashing (LINSKENS and ESSER, 1957).

Chrysanthemum cinerariaefolium VIS. is chosen as an average representative of the other species.

Results

Although *Chrysanthemum cinerariaefolium* pollen could be successfully germinated *in vitro* on solid media containing agar and/or gelatin, the composition of a suitable liquid medium was searched for. Germination percentages obtained with solid media turned out to be irreproducable, perhaps because of different water-restricting conditions due to varying thickness of the layers.

Effect of sucrose

As can be seen in figure 1, good germination of *C. cinerariaefolium* pollen can be obtained at about 1 M sucrose. With small adaptations in sucrose concentration, this medium was suitable for pollen germination of 34 species belonging to the genera *Cichorium, Cosmos, Dahlia, Helianthus, Zinnia, Aster, Inula, Helichrysum, Senecio, Tussilago, Helenium, Tagetes, Gazania, Calendula, Chrysanthemum*, and *Tanacetum*.

Effect of boric acid

In contrast with the solid medium, addition of boric acid was essential in the liquid medium, omission resulting in rupture of the intine. Figure 2 shows that 10–100 μg/ml H_3BO_3 largely prevents bursting and allows for germination. Interaction of boric acid, sucrose and temperature could be established, showing a diminished need of boric acid at lower temperature and higher sucrose concentrations.

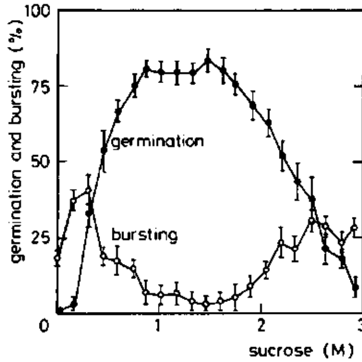

Fig. 1: Germination *in vitro* of *Chrysanthemum* pollen at different sucrose concentrations. The liquid medium also contained 100 μg/ml H_3BO_3 and 2 mM $Ca(OH)_2$ adjusted with concentrated H_3PO_4 at pH = 6.8.

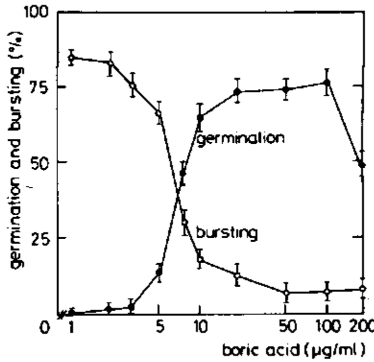

Fig. 2: Effect of boric acid concentration on bursting and germination of *Chrysanthemum* pollen. The liquid medium also contained 1.32 M sucrose and 2 mM $Ca(OH)_2$ adjusted with concentrated H_3PO_4 at pH = 6.8.

Effect of calcium

Like boric acid, Ca is indispensable in the liquid medium, 2 mM $Ca(OH)_2$ adjusted with concentrated H_3PO_4 at pH = 6.8 turned out to be satisfactory. The pH had to be kept within 6.2 and 7.5.

Effect of temperature

In figure 3, the effect of temperature on the germination percentage *in vitro* is demonstrated, 25–32 °C being optimum.

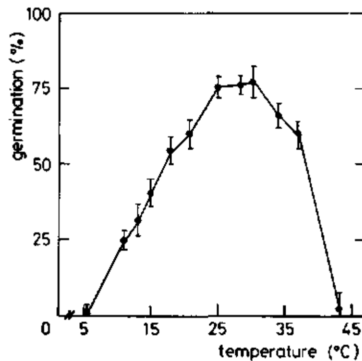

Fig. 3: Effect of temperature upon germination of *Chrysanthemum* pollen. The liquid medium contained 1.32 M sucrose, 2 mM $Ca(OH)_2$ and 100 μg/ml H_3BO_3 pH = 6.8.

Effect of reconditioning

Omitting equilibration in humid air prior to germination *in vitro* frequently caused low germination percentages. Particularly dried and stored pollen lost its germination ability *in vitro* due to considerable leakage of U.V.-absorbing material out of the grains. Equilibration in humid air at 30 °C for at least 15 min. prevented excessive leakage and restored germination ability *in vitro* to a great extent.

Germination *in vitro* and *in vivo* showed identical results, also with different pollen qualities. Apparently, the requirements for optimum germination are realized at 30 °C in a liquid medium containing 0.9–1.4 M sucrose, 100 μg/ml H_3BO_3 and 2 mM $Ca(OH)_2$, adjusted with concentrated H_3PO_4 at pH = 6.8. Tube growth *in vitro*, however, ceased within ten minutes after emergence, resulting in short tubes of 70–100 μ. The protrusions formed were real pollen tubes, staining with aniline blue revealed callose under the ultraviolet microscope, in contrast with the so called instant pollen tubes, which can be obtained by a shock treatment with sulfuric acid to a final concentration of 4 % (LINSKENS and MULLENEERS, 1967).

Germination *in vitro* of fresh pollen samples from plants in the field revealed unexpected fluctuations in daily viability, indicating strong short-term influences of climatic conditions. Effects of relative humidity and temperature during dehiscence were analysed as possible causes of this daily variation.

Effect of RH at dehiscence

Figure 4 shows the influence of the RH at 24 °C upon the viability of fresh pollen during anthesis. The wind velocity had to be at least 0.3 m/sec. in order to maintain the RH, insufficient ventilation allowing for a layer of high humidity to be built up by anther transpiration. High RH during anthesis considerably decreased the vitality of *Chrysanthemum cinerariaefolium* pollen. Pollen of the earlier

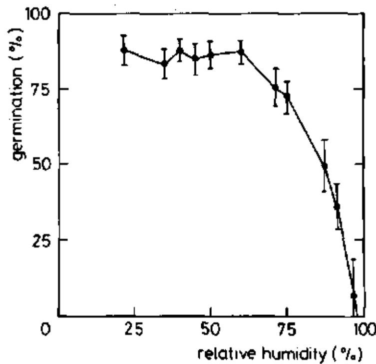

Fig. 4: Effect of relative humidity during dehiscence on germination under optimum conditions *in vitro* of *Chrysanthemum* pollen collected three hrs. after dehiscence at 24 °C.

mentioned genera of *Compositae* showed similar adverse effects. The best pollen quality was obtained at relatively low temperatures (17 °C), high temperatures (35 °C) being detrimental.

Effect of RH during storage

Storage of fresh *Chrysanthemum* pollen turned out to be unfavourable at water-saturated atmospheres, the vitality being limited to hours, particularly at higher temperatures (Figure 5). Longevity could be extended to several days by exposing pollen to lower relative humidities, e.g. 60 % (Figure 6). At high temperatures, e.g. 30°, the beneficial effect of decreased RH was only small. Preservation of vitality for over one year was achieved by drying over NaOH pellets at 5 °C for 20 hours, prior to storage at – 20 °C.

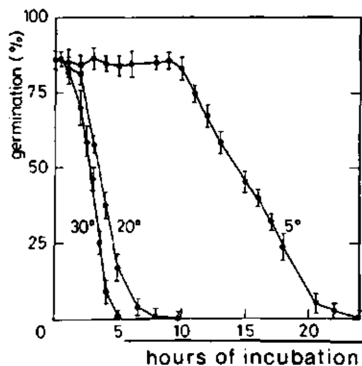

Fig. 5: Vitality decrease of *Chrysanthemum* pollen in the course of incubation in humid air at different temperatures.

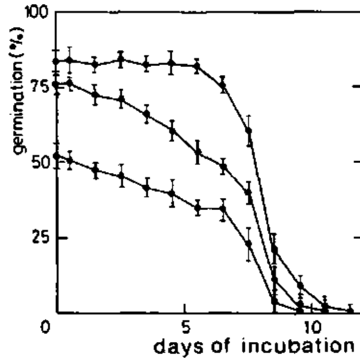

Fig. 6: Vitality decrease at 20 °C and RH = 60 % of three samples of *Chrysanthemum* pollen of different qualitaties.

Discussion

The difficulties with the *in vitro* germination of *Compositae* pollen turn out to be caused by several factors. Relative humidities of 50 % and lower, occasionally occurring in the field, rapidly desiccate pollen. This considerably reduces germination *in vitro* because of leakage of protein and carbohydrate (CHING and CHING, 1964; JENSEN, 1970; DAVIES and DICKINSON, 1971). Membrane integrity can be restored by equilibration of pollen in humid air. Germination media, containing agar or gelatin, may obviate this leakage to some degree by hampering the diffusion of protein . In liquid media, however, omission of equilibration of pollen in humid air, prior to germination, undoubtedly is one of the main causes of irregular germination behaviour of *Compositae* pollen. None of the authors dealing with pollen germination of *Compositae* (PFUNDT, 1910; PODDUBNAJA-ARNOLDI, 1936; KUBO, 1954 and 1955; MATSUBARA and TSUKAMOTO, 1968) applied this method of preliminary equilibration in humid air.

Concerning the germination medium, several components affected germination. Early investigators of pollen physiology avoided the need for calcium and boron by applying media with agar and gelatin, thus reducing the rate of water uptake. Repeated germination of the same sample, however, gave fluctuating results due to the varying consistency in the surface of the medium. Therefore a liquid medium was preferred. Ca and boric acid are then essential, omission causing protein leakage and bursting of grains, respectively.

The results obtained with the medium described in this paper quantitatively agree with those from *in vivo* experiments. Although tube growth ceases readily *in vitro* for unknown reasons, the percentage germination can be looked upon as a measure of germination ability.

This germination method enabled for a frequent observation of the daily variation in vitality of fresh pollen and thus revealed another cause of the irregular germina-

tion of *Compositae* pollen. The apparent fluctuating vitality of fresh pollen originates from the conditions prevailing during dehiscence. High relative humidity and high temperature at anthesis have a detrimental effect on pollen viability, causing the pollen to loose its ability to germinate within a few hours.

This rapid decrease in vitality suggests a rapid activation of the metabolic system. It is interesting, therefore, to compare the metabolic activity of these short-living trinucleate *Compositae* pollen with that of longer living pollen of other plant families (HOEKSTRA and BRUINSMA, 1975).

Also under storage conditions high relative humidities and temperatures are detrimental. However, *Compositae* pollen sampled and tested under the optimal circumstances, described in this paper, can be successfully stored under dry and cold conditions.

References

BREWBAKER, J. L.: Biology of the angiosperm pollen grain. Ind. J. Gen. Plant Brd. *19*, 121–133 (1959).

BREWBAKER, J. L., and B. H. KWACK: The essential role of calcium ion in pollen germination and pollen tube growth. Amer. J. Bot. *50*, 859–865 (1963).

CHING, T. M., and K. K. CHING: Freeze-drying pine pollen. Plant Physiol. *39*, 705–709 (1964).

DAVIES, M. D., and D. B. DICKINSON: Effects of freeze drying on permeability and respiration of germinating lily pollen. Physiol. Plant. *24*, 5–9 (1971).

DICKINSON, D. B.: Permeability and respiratory properties of germinating pollen. Physiol. Plant. *20*, 118–127 (1967).

HOEKSTRA, F. A., and J. BRUINSMA: Respiration and vitality of binucleate and trinucleate pollen. Physiol. Plant. (in press) (1975).

HOLMAN, R. M., and F. BRUBAKER: On the longevity of pollen. Univ. Calif. Publ. Bot. *13*, 179–204 (1926).

JENSEN, C. J.: Some factors influencing survival of pollen on storage procedures. FAO/IUFRO section 22, Working group meeting on the sexual reproduction of forest trees, Finland, 1970.

JOHRI, B. M., and I. K. VASIL: Physiology of pollen. Bot. Rev. *27*, 325–381 (1961).

KUBO, A.: The unstable germination ability of pollen grains of *Cosmos bipinnatus* CAV. J. Sci. Hiroshima Univ. ser. *B 6*, 237–250 (1954).

– Successful artificial method of germination of *Compositae* pollen. J. Sci. Hiroshima Univ. ser. *B 7*, 23–43 (1955).

LICHTE, H. F.: Über die Physiologie von Angiospermenpollen und ihre Bedeutung für die Pflanzenzüchtung. Angew. Bot. *31*, 1–28 (1957).

LINSKENS, H. F., and K. L. ESSER: Über eine spezifische Anfärbung der Pollenschläuche im Griffel und die Zahl der Kallosepfropfen nach Selbstung und Fremdung. Naturwiss. *44*, 16–17 (1957).

LINSKENS, H. F., and J. M. L. MULLENEERS: Formation of «instant pollen tubes». Acta Bot. Neerl. *16*, 132–142 (1967).

MATSUBARA, S., and Y. TSUKAMOTO: Studies on germination of *Chrysanthemum* pollen. III Substance promoting germination. Plant and Cell Physiol. *9*, 565–572 (1968).

NEBEL, B. R., and M. L. RUTTLE: Storage experiments with pollen of cultivated fruit trees. J. Pomol. Hort. Sci. *14*, 347–359 (1936).

O'KELLEY, J. C.: External carbohydrates in growth and respiration of pollen tubes *in vitro*. Amer. J. Bot. *42*, 322–327 (1955).

PFUNDT, M.: Der Einfluß der Luftfeuchtigkeit auf die Lebensdauer des Blütenstaubes. Jahrb. Wiss. Bot. *47*, 1–40 (1910).

PODDUBNAJA-ARNOLDI, V.: Beobachtungen über die Keimung des Pollens einiger Pflanzen auf künstlichen Nährboden. Planta *25*, 502–529 (1936).

SCHMUCKER, TH.: Bor als physiologisch entscheidendes Element. Naturwiss. *20*, 839 (1932).

STANLEY, R. G., and H. F. LINSKENS: Enzyme activation in germinating *Petunia* pollen. Nature *203*, 542–544 (1964).

VISSER, T.: Germination and storage of pollen. Mededel. Landbouwhogeschool Wageningen *55*, 1–68 (1955).

Hoofdstuk II

Respiration and Vitality of Binucleate and Trinucleate Pollen

By

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Abstract

The respiration and vitality of ungerminated bi- and trinucleate pollen were studied in order to determine the influence of relative humidity and temperature on metabolic activity. The gas exchange, germination capacity and staining with tetrazolium bromide were followed under standardized conditions.

A constant respiration rate occurred under conditions of high relative humidity (97%). Per mg pollen, the trinucleate grains of *Compositae* and *Gramineae* respired 2 to 3 times as intense as 6 species of binucleate grains. Per unit of pollen protein the differences were even larger. In contrast to binucleate pollen, the longevity of trinucleate pollen was very short and the ability to germinate was lost twice as fast as the respiration capacity. This limits the use of tetrazolium bromide as an indicator of viability.

At reduced relative humidities respiration was strongly restricted, but the longevity of bi- and trinucleate pollen considerably increased.

Pollen of *Gramineae*, however, was very sensitive to changes in relative humidity; short exposure to low relative humidity decreased both the vitality and the capacity to respire.

Introduction

Pollen of angiosperms is bi- or trinucleate at the time of dehiscence. Binucleate grains contain one generative and one vegetative nucleus; the generative one undergoes a second mitosis during pollen tube growth. The trinucleate type, on the contrary, contains one vegetative and two generative nuclei, the second mitosis occurring already prior to anthesis.

Brewbaker (1957) investigated 199 plant families, 130 of which turned out to have binucleate pollen and 48 trinucleate pollen; in 21 families both types were found. A connection was established between several physiological properties and the number of nuclei of the pollen grain (Brewbaker 1959). Germination *in vitro* of the trinucleate type was difficult and irregular, very short pollen tubes being formed. High amounts of sugar had to be applied in the germination medium to prevent bursting of the grains, which retained their viability during a very short period only. As far as incompatibility is concerned, the site of inhibition turned out to be linked with the number of nuclei, genera with trinucleate pollen generally showing inhibition of tube growth at the stigma. Binucleate pollen, on the contrary, germinated readily and produced long tubes in media containing low sugar concentrations. The pollen could easily be stored without loss in vitality. The incompatibility is gametophytically determined with the site of inhibition in the style.

An earlier study on trinucleate pollen of *Compositae* revealed a relation between decrease of vitality and increase of temperature (Hoekstra and Bruinsma 1975). In addition, vitality was seriously affected when high relative humidities were applied at anthesis. The two factors turned out to cause the strong daily fluctuations in germination ability. The rapid loss in vitality of this trinucleate pollen suggests an easy activation of the metabolic system before the onset of germination. In order to investigate this, the respiratory behaviour of bi- and trinucleate pollen grains was analysed in connection with their capacity to germinate and their staining with tetrazolium bromide as a marker of vitality.

Materials and Methods

Pollen collection, staining, and germination

Pollen was collected from freshly opened flowers grown in climate chambers at 23°C, low relative humidity (RH), 50–60%, and an air flow of at least 0.3 m/s. The pollen was dried over NaOH pellets prior to storage at −20°C.

The percentage of viable pollen was examined by adding 1 mg/ml MTT (=3,(4,5-dimethylthiazolyl-2)2,5-diphenylmonotetrazolium bromide) to the germination medium. This vital stain reacts with dehydrogenases, the formazan

complex produced colouring the grains. After 1 h at 26°C the percentage of stained grains was microscopically determined.

Germination *in vitro* was preceded by equilibrating the pollen in humid air (RH = 100%) for 30 min at 30°C. The medium consisted of 148 μg/ml $Ca(OH)_2$, adjusted with concentrated H_3PO_4 to pH 6.8, 100 μg/ml H_3BO_3, and sucrose concentrations varying from 0.94 *M* to 1.32 *M* for the following *Compositae*: *Aster tripolium* L., *Calendula officinalis* L., *Chrysanthemum cinerariaefolium* Vis., *Chrysanthemum segetum* L., *Cosmos bipinnatus* Cav., *Helenium autumnale* L., *Helichrysum bracteatum* Willd., *Inula squarrosa* L., *Senecio jacobaea* L., *Tanacetum vulgare* L., and *Tussilago farfara* L. Pollen of *Gramineae* was not germinated *in vitro*. For 6 species with binucleate grains the following medium was used: 300 μg/ml $Ca(NO_3)_2 \cdot 4H_2O$, 20 μg/ml H_3BO_3 and 0.53 *M* sucrose for *Corylus avellana* L., 0.30 *M* for *Nicotiana alata* Lk. et Otto and *Lycopersicon peruvianum* Mill., 0.20 *M* for *Typha latifolia* L., and 0.15 *M* for *Impatiens balsamina* L. and *Narcissus poeticus* L.

Pollen was germinated by incubating 0.5 mg in 150 μl medium on a slide under humid conditions at 30°C. Trinucleate pollen germinated in about 10 min, binucleate pollen in up to 2 h. After staining with aniline blue, the grains were examined under an ultraviolet microscope (Linskens and Esser 1957).

For the control of tube growth *in vivo*, styles of cut flowers were pollinated at 30°C. After 2 h in the case of *Compositae* flowers, and the next day in the case of flowers with binucleate pollen, the styles were excised and macerated in 1 *N* NaOH prior to staining in aniline blue and squashing.

Measurement of respiration

The respiratory activity was analysed with samples of 5 mg pollen, incubated in 6.0 ml vessels type no. K749000, Kontes Glass Cy., Vineland, N.J., U.S.A., fitted with Microflex valves no. K749100 to avoid gas leakage. This amount of pollen did not inhibit its own respiration by accumulation of CO_2. The pollen sample was placed on a filter paper disk, 10 mm diameter. A high relative humidity of 97% was maintained with 0.2 ml glycerol : water = 15:85 (w/w) on a rolled 3 × 5 cm filter paper.

CO_2, O_2 and N_2 concentrations in the flasks were determined with a F&M Dual model 700-00 gas chromatograph at 105°C, using a katharometer detector at 135°C. One stainless steel column, 120 cm, ⅛ in diameter, was packed with silicagel 30–60 mesh, the other, 200 cm, ¼ in diameter, with Becker Molecular Sieve 5A 45–60 mesh. Sample size was 60 μl, flow rate 28 ml/min with helium as carrier. Retention times for CO_2, O_2 and N_2 were 60, 160 and 240 s, respectively. According to Leijten (1967), a linear relation was established between the amount of a component and the peak area.

Protein content was determined after Lowry *et al.* (1951), using samples of 15 mg pollen, extracted with 2 ml 3% NaCl in a Potter glass homogenizer at 0°C. To avoid interference from phenolic compounds, the protein from 100 μl extract was precipitated with 0.9 ml 10% trichloroacetic acid and dissolved in 0.3 ml 1 *N* NaOH.

Results

(A) *Respiration and vitality at 97% relative humidity*

Although variations in incubation temperatures were limited within 0.5°C, a reduced humidity of 97% had to be applied to avoid condensation of water on the pollen surface. At six different temperatures, respiration was followed simultaneously with germination as a measure of vitality. Figure 1 represents the CO_2 production and the decrease in vitality of the trinucleate pollen of *Aster tripolium* and of the binucleate pollen of *Corylus avellana*. The oxygen uptake usually matched the CO_2 production, the RQ values being mostly about 1.0 (Table 1). CO_2 production and O_2 uptake occurred at initially constant rates. The respiration of binucleate *Corylus* pollen was much lower than that of trinucleate *Aster* pollen at all temperatures, and its longevity much more pronounced, except at temperatures over 26°C (Figure 1).

According to its germination capacity, *Aster* pollen lost its vitality about twice as fast as its capacity to respire. The germination capacity of *Aster* pollen was also measured *in vivo*, with similar results. This rapid loss in viability of still normally respiring pollen grains was found to occur in all *Compositae* tested (Table 1). Dehydration of pollen which had just lost its vitality preserved its metabolic activity. For example, *Cosmos bipinnatus* pollen, normally respiring for 7 h at a rate of 0.48 μmol CO_2/mg × h at 30°C and RH = 97%, when desiccated after 1.5 h under these conditions over NaOH pellets at 5°C, showed upon reincubation a reduced constant respiration rate of 0.20 μmol/mg × h during 5 h, without practically any germination. Preliminary incubation for 3 h, however, completely destroyed the respiration capacity of the reincubated material.

Staining with tetrazolium bromide (MTT) gave the highest percentage of formazan-coloured grains and the most intensive colour during the linear part of the respiration curves. As might be expected, no more formazan complex was formed as soon as the CO_2 production came to an end. Because most pollen samples lost their vitality much faster than their capacity to respire (Table 1), MTT obviously is not always suitable as a vital stain for pollen. However, a pollen sample consists of grains from many flowers. Staining with MTT enables to discern the percentage of pollen in the sample responsible for the CO_2 production, a linear correlation being found between the respiration rate and the percentage of stained grains (Figure 2). In this way, the MTT-staining allowed for a

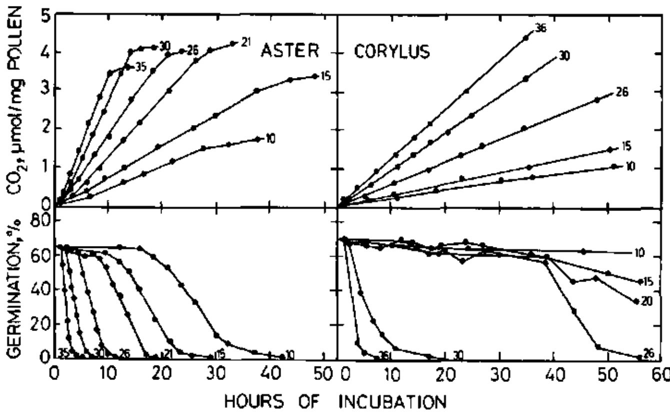

FIG. 1

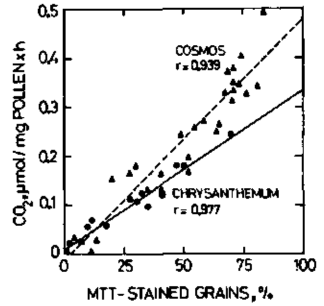

FIG. 2

Figure 1. *Respiration of trinucleate Aster and binucleate Corylus pollen at 97% relative humidity and at different temperatures, and the vitality of this pollen as determined by germination of samples at 30°C in vitro at intervals during the experiments.* Single determinations.
Figure 2. *Correlation between rate of CO_2 production and percentage of MTT-stained pollen grains.* Results of samples from different collections. Single determinations.

Table 1. *Respiration and longevity of pollen species at 30°C and relative humidity = 97%.*

Species	CO_2, µmol per h × mg pollen	CO_2, µmol per h × mg protein	RQ	Duration until cease of respiration, h	Interval until 50% of initial germination, h
Binucleate					
Corylus avellana	0.11	1.0	0.73	>40	5.0
Impatiens balsamina	0.10	—	—	>30	24.0
Narcissus poeticus	0.10	0.5	0.95	>50	35.0
Nicotiana alata	0.14	0.8	1.07	30	15.5
Lycopersicon peruvianum	0.14	1.1	0.98	40	—
Typha latifolia	0.12	—	1.00	>24	—
Trinucleate					
Aster tripolium	0.37	4.0	0.87	13.5	3.7
Calendula officinalis	0.61	—	1.00	4.5	—
Chrysanthemum segetum	0.29	—	0.95	4.5	—
C. cinerariaefolium	0.34	5.7	1.02	13.0	2.7
Cosmos bipinnatus	0.48	—	0.95	7.0	2.2
Helenium autumnale	0.35	—	1.59	5.5	—
Helichrysum bracteatum	0.31	—	1.04	5.5	1.7
Inula squarrosa	0.36	—	1.01	9.0	2.2
Senecio jacobaea	0.31	—	1.35	7.5	—
Tanacetum vulgare	0.41	—	—	4.0	—
Tussilago farfara	0.42	4.9	1.10	7.5	2.2
Triticum sp.	0.32	4.9	1.33	3.8	—

correction for the percentage of non-viable pollen grains in the samples.

No correlation could be found between the respiratory activity of different pollen samples of one species and their protein content. Storage at low temperature did not affect the protein content, while only a slight decrease could be observed during incubation in humid air. Because only the MTT-stained grains contribute to the CO_2 production, measurements of respiratory activity per unit of pollen protein were also corrected according to the percentage MTT-staining of the grains.

Figure 3 shows the effect of temperature on the respiration

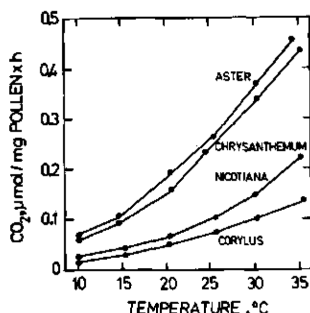

Figure 3. *Respiration rates of trinucleate Aster and Chrysanthemum pollen and binucleate Corylus and Nicotiana pollen at different temperatures at 97% relative humidity.* Triplicate determinations.

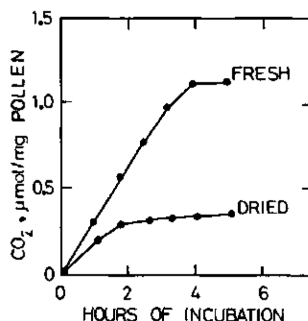

Figure 5. *Effect of predrying for 30 min on Triticum pollen respiration at 30° C and 97% relative humidity.* Single determinations.

rates of bi- and trinucleate pollen. At about 35°C, the rates of CO_2 production were less than could be expected according to the Q_{10} values. Therefore, 30°C is a more favourable temperature to compare respiration and viability of the two types of pollen. In Table 1 the rates of CO_2 production of 6 species with binucleate pollen and of 11 species with trinucleate pollen are compared. The respiration of trinucleate pollen was always higher than that of binucleate pollen, per mg pollen about three times as large on an average. Per unit of pollen protein the difference became more accentuated.

(B) *Influence of relative humidity on respiration and vitality*

Pollen respiration requires a high relative humidity. As can be seen in Figure 4, the respiratory activity considerably decreased at reduced RH, no evolution of CO_2 could be detected at RH = 77%. On the contrary, the vitality was much longer maintained at reduced humidities, particularly at the lower temperature. Apparently respiratory activity and decrease in vitality are closely linked.

Storage of *Gramineae* pollen, particularly, is known to be difficult. Freezing procedures cannot be applied because of the high water content of the fresh pollen, while preliminary drying also terminates the germination ability.

The respiratory behaviour of *Triticum* pollen was tested by incubating one part of a freshly collected sample directly at 30°C and RH = 97%, the other part being kept under laboratory conditions (20°C and RH = 50%) for 30 min prior to incubation. Figure 5 shows the respiratory behaviour of both samples, of which high percentages of MTT-stained grains were obtained. Within 2 h of incubation, however, the predried pollen no longer stained with MTT. Drying of *Gramineae* pollen, therefore, has not only a detrimental effect on vitality, but also affects the respiratory system.

Discussion

Dickinson (1965, 1966, 1967, 1968), and Davies and Dickinson (1971) described the respiratory behaviour of the binucleate *Lilium* pollen during germination *in vitro*,

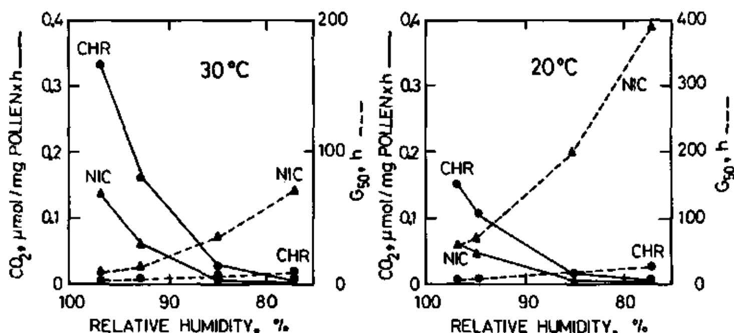

Figure 4. *Respiration rates and longevity of trinucleate Chrysanthemum* (CHR) *and binucleate Nicotiana* (NIC) *pollen at different relative humidities.* Longevity is expressed in hours until 50% of initial germination (G_{50}). Triplicate determinations.

but the respiration of "dry" pollen has not been studied until now. Knowlton's data (1922), concerning storage, suggest that it might be interesting to measure the respiratory activity of trinucleate pollen.

Accelerated respiration can be obtained by incubating pollen under conditions of high relative humidity. The water vapour will be rapidly taken up and subsequently the metabolic activity starts. Respiration, however, involves a decrease in vitality, short-living pollen having a high rate of respiration as compared with longer living pollen. Both types correspond with the tri- and binucleate nature of the grains, respectively.

The respiration of pollen in humid air did not follow the three-phase pattern of germinating lily pollen as described by Dickinson. Conversion of his data for lily pollen into μmol/mg $\times$ h, allowing a comparison, shows that the respiration rate in the germination medium corresponds with the respiration rate of *Compositae* pollen in humid air. It might have been of interest to compare these data with those from germinating *Compositae* pollen. Unfortunately, however, no reasonable measurements of the respiratory activity could be obtained, tube growth ceased within 10 min after emergence. The formed protrusions were real pollen tubes, staining with aniline blue revealed callose under the UV microscope.

As a measure of vitality, staining with tetrazolium bromide (MTT) is of limited value only because it involves the activity of respiratory enzyme. Since the capacity to germinate decreases much faster than that to respire, particularly with trinucleate pollen, staining can still be obtained with grains that lost their germination ability already.

Under natural conditions, respiratory activity and deterioration of vitality can easily occur. Pollen samples from different origins showed different germination percentages and durations of respiration, indeed. Generally, pollen with a reduced germination produced less carbon dioxide during a shorter period of time.

Data concerning the respiratory activity per unit of pollen protein have to be considered with reservation because the Lowry method fails to discriminate between enzymes and structural proteins. Measurements on isolated mitochondria might allow for safer interpretations. However, on a basis of protein content the respiratory data were even more uniform than expressed per unit of pollen weight, accentuating the metabolic difference between bi- and trinucleate pollen.

Pollen looses its vitality proportionally to temperature, which again demonstrates that respiration is related to the processes leading to the functional death of the grain. Reducing the relative humidity, however, which strongly reduces respiration, does not simply remove the decrease in vitality. In *Gramineae* pollen, particularly, reduced humidity itself is very detrimental to the vitality of the pollen.

The present study is a first attempt to analyse the relations between external conditions, metabolic activity, and longevity of bi- and trinucleate pollen. Tentatively, because of their rapid activation and decline, trinucleate pollen, in which the generative nucleus has divided already, can be looked upon as a less dormant dispersal organ than the slower developing binucleate grain.

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References

Brewbaker, J. L. 1957. Pollen cytology and selfincompatibility systems in plants. — J. Hered. 48: 271–277.

— 1959. Biology of the angiosperm pollen grain. — Indian J. Genet. Plant Breed. 19: 121–133.

Davies, M. D. & Dickinson, D. B. 1971. Effects of freeze drying on permeability and respiration of germinating lily pollen. — Physiol. Plant. 24: 5–9.

Dickinson, D. B. 1965. Germination of lily pollen: respiration and tube growth. — Science 150: 1818–1819.

— 1966. Inhibition of pollen respiration by oligomycin. — Nature 210: 1362–1363.

— 1967. Permeability and respiratory properties of germinating pollen. — Physiol. Plant. 20: 118–127.

— 1968. Rapid starch synthesis associated with increased respiration in germinating lily pollen. — Plant Physiol. 43: 1–8.

Hoekstra, F. A. & Bruinsma, J. 1975. Viability of Compositae pollen: germination in vitro and influences of climatic conditions during dehiscence. — Z. Pflanzenphysiol. 76. In press.

Knowlton, E. H. 1922. Studies in pollen with special reference to longevity. — Cornell Univ. Agric. Exp. Stn. 52: 747–794.

Leijten, J. F. 1967. Clinical gas analysis based on gas chromatography. — Thesis Univ. Nijmegen, Netherlands.

Linskens, H. F. & Esser, K. L. 1957. Über eine spezifische Anfärbung der Pollenschläuche im Griffel und die Zahl der Kallosepfropfen nach Selbstung und Fremdung. — Naturwissenschaften 44: 16–17.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. I. 1951. Protein measurement with the Folin-phenol reagent. —J. Biol. Chem. 193: 265–275.

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Hoofdstuk III

Mitochondrial Development and Activity of Binucleate and Trinucleate Pollen during Germination in Vitro

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Abstract. Bi- and trinucleate pollen generally differ in the extent of their mitochondrial development at anther dehiscence and in the rate of their attainment of maximum-phosphorylative capacity during germination in vitro, as judged from experiments with representatives of both groups.

The typically trinucleate pollen of *Aster tripolium* L. immediately respired at a high rate, maintaining a high energy charge. Mitochondria attained maximum electron-transducing capacity within 2 min of incubation, while tube growth started within 3 min. In contrast, the binucleate pollen of *Typha latifolia* L. only gradually reached a relatively low rate of respiration, concomitant with a temporary decrease in energy charge, upon immersion in the germination medium. Development of the mitochondrial, electron-transducing system occurred in about 75 min, after which the first pollen tubes emerged. Starting from a poor differentiation, mitochondria became increasingly normal in appearance as germination proceeded.

The binucleate pollen of *Nicotiana alata* Link et Otto and *Tradescantia paludosa* Anders. et Woods. showed intermediate characteristics: *Nicotiana* resembled *Typha* but mitochondria developed at a higher rate; *Tradescantia* germinated more rapidly and resembled the trinucleate pollen of *Aster*.

Inhibitors of mitochondrial or cytoplasmic protein synthesis failed to affect the development of the mitochondrial, respiratory capacities during pollen germination. It is concluded that the duration of the lag period is determined by the level and rate of mitochondrial development and not by the division of the generative cell.

Key words: Cytochromes – Energy charge – Germination (pollen) – Mitochondrial development – Pollen – Protein synthesis.

Abbreviations: BSA = bovine serum albumine; CAP = D(−) threo chloramphenicol; CHI = cycloheximide; DNP = 2-4 dinitrophenol; EBr = ethidium bromide; EC = energy charge; EGTA = ethyleneglycol-bis (2-aminoethyl ether) N,N′-tetra-acetic acid; EM = electron microscope; ETC = electron transfer chain; HEPES = N-2-hydroxyethyl piperazine N′-2-ethane sulfonic acid; LSD = least significant difference; PVP = polyvinyl pyrrolidone; RCR = respiratory control ratio; RH = relative humidity; TCA = tricarboxylic acid; TES = N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid; URCI = uncoupler respiratory control index (Hunter et al., 1976)

Introduction

Brewbaker (1957, 1959, 1967) extensively surveyed the occurrence of bi- and trinucleate pollen species among plant families. Seventy percent were found to shed their pollen in the binucleate stage and the rest in the trinucleate stage, except some families in which both types occur. Trinucleate species were encountered exclusively in phylogenetically-recent plant families (Brewbaker, 1957; Pandey, 1960).

This type, in which the generative cell has already divided prior to dehiscence, is generally characterized by a syndrome of physiological phenomena. In contrast to binucleate pollen, trinucleate are difficult to germinate in vitro. They often require water-restricting conditions (Bar-Shalom and Mattsson, 1977) or high concentrations of sucrose, and develop only short tubes. Vitality is easily lost, even in conditions routinely used for storage. In vivo, they germinate mostly on "dry" stigmas without exudate (Heslop-Harrison and Shivanna, 1977). In incompatibility reactions, trinucleate pollen is generally rejected on the stigma. The genetic basis of the incompatibility system is either sporophytic, or gametophytic based on a complex, S-gene system, as in *Gramineae* (Lundqvist, 1975). Whereas the incompatibility of the binucleate species is always gametophytically determined.

Trinucleate pollen of *Compositae* is found to loose its vitality in about 3 h upon incubation in an atmosphere of 97% RH at 30° C (Hoekstra and Bruinsma, 1975a). This rapid loss of vitality is accompanied by a respiratory activity 2 to 3 times higher than that of six species of binucleate grains (Hoekstra and Bruinsma, 1975b).

In the present investigation, in order to clarify the nature of this difference, mitochondria from bi- and trinucleate pollen were isolated and characterized, and their development during germination in vitro was followed.

Materials and Methods

Pollen Sources, Collection, Storage, Pretreatments, and Germination in Vitro

Four plant species were selected for their widely-differing rates of pollen respiration in humid conditions (Hoekstra and Bruinsma, 1975b): the binucleate species *Typha latifolia* L., *Nicotiana alata* Link et Otto, and *Tradescantia paludosa* Anderson et Woodson, and the typically trinucleate species *Aster tripolium* L.

Pollen was collected at anther dehiscence, desiccated over NaOH pellets at 4° C to 5–10% H_2O and stored at −20° C until use.

Prior to germination in vitro, pollen was washed three times with diethyl ether, dried by evaporation, and incubated in an atmosphere of 97% RH for 12 h at 4° C. This washing procedure enhanced germination and tube growth (Iwanami and Nakamura, 1972; Iwanami, 1973), and microbial contamination of the pollen was strongly reduced. The ether washings did not affect the respiratory capacities of the mitochondria from these pollen species.

Germination in vitro was performed by suspending about 500 mg of pollen in 25 ml of the liquid medium. The wide-necked Erlenmeyer flasks were vigorously shaken at 24° C. The composition of the medium was, for *Typha*: 0.2 M sucrose, salts according to Brewbaker and Kwack (1963), and 1.6 mM H_3BO_3; for *Nicotiana*: 0.3 M sucrose, 0.3 mM $Ca(NO_3)_2 \cdot 4H_2O$ and 0.5 mM H_3BO_3; for *Tradescantia*: 0.15 M sucrose, 2.1 mM $Ca(NO_3)_2 \cdot 4H_2O$, and 1.6 mM H_3BO_3; and for *Aster*: 1.0 M sucrose, 2.1 mM $Ca(NO_3)_2 \cdot 4H_2O$, and 1.6 mM H_3BO_3. Tube emergence and growth were examined by light microscopy. Only highly-viable pollen samples were used for the isolation of mitochondria.

Preparation of Mitochondria

Germinating pollen (500 mg) was collected on millipore filters and homogenized in a Teflon-glass, Potter homogenizer in 10 ml of extraction buffer. Non-germinated controls were presoaked in the cold germination medium (4° C) for 2 min prior to collection and homogenization. The extraction medium consisted of 0.3% (w/v) fatty acid-poor BSA in 10 mM TES-KOH, pH 7.2, and was supplemented with 0.3 M mannitol, 0.4 M mannitol, 0.3 M sucrose + 0.3 M mannitol, and 1.0 M mannitol + 0.2 M sucrose + 10 mM cysteine + 0.5% (w/v) PVP-insoluble, for pollen of *Typha*, *Nicotiana*, *Tradescantia* and *Aster*, respectively. Finally EGTA, previously neutralized with KOH to pH 7.2, was added to each medium to a concentration of 1 mM.

The homogenate was immediately clarified by successive filterings through a 37 μm pore-size nylon and 12 and 8 μm pore-size millipore filters. The filtrate was centrifuged at 1,500 *g* for 10 min. The supernatant was further centrifuged at 35,000 *g* for 2 min, with the exception of the *Aster* mitochondria, with which centrifugation was extended to 10 min to compensate for the higher viscosity and density of the isolation medium. The supernatant was removed by suction, and the pellet resuspended in a few drops of the grinding medium without cysteine and PVP. No further washings were performed. All steps were carried out at 0–4° C.

Respiration Measurements

Mitochondrial O_2 consumption was measured polarographically with a Clark-type O_2 electrode (Yellow Springs Instrument Comp., Yellow Springs, Ohio, USA) inserted in a 3 ml glass, reaction chamber with continuous magnetic stirring at 24° C. Aliquots of the mitochondrial fraction were mixed with the reaction medium consisting of 0.1% (w/v) BSA, 10 mM TES-KOH, pH 7.2, 5 mM KH_2PO_4, and 5 mM $MgCl_2$ as the basal medium, supplemented with 0.5 M, 0.5 M, 0.7 M, and 1.0 M mannitol in case of *Typha*, *Nicotiana*, *Tradescantia*, and *Aster*, respectively. As for the isolation, 1 mM of neutralized EGTA was finally added to each medium. Respiratory rates were calculated from a recorder trace on the basis of 241 μM O_2 in the aerated medium at 24° C. The rates were expressed as nmol O_2 taken up per min by mitochondria, which were isolated from 100 mg of dry pollen. The ADP/O ratio was calculated as the quotient of the amount of ADP added to the reaction medium and the extra O_2 uptake which resulted from this addition (Lehninger, 1970). The respiratory control ratio (RCR = ratio of respiration state 3: respiration state 4) was calculated according to Chance and Williams (1956). Respiratory substrates and inhibitors, from Sigma Chemical Comp., St Louis, Mo., USA, were injected into the reaction chamber through a slit in the Clark probe, to a final concentration of 1 mM for NADH, and 10 mM for succinate, pyruvate, malate, and α-ketoglutarate. Oligomycin, antimycin A, and DNP were administered in acetone solutions to give a final concentration of 5 μg ml^{-1}, 5 μM, and 0.1–0.5 mM, respectively. The acetone concentration in the mitochondrial suspension was kept below $2^0/_{00}$ to avoid any side effects.

O_2 consumption of intact pollen, incubated in the germination medium, was determined with the same electrode at 24° C. Aliquots of germinating pollen, 20 mg ml^{-1}, were transferred to the 3 ml reaction cell and diluted four times with fresh medium.

Extraction and Assay of Adenylate Phosphates

Fifty mg of pollen, incubated on a 2×4 cm nylon cloth, with a pore diameter of 37 μm, was quantitatively transferred into 15 ml of 0.3 M ice-cold $HClO_4$ and extracted for 45 min at 4° C with magnetic stirring. Homogenization did not improve the yield of adenylate phosphates and was, therefore, omitted. The pollen incubated in the germination medium was mixed with a more concentrated $HClO_4$ solution to attain the same final molarity of 0.3 M. The acid-precipitable material was centrifuged at 5,000 *g* for 10 min and 10 ml of the supernatant was neutralized with 10 M KOH and 0.5 M HEPES to a final buffer concentration of 20 mM at pH 7.6. After standing in the cold for 15 min, the $KClO_4$ was removed by centrifugation. The two separate centrifugations were necessary to avoid recovery of enzymatic activity upon neutralization, which has resulted in considerable losses of ATP (Davison and Fynn, 1974; Swedes et al., 1975). After diluting the neutralized extracts 100 to 200 times with a buffer containing 20 mM $MgSO_4$ and 20 mM HEPES, pH 7.6, ATP was assayed by an adapted luciferase reaction, using a Nuclear Chicago Mark I liquid scintillation spectrometer with the instrument setting as indicated by Van Dyke (1974).

Thirty µl of a concentrated firefly luciferin-luciferase extract (Boehringer, Mannheim), which was continuously kept on ice, was injected into a scintillation vial containing 0.5 ml of the diluted extract and thoroughly mixed. Exactly 10 s after injection, the bioluminescence was measured for 6 s at 18° C. (ATP+ADP), and (ATP+ADP+AMP) were determined similarly after enzymatic conversion of ADP and AMP into ATP (Pradet, 1967; Ching and Ching, 1972). ADP and AMP were determined by difference. The recovery of pure adenylate phosphates throughout the entire extraction procedure ranged from 97–108% for ATP, 99–103% for ADP, and 93–100% for AMP. These results were in accordance with those obtained by Swedes et al., (1975). Repeated quenching and rapid decay in the activity of the crude enzyme preparation necessitated internal standardization. After St. John (1970), samples with and without an internal standard were alternately counted. In each extract, the ATP, (ATP+ADP), and (ATP+ADP+AMP), were assayed 5 times. (The standard error of the mean refers to triplicate extractions.) From these values, the adenylate energy charge, defined as EC = {(ATP)+(ATP+ADP)}: 2 (ATP+ADP+AMP), (Atkinson, 1968) was calculated. The standard error of the mean for the EC was calculated using the appropriate methods of the propagation of errors, and ranged from 0.03 to 0.04 EC units.

Cytochrome Spectra

Measurement of cytochromes in pollen grains was performed by determining the difference spectra of homogenates at the temperature of liquid N_2. Two g of non-germinated pollen were suspended in 20 ml of the germination medium immediately frozen and completely disrupted in a precooled X-press cell desintegrator (LKB); the homogenate was clarified by centrifugation at 100 *g* for 5 min. Homogenates from germinated pollen were obtained in the same way from pollen suspensions concentrated by filtration to the same density; the filtrate did not contain significant amounts of cytochromes.

One part of the homogenate was reduced by the addition of dry $Na_2S_2O_4$, the other part was oxidized with 30% H_2O_2; the germination medium was treated similarly as a blank. Oxidized and reduced samples were placed in a 1.5 mm light-path, plexiglass cuvette and cooled in liquid N_2 during the scanning of the spectrum. Spectra were recorded with a specially designed, single-beam spectrophotometer, which was connected with a Laben multichannel analyzer by using a fixed-slit width, corresponding with a band width of 6 nm, and an E-Prom photomultiplier tube-mediated, base-line correction (Jupijn and Desmet, 1979). The device was equipped with an end-on PM tube to minimize the effects of light scattering. Calculations of reduced minus oxidized difference spectra were made with a Digital PDP 11 computer after appropriate subtraction of a blank difference spectrum.

Cytochromes of mitochondria, isolated from germinated pollen, were identified according to Hackett (1964) at the temperature of liquid N_2. The absorbancy of cytochromes *b* and *c* was measured from the tangent-line, connecting points of the spectrum at about 540 and 570 nm; that of cytochrome aa_3 from the line, connecting points of the spectrum at 580 and 610 nm. Quantities of cytochromes were roughly estimated on the basis of the molar-extinction coefficients given by Chance and Williams (1956), and corrected for the about 7 times intensification of absorption peaks at 77°K (Lance and Bonner, 1968; Dizengremel, 1975).

Cytochrome c Oxidase Activity

The activity of cytochrome *c* oxidase (EC 1.9.3.1) was measured spectrophotometrically with an appropriate amount of mitochondria diluted in 2 ml of 0.01 M phosphate buffer, pH 7.2, and reduced cytochrome *c* (Boehringer, Mannheim) to a final concentration of about 45 µM. Facultatively, 0.5 M mannitol was added to the phosphate buffer to maintain a high osmolarity. The oxidation of reduced cytochrome *c* was followed for 5 min using a Hitachi Perkin-Elmer Model 356 dual wavelength spectrophotometer at 550 nm with the reference beam at 540 nm (Wilson and Bonner, 1971). Finally, the optical density of the oxidized cytochrome *c* was determined by adding a small drop of saturated $K_3Fe(CN)_6$ solution. Activities were calculated from the absorbance changes as the first-order, rate-constant ks^{-1} per mitochondria isolated from 1 g pollen for the 2 ml reaction mixture.

Electron Microscopy

Isolated mitochondria were fixed with neutralized glutaraldehyde, pH 7.2, at the final concentration of 2% (v/v) in 10 ml of grinding buffer, without cysteine and PVP. After 60 min at 4° C, mitochondria were pelleted at 12,000 *g* for 10 min and the pellet mixed, either with a drop of warm 3% (w/v) agar or with 4 ml of 0.1 M phosphate buffer, pH 7.2. With the latter, an additional high-speed centrifugation at 160,000 *g* for 60 min was performed, to obtain a dense pellet. After several cycles of washing in the 0.1 M phosphate buffer, pH 7.2, the pellet or agar was postfixed in a 0.75% (w/v) $KMnO_4$ solution in the same buffer for 40 min at 0° C. Fixed material was washed, dehydrated in ethanol and propylene oxide, embedded in Epon-812 (Luft, 1961), sectioned, stained with lead citrate (Reynolds, 1963), and examined in a Philips 300 electron microscope.

Results

Account of the Isolation Method

Since the first intact plant mitochondria, showing respiratory control, were isolated (Bonner and Voss, 1961), many different isolation media have been described (e.g. Hanson and Hodges, 1967). Combinations were selected for the isolation of pollen mitochondria. Although final prescriptions for isolation are given in Materials and Methods, we would like to emphasize the improvements made during our studies. Apart from the beneficial effect of fatty acid-poor BSA (Hanson and Hodges, 1967), respiratory control was enhanced by the rapid filtering done through millipore filters, which resulted in a rapid separation of cell debris from the mitochondrial suspension, as proposed earlier by Palmer (1967) and Sarkissian and Srivastava (1968). Repeated washing of mitochondria did not significantly improve the results and was, therefore, omitted. Except in the case of *Aster*, insoluble PVP and cysteine were also left out, since they gave no beneficial effects. The replacement of EDTA with EGTA, that selectively chelates Ca with a high stability coefficient (Marhol and Cheng, 1970), was an important improvement. It increased the RCR by about 30% with NADH as the substrate. However, the interpretation of the results

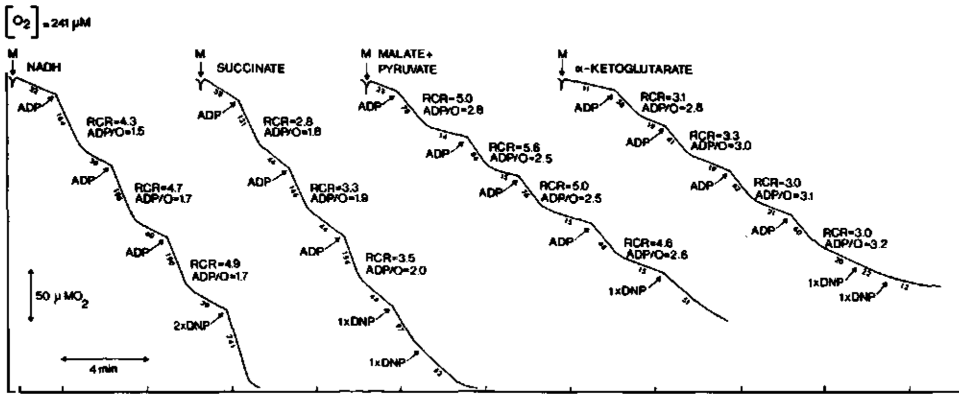

Fig. 1. Polarograph traces of the oxidation of different substrates by mitochondria isolated from *Typha* pollen after 3 h germination. ADP and DNP were added to give a final concentration of 100 µM. Numbers represent rates of O_2 uptake in nmol per min per amount of mitochondria, isolated from 100 mg dry pollen; M, addition of mitochondria. ADP/O ratios and RCR values are also indicated

would in no way have been different if EDTA had been used instead.

Oxidation of Different Substrates

First, isolated mitochondria were characterized by studying their oxidative phosphorylation. Figure 1 shows the rates and efficiencies of oxidation of different substrates by mitochondria isolated from binucleate *Typha* pollen which was incubated for 3 h in the germination medium. The relatively high RCR values are indicative of the good condition of these mitochondria (Palmer, 1976).

Effects of the uncoupler DNP on the rate of O_2 uptake were also studied with these different substrates. At 100 µM, DNP is supposed to completely uncouple phosphorylation from the electron transfer, enhancing O_2 uptake to a large extent. However, it may also act as an inhibitor of respiration through the limitation of substrate transport due to collapse of the proton gradients (Day and Hanson, 1977). Such inhibition is clearly demonstrated for the three TCA-cycle substrates in Figure 1, which shows a DNP-mediated decrease in the rate of O_2 uptake compared to the ADP stimulated rate. Since exogenous NADH fully relieved the uncoupler-inhibited oxidation of the TCA-cycle substrates, confirming Day and Hanson's (1977) results, this substrate is not likely to interfere with the ETC. Moreover, the rate of NADH oxidation after addition of DNP always exceeded the ADP-mediated rate. Because the rate-limiting steps evidently occur before the entry of electrons from exogenous NADH into the ETC, from the onset of germination in vitro, mitochondrial development was followed by determining the capacity of the ETC with NADH as the substrate.

Table 1. Differential respiratory and functional characteristics of 3 binucleate and 1 typical trinucleate pollen species in humid conditions (RH=97%, 30° C) and in the germination medium (24° C)

	Species			
	binucleate			trinucleate
	Typha	*Nicotiana*	*Tradescantia*	*Aster*
Incubation in humid air				
Rate of O_2 uptake (µmol h^{-1} mg^{-1})	0.06	0.11	0.26[a]	0.34
Interval until 50% of initial viability (h)	>35	15.5	10[b]	3.7
Incubation in germination medium				
Rate of O_2 uptake (µmol h^{-1} mg^{-1})	0.40	0.65	1.10	1.00
Interval until constant rate of respiration (min)	12	7	< 0.5	<0.5
Interval until first outgrowth of pollen tubes (min)	70	35	6	3

[a] Measured at 22° C, but computed for 30° C
[b] Measured at 22° C

Activity and Structural Integrity of Mitochondria During Germination

Table 1 summarizes the respiratory and longevity characteristics in humid air of the 4 pollen species.

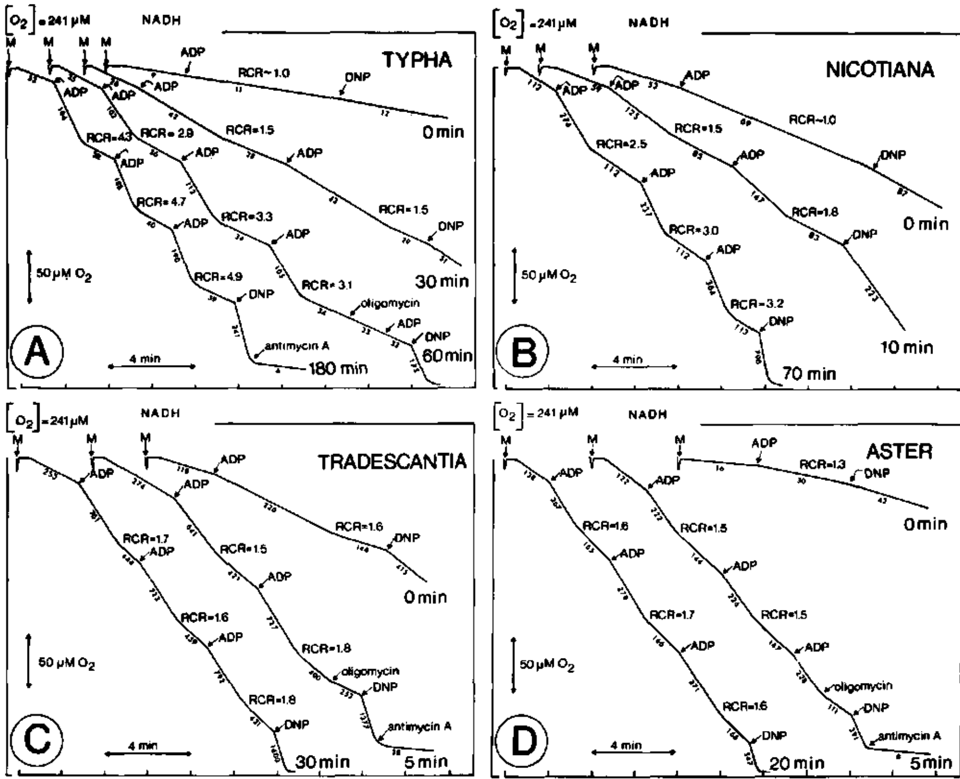

Fig. 2A–D. Polarograph traces of O_2 consumption by mitochondria isolated from 4 pollen species at intervals during incubation in the germination medium. Numbers along the traces are rates of O_2 uptake in nmol per min per amount of mitochondria isolated from 100 mg dry pollen, NADH being substrate; M, mitochondria. ADP was administered at each cycle to a final concentration of 100 μM, DNP up to maximum stimulation of O_2 uptake (mostly about 200 μM), oligomycin 5 μg ml^{-1}, and antimycin A at 5 μM

Typha and *Nicotiana* behave as a typical binucleate, and *Aster* as a typically trinucleate species. Because of its relatively high respiration and rapid decrease of vitality, the binucleate pollen of *Tradescantia* forms a transition to the trinucleate character.

Mitochondria isolated from non-germinated *Typha* pollen had no respiratory control (Fig. 2A), even at much higher densities than presented in the figure. In the course of germination, however, this property improved significantly, until, after 3 h, an RCR of about 4.9 was reached. Figures 2B, C, and D show the much more rapid development of respiratory control of *Nicotiana*, and particularly of *Tradescantia* and *Aster*. The high rates of initial and state-4 respiration indicate the extreme difficulty of the isolation of properly-functioning mitochondria from the latter two. The nature of this rapid, basal respiration was analyzed using the antibiotics oligomycin and antimycin A. The minor contribution of phosphorylative processes to this O_2 uptake was clearly demonstrated by the relatively small inhibition by oligomycin, whereas the strong inhibition by antimycin A pointed to the exclusive flow of electrons through the main ETC (Fig. 2, C and D). Mitochondria of trinucleate *Zea mays* pollen exhibited a similar, loose coupling of phosphorylation and high rate of electron transport.

These high rates occur after a few minutes, as shown by the URCI determinations. The increase of the URCI, in the course of germination in vitro, is shown in Figure 3. Maximum URCI values for *Aster* and *Tradescantia* are reached within 2 min after immersion in the medium, in about 30 min for *Nicotiana*, and 75 min for *Typha*. These periods coincide strikingly with the lag periods of germination in vitro, i.e. the periods up to the first outgrowth of pollen

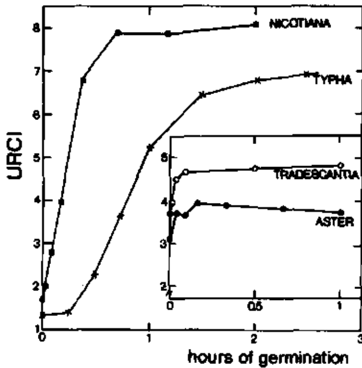

Fig. 3. Change in respiratory capacities of mitochondria isolated from different pollen species during incubation in the germination medium, expressed as uncoupler respiratory control index (URCI). The index is expressed as the ratio of the rates of NADH respiration in the presence of oligomycin + DNP, and oligomycin alone. DNP was introduced into the reaction chamber up to a concentration which gave maximum O_2-uptake after addition of 0.7 µmol ADP and subsequent inhibition of phosphorylation by oligomycin. Each point is the result of two determinations

Table 2. Cytochrome c oxidase activity of mitochondria isolated from *Typha* pollen at intervals during germination in vitro. The percentage intact mitochondria is the counterpart of the injured fraction that is calculated as the ratio of the activities of non-shocked and osmotically shocked ones. The calculated RCR is composed assuming full respiratory capacity at the onset of germination of the intact mitochondria (as at 180 min) and the injured ones at the basal level (RCR = 1)

min of incubation	Activity $k(s^{-1}\,g^{-1})$		Percentage intact mitochondria	RCR	
	shocked 15 mOsm	non-shocked 500 mOsm		calculated	measured
0	0.44	0.26	40	2.9	1.0
15	0.56	0.32	42	3.0	1.1
30	0.80	0.38	52	3.5	1.5
60	0.97	0.19	80	4.8	3.1
180	1.02	0.19	81	5.8	4.9

Table 3. Stoichiometry of electron carriers in *Typha* pollen mitochondria isolated after 0 and 2 h incubation in the germination medium. The subscript refers to the position of the peak in the low temperature difference spectrum. Identification and quantitation of cytochromes as indicated in Materials and Methods. The concentration of cytochrome aa_3 is taken as unity. Each value is the average from three different isolations

h in germination medium	Relative concentration of cytochromes				
	cyt aa_3	cyt b_{553}	cyt b_{557}	cyt b_{562}	cyt c
0	1.00	1.34	1.21	0.95	1.06
2	1.00	1.46	1.24	1.01	1.82

tubes can be observed (Table 1). These lag periods can be regarded as intrinsic since attemps to shorten them by application of such lectins as concanavalin A and phytohaemagglutinin-M, as described for lily pollen by Southworth (1975), were not successful.

In order to distinguish whether the poor respiratory control of mitochondria from fresh *Typha* pollen is an intrinsic property or due to membrane disruption during isolation, the oxidation of exogenous reduced cytochrome *c* was studied. Since the outer mitochondrial membrane is thought to be impermeable for cytochrome *c* (Pfaff et al., 1968; Woytczak and Zaluska, 1969; Van der Plas et al., 1976), an extremely limited oxidation is to be expected by the inner membrane-located cytochrome *c* oxidase (Douce et al., 1972; 1973) for intact mitochondria. The enzyme activity of freshly-isolated mitochondria as a percentage of the activity of osmotically shocked ones may then be considered as the injury suffered during isolation. Table 2 shows the improvement in integrity of mitochondria isolated from *Typha* pollen at intervals during germination in vitro. The calculated RCR is the ratio which should be obtained only when integrity problems are involved. From the course in the discrepancy between the measured and calculated RCR values, the actual development of the mitochondrial ETC during germination in vitro can be followed. Attempts to improve the integrity of mitochondria from the non-germinated pollen by varying the osmolarity and pH, and application of kinetin in the isolation medium (Banerji and Kumar, 1975), were not successful.

Cytochrome Spectra

Results of low-temperature difference spectrometry indicated differences in the relative concentrations of electron carriers in mitochondrial fractions from germinated and non-germinated *Typha* pollen (Table 3). Only half of the cytochrome *c* present in mitochondria from germinated pollen was found in those isolated from the non-germinated sample. This can be interpreted as leakage from fresh pollen mitochondria as a result of reduced integrity. Alternatively, however, if this low value were an intrinsic characteristic of these mitochondria, then it might explain their low respiratory activity. In order to distinguish between these alternatives, whole pollen grains were completely disrupted and homogenized, allowing for a complete recovery, and cytochrome spectra of

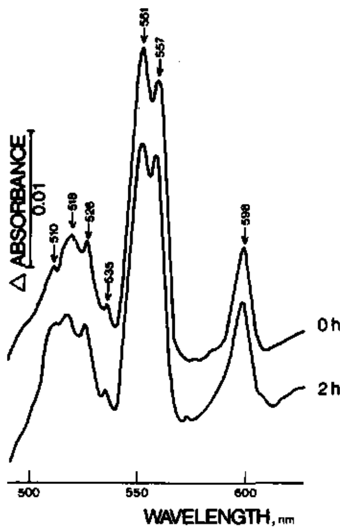

Fig. 4. Difference spectra of oxidized and reduced samples from the crude homogenate (X-press) of non-germinated and 2 h germinated *Typha* pollen (100 mg dry pollen ml^{-1}) at liquid nitrogen temperature

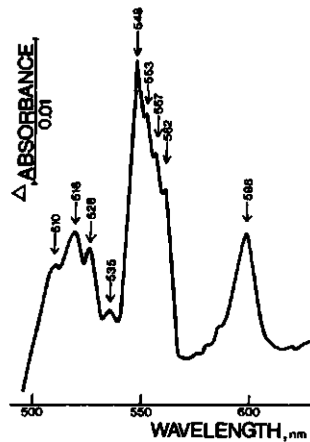

Fig. 5. Low temperature difference spectrum of oxidized and reduced mitochondria isolated after 2 h germination of *Typha* pollen, using the normal Potter homogenization procedure. Identification of cytochromes as indicated in Materials and Methods

Table 4. Cytochrome content of *Typha* pollen during germination in vitro as the absorbance in the crude homogenate (100 mg dry pollen per ml) at liquid N_2 temperature. Mean from 4 different homogenates

h of germination	Cytochrome content in absorbance units		
	cyt $bc_{(551)}$	cyt $b_{(557)}$	cyt aa_3
0	0.028	0.025	0.010
$^1/_2$	0.029	0.027	0.011
2	0.024	0.023	0.010
LSD (P=0.05)	0.004	0.003	0.002

Table 5. Adenylate energy charges in 4 pollen species during germination in vitro. Mean values from three extractions. EC values calculated with the technique of propagation of errors. The standard error of the mean varied from 0.03 to 0.04

Species	min of germination in vitro (24° C)			
	0	2	5	25
Typha latifolia	0.86	0.56	0.66	0.85
Nicotiana alata	0.40	0.68	0.69	0.80
Tradescantia paludosa	0.46	0.84	0.84	0.80
Aster tripolium	0.90	0.91	0.91	0.80

the crude extracts were analyzed (Fig. 4). α-Bands, present at 598, 557 and 551 nm, correspond with cytochrome oxidase, cytochrome b_{557} and a non-separated combination of cytochromes *b* and *c*, respectively (Lance and Bonner, 1968; Ikuma, 1972). The β-bands (510–535 nm) are in accordance with the same references. Changes in band position in the course of germination were not noticed and no increase in the contents could be established (Table 4). The low cytochrome *c* content in fresh pollen mitochondria must, therefore, be ascribed to the reduced integrity of the outer mitochondrial membrane. The complex of cytochromes at 551 nm was found only in crude pollen extracts, and may, in part, have a non-mitochondrial origin (Jesaitis et al., 1977). Iso-

Table 6. Effect of cycloheximide (CHI), D(−)threo chloramphenicol (CAP), and ethidium bromide (EBr) on development of mitochondrial respiratory capacities and tube growth of *Typha* pollen after 2 h germination. Mean values of four isolations

Inhibitor	DPN stimulated rate of O_2 uptake	URCI	Tube growth
	nmol min^{-1} 100 mg^{-1}		
control	224	6.03	+
CHI (100 μg ml^{-1})	223	5.96	−
CAP (250 μg ml^{-1})	261	6.47	+
CAP (1 mg ml^{-1})	239	5.52	−
EBr (25 μg ml^{-1})	237	6.09	+
LSD (P=0.05)	28	0.64	

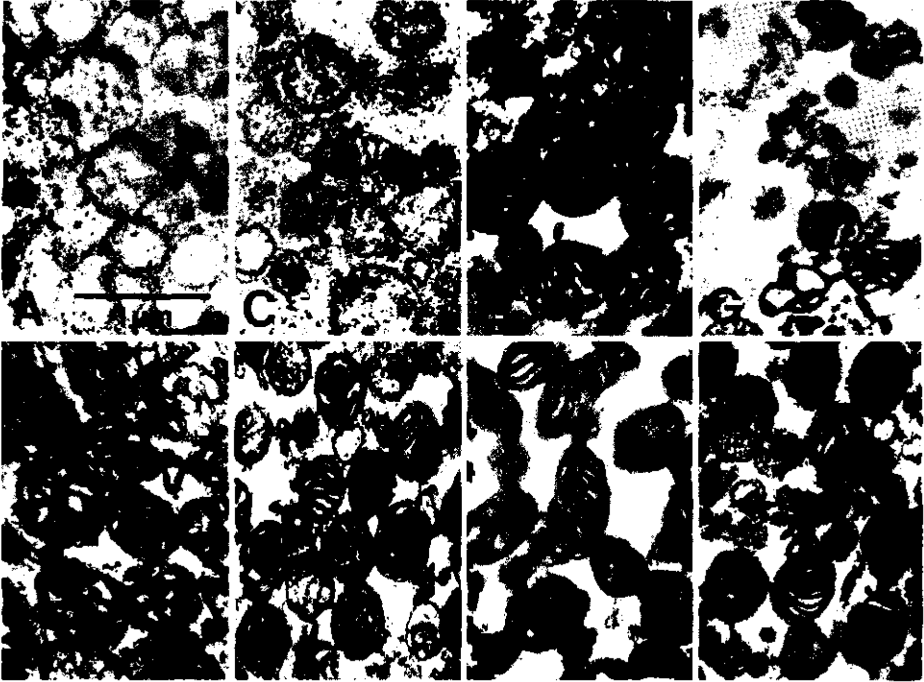

Fig. 6A–H. Electron micrographs of the mitochondrial fraction isolated from pollen in the non-germinated or germinated condition. **A** and **B** non-germinated and germinated (2 h) *Typha* pollen; **C** and **D** non-germinated and germinated (1 h) *Nicotiana* pollen; **E** and **F** non-germinated and germinated (15 min) *Tradescantia* pollen; **G** and **H** non-germinated and germinated (10 min) *Aster* pollen. Magnification, ×22,500

lated mitochondria clearly showed a cytochrome *c* peak at 548 nm; three cytochrome *b* peaks at 553, 557 and 562 nm; and a cytochrome aa_3 peak at 598 nm (Fig. 5), identified according to Hackett (1964).

Onset of Respiration and Change in Adenylate Energy Charge (EC)

During germination, together with the rise in URCI of their isolated mitochondria, pollen of *Typha* and *Nicotiana* exhibited a rise in respiration rate. Those of *Tradescantia* and *Aster*, however, respired maximally at the first measurement. The intervals up to the stabilization of respiration and related rates of O_2 uptake are given in Table 1. Although with lower accuracy, the above-mentioned rise in rate of O_2 uptake was also observed with the Warburg method.

Such a slow rise in respiration may originate from a slow rise in the rate of anabolic processes or from a slowly developing mitochondrial system. In the first case, the ATP requiring, biosynthetic processes remain in equilibrium with the ATP generating systems, resulting in a high EC in the cell. In the latter case, a disparity between generating and utilizing systems can be anticipated, with a concomitant low EC. Measurement of the content of adenylate phosphates during the early phase of germination in vitro resulted in the EC's given in Table 5. Parallel with the slow rise in respiration rate, *Typha* pollen was found to exhibit a temporary, sharp decrease in the EC. After 25 min, the EC had returned to the normal, high level again, which demonstrates that, initially, the ATP generating systems failed to cope with the need. By contrast, the trinucleate pollen of *Aster* immediately reached a high rate of respiration upon immersion in the medium, and the EC remained high. This is an indication of the presence of highly organized mitochondria at anthesis. The other two pollen species behaved intermediately, with anabolic processes already occurring at a considerable rate with the onset of germination.

Effect of Protein Synthesis Inhibitors on the Development of Mitochondria

Many pollen species perform protein synthesis during germination in vitro (Mascarenhas, 1975). *Typha* pollen was found to incorporate approximately 5.5 pmol leucine min^{-1} mg^{-1} into proteins (Hoekstra and Bruinsma, 1979). Since it takes *Typha* mitochondria about 75 min to attain to maximum respiratory control, protein synthesis might be a limiting factor.

Cycloheximide (100 µg ml^{-1}), an inhibitor of cytoplasmic protein synthesis, completely abolished the incorporation of [^{3}H]leucine into acid-insoluble protein (Hoekstra and Bruinsma, 1979) and prevented outgrowth of pollen tubes. However, cycloheximide did not affect either the rise in the URCI, as presented in Figure 3, or the uncoupler-stimulated rate of oxygen uptake (Table 6). Similar results were obtained with D(−)threo choramphenicol, an inhibitor of mitochondrial protein synthesis administered in two concentrations, 250 µg ml^{-1} and 1000 µg ml^{-1}, the latter of which markedly inhibited germination (Table 6). As expected, the inhibitor of mitochondrial DNA synthesis, ethidium bromide, had no effect.

Electron Microscopy (EM)

That differences exist in mitochondrial development among fresh pollen species at dehiscence, was further supported by EM observations. In isolations from non-germinated *Typha* and *Nicotiana* pollen, which showed high activity of the mitochondrial marker-enzyme, cytochrome oxidase, particles with only a faint electron density and a scanty matrix material could be observed (Fig. 6A and C). Moreover, these particles were so fragile, that the preparation had to be embedded in agar instead of being pelleted by centrifugation at 160,000 *g*. However, complex, cristae-like structures were visible in particles isolated from *Typha* and *Nicotiana* pollen after germination (Fig. 6B and D). By contrast, mitochondria prepared from *Tradescantia* and *Aster* (Fig. 6E, F, G, and H) always showed highly-organized inner structures. The slight differences between non-germinated and germinated states probably originate from swelling and contraction phenomena (Malone et al., 1974; Pomeroy, 1976).

Discussion

Since Dickinson (1966, 1967) discussed the probability of oxidative phosphorylation in germinating pollen, no successful isolation of mitochondria, showing in vitro phosphorylation of ADP, has been made, until now. The general inability to generate ATP of mitochondria, isolated from non-germinated pollen, will certainly have contributed to this failure, particularly because species with relatively low-developed mitochondria have been routinely used in pollen research. In the present paper, the preparation of highly-functional mitochondria is demonstrated from three binucleate pollen species and from one typical trinucleate species.

Mitochondrial preparations from *Typha latifolia* pollen, germinating for 3 h, showed good respiratory control with all the substrates tested. With the oxidation of malate/pyruvate and α-ketoglutarate, ADP/O ratios approximated fairly well the theoretical values of 3 and 4, respectively, suggesting the operation of 3 sites of phosphorylation in pollen mitochondria. As might be expected for plant mitochondria (Ikuma and Bonner, 1967), the oxidation of exogenous NADH and succinate occurred at a lower efficiency, approximating the theoretical value of 2. This demonstrates the suitability of media for the isolation and assay of pollen mitochondria. The excess of Ca, necessary for optimal germination in vitro, was chelated by the non-penetrant EGTA (Reed and Bygrave, 1974), restoring coupling and permeability, and improving the Ca:Mg ratio (Hunter et al., 1976). Inhibition by EGTA of oxidation of exogenous NADH (Coleman and Palmer, 1971) did not occur.

Polarograph traces of mitochondrial respiration from preparations of germinating *Typha* and *Nicotiana* pollen show the orthodox pattern, with high rates of state-3 respiration and low rates of both initial and state-4 respiration. Isolations from germinating *Aster* and *Tradescantia* pollen, however, showed high rates of initial and state-4 respiration soon after the beginning of incubation in the germination medium. This uncoupling was probably brought about by the isolation procedure. A slow start of mitochondrial activity upon germination in vitro was visible in *Nicotiana* and *Typha* pollen. The gradual development of their electron-transducing system in the course of germination in vitro cannot solely be ascribed to differences in mitochondrial integrity, although the improvement in mitochondrial functioning coincides with improved resistance of the particles to injury during homogenization (Table 2).

Maximum stimulation of O_2 uptake by uncoupling, in the presence of NADH as a substrate, proved effective for estimating the electron-transducing capacity of a mitochondrial preparation. The URCI provided a means to determine this capacity independent of the amount of mitochondria present in the probe. In Figure 3, it is the shape of the curve that

counts rather than its height, because partly uncoupled, basal respiration also contributes to the URCI. When the URCI becomes constant, mitochondrial development is completed. In contrast to *Typha* and *Nicotiana* pollen, those of *Tradescantia* and *Aster* showed virtually no mitochondrial development from the onset of germination in vitro. The latter two are, therefore, equipped with fully-differentiated mitochondria already in the freshly-dehisced condition.

The rapid increase in O_2 uptake of the intact germinating *Typha* and *Nicotiana* pollen grains contrasts with the prolonged rise of URCI of their mitochondria. This can be understood by comparing the energy demand at the level of the individual mitochondrion prior to, and at the time of outgrowth of the pollen tube. Taking germinating *Typha* pollen as an example, it was calculated that, on a volume basis, the total content of the grain would fit in about 300 µm of the pollen tube. Therefore, mitochondria must be separated spatially from each other. It is considered unlikely that the local requirement for ATP is compensated for by the forward plasma streaming of about 50 µm min^{-1} (Jaffe et al., 1975). Assuming the energy demand to be localized mostly at the tip of the tube, with a total demand for ATP of approximately 30 nmol min^{-1} mg^{-1} pollen, at a pool turnover of at least 7 per min (Hoekstra and Bruinsma, 1979), it is obvious that the phosphorylative capacity of the individual mitochondrion, during tube growth, must be far larger than after 12 min incubation in the germination medium. This also explains why the periods in which mitochondria develop, correspond so strikingly with the lag period of germination in vitro. Apparently tube growth cannot start until the mitochondria are fully developed.

Decisive results have been obtained by direct extraction and assay of adenylate phosphates from the germinating grains. The temporary, sharp decrease in the EC in germinating *Typha* pollen shows that, in intact pollen, the mitochondria were not able to cope with the demand for ATP, confirming the results of the isolation experiments. Fully functioning mitochondria must be present in *Aster* pollen, since their EC remained high from the start of incubation in the germination medium. Pollen of *Nicotiana* resembled those of *Typha* with respect to their attainment of a high EC, but the discrepancy between ATP generating and utilizing systems was already evident in the non-germinated condition. Non-germinated pollen of *Tradescantia* also had a low energy charge. Since this pollen species respired maximally at the start of incubation and, moreover, the EC increased to 0.84 within two min of incubation, we may consider the low ATP content of the non-germinated sample as a protection against rapid loss of vitality, rather than as a sign of limited mitochondrial differentiation. Electron micrographs of particles in the mitochondrial pellet of non-germinated *Tradescantia* pollen revealed, indeed, recognizable mitochondria with well-developed, complex inner structures. No such well-defined particles were visible on electron micrographs of pellets from non-germinated pollen of *Typha* and *Nicotiana*, since inner structures were recognizable but poorly differentiated. In *Tradescantia* pollen, these poorly structured vesicles are present in the developing young microspore prior to dehiscence, as observed by Maruyama (1968).

Striking similarities occur in the early stages of germination of slowly-germinating pollen and seeds. Together with an increasingly normal appearance of mitochondria as seed germination proceeds (Mayer and Shain, 1974; Webster and Leopold, 1977), respiratory control ratios also rise, and phosphorylative capacities are improved (Sato and Asahi, 1975; Morohashi and Shimokoriyama, 1975). Mitochondrial development could not be ascribed solely to hydratation, since active, temperature-dependent differentiation occurred (Nawa and Asahi, 1973a; Morohashi and Shimokoriyama, 1977). As for germinating pea seeds (Nawa and Asahi, 1973b), we conclude from our experiments with protein-synthesis inhibitors that, notwithstanding their development, mitochondria do not need de novo synthesis as a prerequisite for acquisition of the capacity to generate ATP. Accordingly, all the cytochromes were found to be already present in the non-germinated grains of *Typha*. Mitochondria probably become functional and stable through assembly of pre-existing proteins into their membranes, prior to the outgrowth of the pollen tube.

The differences in the rate of respiration between intact bi- and trinucleate pollen in humid air, as described earlier (Hoekstra and Bruinsma, 1975b), can now largely be explained by the condition of their mitochondria. Some rapidly germinating binucleate species, e.g. *Tradescantia*, however, are exceptional in that they are equipped with highly organized mitochondria, but exhibit a relatively low rate of respiration. However, this pollen has low energy charges that reach normal values as soon as the grains become wet. We conclude that, compared to the trinucleate system, longevity is certainly favoured by the apparent blockade of the respiratory system, since uncontrolled loss of substrate and nonsense biosyntheses are prevented.

This paper demonstrates that rapidly-germinating pollen must be equipped with fully-developed mitochondria already at dehiscence, which is in contrast to slowly-germinating types. The latter, evolutionarily more primitive types (Hoekstra and Bruinsma, 1978) as independent, male gametophytes complete the

structural and functional development of their mitochondria as a requirement for the outgrowth of their pollen tubes.

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References

Atkinson, D.E.: The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. Biochemistry **7**, 4030–4034 (1968)

Banerji, D., Kumar, N.: Partial inhibition of the decay of Hill activity in isolated chloroplasts by kinetin. Biochem. Biophys. Res. Commun. **65**, 940–944 (1975)

Bar-Shalom, D., Mattsson, O.: Mode of hydration, an important factor in the germination of trinucleate pollen grains. Bot. Tidsskrift **71**, 245–251 (1977)

Bonner, W.D., Voss, D.O.: Some characteristics of mitochondria extracted from higher plants. Nature **191**, 682–684 (1961)

Brewbaker, J.L.: Pollen cytology and self-incompatibility systems in plants. J.Hered. **48**, 271–277 (1957)

Brewbaker, J.L.: Biology of the angiosperm pollen grain. Ind. J. Gen. Pl. Breed. **19**, 121–133 (1959)

Brewbaker, J.L.: The distribution and significance of binucleate and trinucleate pollen grains in the angiosperms. Am. J. Bot. **54**, 1069–1083 (1967)

Brewbaker, J.L., Kwack, B.H.: The essential role of calcium ion in pollen germination and pollen tube growth. Am. J. Bot. **50**, 859–865 (1963)

Chance, B., Williams, G.R.: The respiratory chain and oxidative phosphorylation. Adv. Enzymol. **17**, 65–134 (1956)

Ching, T.M., Ching, K.K.: Content of adenosine phosphates and adenylate energy charge in germinating ponderosa pine seeds. Plant Physiol. **50**, 536–540 (1972)

Coleman, J.O.D., Palmer, J.M.: Role of Ca^{2+} in the oxidation of exogenous NADH by plant mitochondria. FEBS Lett. **17**, 203–208 (1971)

Davison, J.A., Fynn, G.H.: The assay of ATP by the luciferin-luciferase method. Interference by a bacterial phosphatase enzyme stable to perchlorate treatment. Anal. Biochem. **58**, 632–637 (1974)

Day, D.A., Hanson, J.B.: Effect of phosphate and uncouplers on substrate transport and oxidation by isolated corn mitochondria. Plant Physiol. **59**, 139–144 (1977)

Dickinson, D.B.: Inhibition of pollen respiration by oligomycin. Nature **210**, 1362–1363 (1966)

Dickinson, D.B.: Permeability and respiratory properties of germinating pollen. Physiol. Plant. **20**, 118–127 (1967)

Dizengremel, P.: La voie d'oxydation insensible au cyanure dans les mitochondries de tranches de pomme de terre *(Solanum tuberosum* L.*)* maintenues en survie. Physiol. Vég. **13**, 39–54 (1975)

Douce, R., Christensen, E.L., Bonner, W.D.: Preparation of intact plant mitochondria. Biochim. Biophys. Acta **275**, 148–160 (1972)

Douce, R., Mannella, C.A., Bonner, W.D.: The external NADH dehydrogenases of intact plant mitochondria. Biochim. Biophys. Acta **292**, 105–116 (1973)

Hackett, D.P.: Enzymes of terminal respiration. In: Modern methods of plant analysis, vol. VII, pp. 647–694, Linskens, H.F., Sanwal, B.D., Tracey, M.V., eds. Berlin, Göttingen, Heidelberg: Springer 1964

Hanson, J.B., Hodges, T.K.: Energy-linked reactions of plant mitochondria. In: Current Topics in Bioenergetics, vol. 2, pp. 65–98, Sanadi, D.R., ed. New York, London: Academic Press 1967

Heslop-Harrison, Y., Shivanna, K.R.: The receptive surface of the angiosperm stigma. Ann. Bot. **41**, 1233–1258 (1977)

Hoekstra, F.A., Bruinsma, J.: Viability of *Compositae* pollen: Germination in vitro and influences of climatic conditions during dehiscence. Z. Pflanzenphysiol. **76**, 36–43 (1975a)

Hoekstra, F.A., Bruinsma, J.: Respiration and vitality of binucleate and trinucleate pollen. Physiol. Plant. **34**, 221–225 (1975b)

Hoekstra, F.A., Bruinsma, J.: Reduced independence of the male gametophyte in angiosperm evolution. Ann. Bot. **42**, 759–762 (1978)

Hoekstra, F.A., Bruinsma, J.: In press (1979)

Hunter, D.R., Haworth, R.A., Southard, J.H.: Relationship between configuration, function and permeability in calcium-treated mitochondria. J. Biol. Chem. **251**, 5069–5077 (1976)

Ikuma, H.: Electron transport in plant respiration. Ann. Rev. Plant Physiol. **23**, 419–436 (1972)

Ikuma, H., Bonner, W.D.: Properties of higher plant mitochondria. I. Isolation and some characteristics of tightly-coupled mitochondria from dark-grown mung bean hypocotyls. Plant Physiol. **42**, 67–75 (1967)

Iwanami, Y.: Acceleration of the growth of *Camellia sasanqua* pollen by soaking in organic solvent. Plant Physiol. **52**, 508–509 (1973)

Iwanami, Y., Nakamura, N.: Storage in an organic solvent as a means for preserving viability of pollen grains. Stain Techn. **47**, 137–139 (1972)

Jaffe, L.A., Weisenseel, M.H., Jaffe, L.F.: Calcium accumulations within the growing tips of pollen tubes. J. Cell Biol. **67**, 488–492 (1975)

Jesaitis, A.J., Heners, P.R., Hertel, R.: Characterization of a membrane fraction containing a b-type cytochrome. Plant Physiol. **59**, 941–947 (1977)

Jupijn, G.L., Desmet, G.M.: Description of the use of an E-prom memory for the automatic base line control of a single-beam spectrophotometer. Accepted for publication in J. Physics E: Sci. Instr. 1979

Lance, C., Bonner, W.D.: The respiratory chain components of higher plant mitochondria. Plant Physiol. **43**, 756–766 (1968)

Lehninger, A.L.: Biochemistry. New York: Worth Publishers 1970

Luft, J.H.: Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. **9**, 409–414 (1961)

Lundqvist, A.: Complex self-incompatibility systems in angiosperms. Proc. Roy. Soc. B. **188**, 235–245 (1975)

Malone, C., Koeppe, D.E., Miller, R.J.: Corn mitochondrial swelling and contraction – an alternate interpretation. Plant Physiol. **53**, 918–927 (1974)

Marhol, M., Cheng, K.L.: Simple ion exchange separation of magnesium from calcium and other metal ions using ethyleneglycol-bis (2-aminoethylether) tetra acetic acid as a complexing agent. Anal. Chem. **42**, 652–655 (1970)

Maruyama, K.: Electron microscopic observation of plastids and mitochondria during pollen development in *Tradescantia paludosa*. Cytologia **33**, 482–497 (1968)

Mascarenhas, J.P.: The biochemistry of angiosperm pollen development. Bot. Rev. **41**, 259–314 (1975)

Mayer, A.M., Shain, Y.: Control of seed germination. Ann. Rev. Plant Physiol. **25**, 167–193 (1974)

Morohashi, Y., Shimokoriyama, M.: Development of glycolytic and mitochondrial activities in the early phase of germination of *Phaseolus mungo* seeds. J. Exp. Bot. **26**, 932–938 (1975)

Morohashi, Y., Shimokoriyama, M.: Water content and mitochondrial activities in the imbibitional phase of germination of *Phaseolus mungo* seeds. Z. Pflanzenphysiol. **82**, 173–178 (1977)

Nawa, Y., Asahi, T.: Relationship between the water content of pea cotyledons and mitochondrial development during the early stage of germination. Plant Cell Physiol. **14**, 607–610 (1973a)

Nawa, Y., Asahi, T.: Biochemical studies on development of mitochondria in pea cotyledons during the early stage of germination. Plant Physiol. **51**, 833–838 (1973b)

Palmer, J.M.: Rapid isolation of active mitochondria from plant tissue. Nature **216**, 1208 (1967)

Palmer, J.M.: The organization and regulation of electron transport in plant mitochondria. Ann. Rev. Plant Physiol. **27**, 133–157 (1976)

Pandey, K.K.: Evolution of gametophytic and sporophytic systems of self-incompatibility. Evolution **14**, 98–115 (1960)

Pfaff, E., Klingenberg, M., Ritt, E., Vogell, W.: Korrelation des unspezifisch permeabelen mitochondrialen Raumes mit dem „Intermembran-Raum". Eur. J. Biochem. **5**, 222–232 (1968)

Plas, L.H.W. van der, Jobse, P.A., Verleur, J.D.: Cytochrome c dependent, antimycin-A resistant respiration in mitochondria from potato tuber *(Solanum tuberosum* L.*)*. Influence of wounding and storage time on outer membrane NADH-cytochrome-c-reductase. Biochim. Biophys. Acta **430**, 1–12 (1976)

Pomeroy, M.K.: Swelling and contraction of mitochondria from coldhardened and nonhardened wheat and rye seedlings. Plant Physiol. **57**, 469–473 (1976)

Pradet, A.: Étude des adénosine-5′-mono, di et tri-phosphates dans les tissus végétaux. I. Dosage enzymatique. Physiol. Vég. **5**, 209–221 (1967)

Reed, K.C., Bygrave, F.L.: The inhibition of mitochondrial calcium transport by lanthanides and ruthenium red. Biochem. J. **140**, 143–155 (1974)

Reynolds, E.S.: The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. **17**, 208–212 (1963)

Sarkissian, I.V., Srivastava, H.K.: On methods of isolation of active tightly coupled mitochondria of wheat seedling. Plant Physiol. **43**, 1406–1410 (1968)

Sato, S., Asahi, T.: Biochemical properties of mitochondrial membrane from dry pea seeds and changes in the properties during imbibition. Plant Physiol. **56**, 816–820 (1975)

Southworth, D.: Lectins stimulate pollen germination. Nature **258**, 600–602 (1975)

St. John, J.B.,: Determination of ATP in chlorella with the luciferin-luciferase enzyme system. Anal. Biochem. **37**, 409–416 (1970)

Swedes, J.S., Sedo, R.J., Atkinson, D.E.: Relation of growth and protein synthesis to the adenylate energy charge in an adenine-requiring mutant of *Escherichia coli*. J. Biol. Chem. **250**, 6930–6938 (1975)

Van Dyke, K.: Uses of the liquid scintillation counter for measurement of bioluminescent and chemiluminescent reactions. Packard Techn. Bull. **20**, 1–13 (1974)

Webster, B.D., Leopold, A.C.: The ultrastructure of dry and imbibed cotyledons of soybean. Am. J. Bot. **64**, 1286–1293 (1977)

Wilson, S.B., Bonner, W.D.: Studies of electron transport in dry and imbibed peanut embryos. Plant Physiol. **48**, 340–344 (1971)

Wojtczak, L., Zaluska, H.: On the impermeability of the outer mitochondrial membrane to cytochrome c. Biochim. Biophys. Acta **193**, 64–72 (1969)

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Hoofdstuk IV

Control of Respiration of Binucleate and Trinucleate Pollen under humid Conditions

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ABSTRACT

The equal rates of water vapour absorption by both bi- and trinucleate pollen indicate that their widely-differing rates of respiration have an intrinsic, biochemical basis. This was investigated with various metabolic inhibitors that were previously introduced into dry pollen via anhydrous acetone.

The uncoupler, CCCP, inhibited the O_2 uptake of rapidly respiring pollen and stimulated that of slowly respiring types to similar absolute values, that probably reflect the rates of substrate transport across the mitochondrial membranes.

The extent of inhibition of the O_2 uptake by oligomycin, DCCD, antimycin A, and SHAM, alone and in combinations, indicates that hardly any oxidative phosphorylation and anabolic activities occur in slowly respiring, binucleate pollen species, having low-developed mitochondria and high EC values. The presence of the alternative pathway was insignificant.

In other binucleate pollen species, characterized by recognizable mitochondria and low EC values, a limited ATP synthesis was established. The low EC values point to imbalance between phosphorylative and anabolic activities.

In rapidly respiring, trinucleate pollen, containing well-developed mitochondria, a significant activity of the alternative oxidase was found. The EC values were high notwithstanding the large demand for ATP, mounting to 1.7 µmol h^{-1} mg $pollen^{-1}$.

In some pollen species, oligomycin highly stimulated the flow of electrons through the cytochrome pathway, which made an estimation of the ATP synthesis impossible.

Abbreviations: Butyl-PBD, 2-biphenylyl-(4)-5-(4-tert-butylphenyl)-1,3,4-oxadiazol; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; DCCD, N N'-dicyclohexyl carbodiimide; EC, energy charge; RH, relative humidity; SHAM, salicyl hydroxamic acid.

INTRODUCTION

Angiosperm pollen species can be classified into two groups according to whether they contain 2 or 3 nuclei. Many physiological phenomena have been related to this cytological character (Brewbaker, 1957; 1959; 1967; Heslop-Harrison and Shivanna, 1977). Generally, trinucleate pollen respires at much higher rates in humid air than the binucleate type, which is associated with a rapid loss of vitality (Hoekstra and Bruinsma, 1975 b). The phylogenetically advanced trinucleate pollen is equipped with fully developed mitochondria at dehiscence, enabling it to germinate more rapidly *in vitro* and on the stigma (Hoekstra en Bruinsma, 1978). By contrast, binucleate pollen species show various stages of mitochondrial development, connected with delays in germ tube emergence.

It has been suggested that the differences in the rate of respiration in humid air between bi- and trinucleate pollen are determined by the different conditions of their mitochondria (Hoekstra, 1979). Since longevity is far more divergent than respiration, there may be large differences in the efficiency of phosphorylation. Moreover, the data of Okunuki (1939) concerning the respiration of germinating pollen, indicate the occurrence of the less efficient alternative pathway of electron transport.

We have compared the ways of electron flow and the ATP turnover in the two types of pollen under humid conditions prior to germination. This could only be realized by the use of specific inhibitors acting on intact pollen, since experiments with mitochondria, isolated from non-germinated pollen, generally failed owing to isolation difficulties (Hoekstra, 1979).

MATERIALS AND METHODS

Pollen sources, storage, pretreatments, and germination in vitro

One phylogenetically primitive gymnosperm species, and 5 binucleate and 6 trinucleate angiosperm pollen species were selected for respiration studies (Table 1). Collection, storage, pretreatments, and germination were performed as described earlier (Hoekstra and Bruinsma, 1975 a; Hoekstra, 1979). Only highly viable pollen samples were used in the respiration experiments.

Uptake of water vapour

Dry pollen, with a water content of 5 to 8%, was exposed to an atmosphere of 97% RH, containing tritiated water vapour with a specific activity of 3 µCi g^{-1}. At intervals during the incubation at 30°C, 5-mg samples in small cellulose nitrate cups were transferred to an Intertechnique sample oxidizer and immediately combusted. The water recovered from the instrument was collected in 20 ml of a mixture of 700 ml dioxan, 300 ml toluene, 20 g naphthalene, and 7 g butyl-PBD. The radioactivity was determined by scintillation spectrometry with an efficiency of about 35%, using the channels ratio method of quench correction.

The absorption of tritium-labeled water vapour was a better way to estimate the water uptake than the increase in weight, since comparatively small, well-spread samples could be used, which assured the free accessibility of water molecules to the individual grains.

Measurement of respiration

The procedures of pollen incubation in air of 97% RH and measurement of respiration were described earlier (Hoekstra and Bruinsma, 1975 b), except that some improvements were introduced. Samples of 25 mg pollen were incubated in the vessels instead of 5 mg, to enable the determination of the respiratory activity within an hour. To establish unrestricted O_2 supply, the pollen was spread uniformly over a nylon mesh (37 µm pore size diameter), fixed onto a frame that fitted the vessel. Furthermore, the sensitivity of the gaschromatographic detection was enhanced by an additional amplifier, connected with a Solartron digital volt meter, with a maximum and minimum setting.

Rates of O_2 uptake and CO_2 release were calculated by linear regression analysis of 4 measurements during respiration. The error, as expressed by the correlation coefficient, was negligible compared to errors originating from replications within the treatment. Standard deviations refer, therefore, to sample replications.

Extraction and assay of adenylate phosphates

At intervals during incubation in humid conditions, samples of 50 mg pollen were extracted in 0.3 M perchloric acid as described previously (Hoekstra,

1979). ATP was determined with the firefly luciferin-luciferase enzyme system and a liquid scintillation spectrometer. Analyses of ADP and AMP were performed after enzymic conversion into ATP.

Application of inhibitors

The efficiency of oxidative phosphorylation and the occurrence of different pathways of electron transport in 'dry' pollen, respiring in humid conditions, were studied after infiltration of inhibitors via acetone. The soaking of pollen in some organic solvents has been reported not to affect its vitality (Iwanami, 1973; Iwanami and Nakamura, 1972). Prior to the addition of inhibitors, three washings with diethyl ether were performed to remove the sticky, lipophylic material from the pollen wall. Pollen was dried in air at room temperature and mixed for 10 min with anhydrous acetone containing the inhibitor in the ratio of 2 pollen / 3 solvent (w/v). The acetone was removed by vacuum evaporation and the pollen was mixed and sieved to obtain a homogeneous sample. Prior to determining respiration at 30°C, pollen was incubated in 97% RH for 12 h at 4°C and subsequently for 1.5 h at 30°C, allowing for the internal distribution of the inhibitors. Oligomycin was dissolved at a concentration of 200 µg per ml of acetone, CCCP in the range of 0.2 - 200 µg ml^{-1}, antimycin A at 500 µg ml^{-1}, SHAM at 1.5 mg ml^{-1}, and DCCD at 1 mg ml^{-1}. Controls were treated with comparable amounts of anhydrous acetone. Diethyl ether and acetone washes did not exert any significant effect on the rate of respiration in humid conditions as compared to that of non-treated blanks.

Treatment differences were subjected to an analysis of variance, following Bartlett's test of homogeneity of variance (Snedecor and Cochran, 1967).

Reagents

Antimycin A, CCCP, and oligomycin were purchased from Sigma Chemical Comp., St. Louis, MO, USA, SHAM from Aldrich Chemical Comp., Milwaukee, Wis., USA, and DCCD from E. Merck, Darmstadt, W-Germany.

RESULTS

Uptake of water from the humid environment

Differences in respiratory activities in humid air (30°C, RH = 97%) between binucleate and trinucleate pollen species (Table 1) might depend on differences in the rate at which pollen imbibe water from the environment, or on differences in intrinsic metabolic properties. Figure 1 shows that bi- and trinucleate pollen exhibit the same biphasic pattern of water uptake from the surrounding vapour at approximately the same rate.

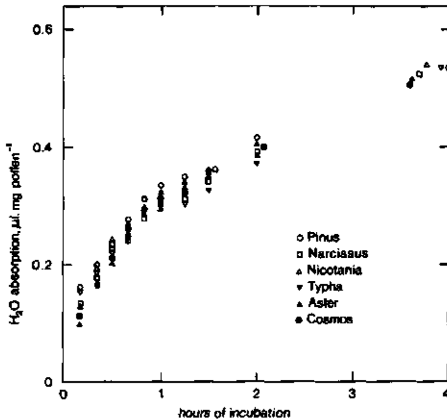

Fig. 1. *Absorption of water by different pollen species from air of 97% RH at 30°C.* The absorption was calculated from the uptake of tritiated water vapour. Values are the averages from two determinations.

Effects of CCCP and oligomycin on respiration

Table 1 shows the effects of CCCP and oligomycin on the O_2 uptake of the rehydrated pollen. The gymnosperm and most of the binucleate pollen species with relatively slow respiration, showed the expected enhancement in respiration upon the addition of the uncoupler CCCP. However, the O_2 consumption of *Tradescantia* and the trinucleate species was inhibited to about the same level as in the afore-mentioned species.

The dose-response curve of CCCP in the respiratory activity of a representative of each of the two groups is shown in Figure 2. Not only *Nicotiana* shows a stimulation of respiration, also the O_2 uptake of the trinucleate *Cosmos* is slightly increased at low concentrations of CCCP. This indicates that the uncoupler is able to produce the expected stimulation of O_2 utilization in rapidly respiring pollen, too. This stimulation, however, is abolished by a much stronger inhibitory effect at concentrations higher than 2 µg ml^{-1}.

Table 1. *Effects of the uncoupler CCCP (20 µg per ml acetone) and oligomycin on the rate of O_2 consumption of pollen in air of 97% RH at 30°C.* Each value is the average from at least 4 replicates.

Species	rate of O_2 uptake (nmol $h^{-1}mg^{-1}$)		
	control	CCCP	oligomycin
Gymnosperms			
Pinus sylvestris L.	71	103[b]	64
Angiosperms			
binucleate			
Narcissus poeticus L.	61	111[b]	66
Typha latifolia L.	65	129[b]	66
Alnus glutinosa Gärtn.	109	163[b]	130[b]
Nicotiana alata Link et Otto	113	232[b]	189[b]
Tradescantia paludosa Anders. et Woods.[a]	256	90[b]	140[b]
trinucleate			
Aster tripolium L.	340	76[b]	521[b]
Tussilago farfara L.	401	100[b]	128[b]
Tanacetum vulgare L.	403	101[b]	114[b]
Aster novi-Belgii L.	411	-	75[b]
Chrysanthemum leucanthemum L.	412	-	130[b]
Cosmos bipinnatus Cav.	589	192[b]	175[b]

a) measured at 22°C, and computed for 30°C
b) significantly different from untreated control at 5% level

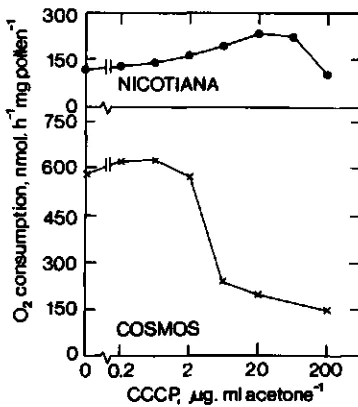

Fig. 2. *Effect of CCCP on the rates of O_2 consumption of trinucleate Cosmos and binucleate Nicotiana pollen incubated in humid air* (RH = 97%, 30°C). Single determinations.

Oligomycin severely inhibited the O_2 uptake of most of the rapidly respiring pollen species (Table 1), indicating an active oxidative phosphorylation. In

addition, increased RQ values pointed to a stimulation of glycolytic activity, which is a well known side effect of this inhibitor of oxidative phosphorylation.

In *Aster tripolium* and in some binucleate species, oligomycin caused stimulation of the O_2 uptake, leaving the question of operation of oxidative phosphorylation undecided. The O_2 uptake of the other, slowly respiring types was not significantly affected by oligomycin. This may be due to a lack of penetration of the inhibitor. To further analyze these oligomycin effects, ATP measurements were performed simultaneously.

Effects of oligomycin on the ATP content

As could be expected for most of the Compositae species and *Tradescantia*, where oligomycin largely inhibits the O_2 utilization, the ATP content steadily decreased (and the ADP and AMP contents proportionally increased) (Figure 3).

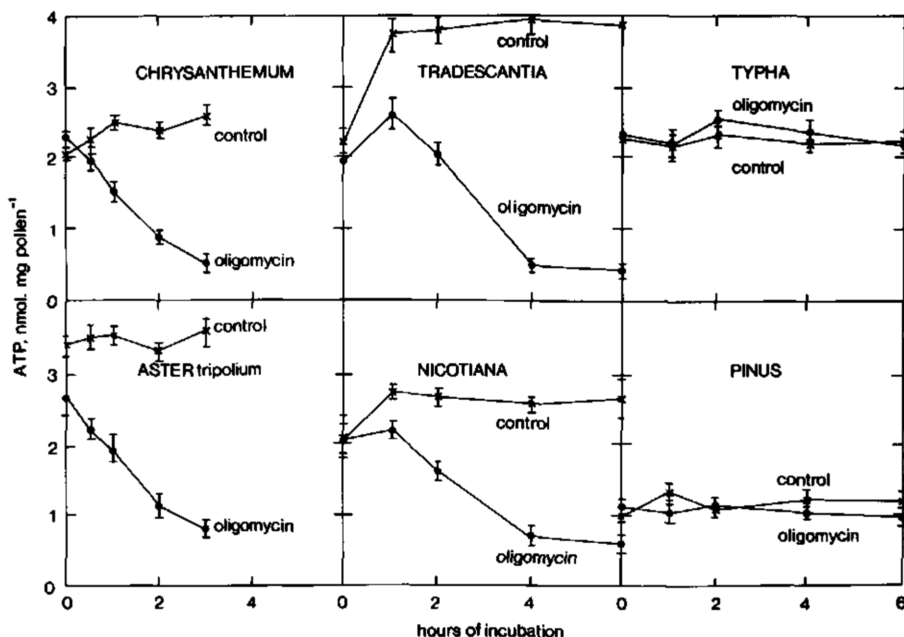

Fig. 3. *Effect of oligomycin on the ATP content of pollen, respiring in humid air* (RH = 97%, 30°C). Each point is the result of triplicate extractions. The standard error of the mean is indicated by bars.

Such decreases in ATP content also occurred in pollen species with an increased rate of oligomycin-mediated O_2 uptake, e.g. *Aster tripolium*, *Alnus*, *Nicotiana*. On the contrary, the ATP level did not change in pollen of *Typha* and *Pinus* with or without oligomycin, which indicates that the occurrence of oxidative phosphorylation remains obscure for at least 6 h from the start of incubation. After 8 h, however, a slight reduction in ATP level was sometimes noticed. With DCCD, a compound acting similarly as oligomycin, this decrease occurred somewhat sooner.

Adenylate energy charge during respiration

The pool sizes of the different adenylate phosphates and the resulting EC values during respiration are shown in Table 2. In *Pinus* and *Typha* pollen, with low rates of respiration and only a slight indication of ATP turnover, the high EC values indicate that the low anabolic activities and the energy-generating sequences are in balance. By contrast, the high EC values of the actively respiring *Aster* and *Chrysanthemum* pollen result from the capacity of the fully developed mitochondria to cope with the large demand for ATP. The low EC values in *Nicotiana* and *Tradescantia*, on the contrary, indicate that the ATP-generating processes are limiting. In these two species, high amounts of ADP and AMP occurred. Artificially, low EC values were obtained when oligomycin induced a decrease in ATP level (Figure 3), with a proportional increase of ADP and AMP.

Table 2. *Amounts of adenylate phosphates after 4 h respiration in humid air* (RH = 97%, 30°C). EC = ATP + ½ADP / ATP + ADP + AMP.

Species	Adenylate phosphates ($nmol\ mg^{-1}$)			EC[a]
	ATP[a]	ADP[a]	AMP[a]	
Pinus sylvestris	1.3 ± 0.0	0.7 ± 0.1	0.0 ± 0.2	0.84 ± 0.07
Typha latifolia	2.1 ± 0.0	1.1 ± 0.1	0.1 ± 0.2	0.80 ± 0.05
Nicotiana alata	1.5 ± 0.2	3.9 ± 0.3	0.6 ± 0.3	0.58 ± 0.03
Tradescantia paludosa	3.3 ± 0.3	3.6 ± 0.4	4.3 ± 0.5	0.46 ± 0.03
Aster tripolium	3.6 ± 0.1	0.6 ± 0.1	0.0 ± 0.2	0.93 ± 0.02
Chrysanthemum leucanthemum	2.7 ± 0.1	1.0 ± 0.1	0.0 ± 0.1	0.86 ± 0.04

a) ± standard error of the mean of triplicate extractions

The alternative pathway

The different behaviour of 2 binucleate and 2 trinucleate pollen species upon treatment with inhibitors of the two respiratory pathways and combinations thereof are shown in Table 3. Because of its specificity for the cytochrome pathway and its solubility in acetone, antimycin A was preferred over cyanide.

A combination of sufficiently high concentrations of SHAM and antimycin A is supposed to block the flow of electrons through the two pathways completely. The difference between this complex inhibition and that of antimycin A alone is indicative of the presence of the antimycin A-resistant, alternative respiration. The nature of the oligomycin stimulation of the O_2 uptake was analyzed by comparing it with the effects of SHAM plus oligomycin and antimycin A plus oligomycin. The combination containing SHAM should at least partly abolish a suspected stimulation of the alternative pathway, whereas the combination with antimycin A should completely inhibit such of the cytochrome pathway. SHAM alone was used for comparison with the combined treatments.

The trinucleate *Cosmos* pollen exhibited the pattern typical of a cell able to show activity of the alternative oxidase, amounting at the highest to 28% of the total respiration. The inhibitory effect of SHAM alone is also consistent with the occurrence of the alternative pathway.

In *Aster* pollen, this activity was hardly significant (8%). The combination

Table 3. *Effects of inhibitors of oxidative phosphorylation, the cytochrome pathway, and the alternative pathway, and combinations thereof, upon the O_2 utilization of binucleate and trinucleate pollen species during incubation in humid conditions* (RH = 97%, 30°C). Each value is the average from 3 determinations. AA stands for antimycin A.

Species	Percentage inhibition						
	oligomycin	AA	SHAM + AA	SHAM	SHAM + oligomycin	AA + oligomycin	LSD *P*=0.05
binucleate							
Typha latifolia	-5	77	85	-5	-2	85	10
Nicotiana alata	-71	38	47	-43	-146	39	10
trinucleate							
Aster tripolium	-57	85	93	-2	-41	86	6
Cosmos bipinnatus	71	70	98	40	81	72	6

of SHAM and oligomycin demonstrates that oligomycin does not induce the alternative pathway. Accordingly, the extent of inhibition by antimycin A, and antimycin A plus oligomycin, show that the stimulation brought about by oligomycin alone is probably due to uncoupling, since the electrons must have passed through the main chain.

The alternative oxidase activity was insignificant in the binucleate pollen. This insignificance was also due to the relatively low rates of respiration. In *Nicotiana* pollen oligomycin, as well as SHAM, caused a considerable O_2 consumption, resulting from the flow of electrons through the cytochrome pathway. However, the combination of antimycin A and SHAM only partly inhibited the O_2 utilization, probably due to a third oxidative pathway of a non-mitochondrial origin.

DISCUSSION

Although the absorption of water vapour is essential for proper swelling and germination (Gilissen, 1977), its rate apparently does not determine the differences in respiratory activity between the various pollen species. Intrinsic biochemical properties, therefore, form the basis of the observed differences.

The further experiments attempt to give information as to how far respiration in humid air of 2- and 3-celled pollen leads to phosphorylation, and how the electron transport is controlled. For this purpose, various metabolic inhibitors were previously infiltrated in the intact grains, and respiration and EC were measured.

Application of CCCP was meant to examine as to how far respiration could be stimulated, a substantial increase indicating that a proton gradient across the inner mitochondrial membrane exists. This is the case with the slowly respiring pollen species, however, it needs not necessarily involve oxidative phosphorylation. Respiration of the rapidly respiring pollen species was severely inhibited by CCCP, with O_2 consumptions of about 100-200 nmol h^{-1} mg^{-1}, similar as with the slowly respiring types. These results can be explained by the fact that the respiratory substrates, succinate, malate and pyruvate, are actively transported across the inner mitochondrial membrane (Day and Hanson, 1977 a; 1977 b). True uncouplers such as CCCP bring about a collapse of the proton gradient across the membrane and stimulate ATPase activity, resulting in an impaired transport of these substrates, and inhibition of respiration under conditions of high energy

demand. Such high energy demand occurred in the trinucleate Compositae pollen and, to a lesser extent, in the binucleate pollen of *Tradescantia*. The similar rates of O_2 uptake, therefore, may well reflect diffusion characteristics of substrates across the membrane. In the rapidly respiring pollen this means inhibition of respiration, probably due to substrate limitation, and in the slowly respiring ones a stimulation caused by an enhanced rate of electron flow.

Inhibitors of oxidative phosphorylation turned out to be far more suitable for establishing the turnover of ATP. Oligomycin and DCCD are known to inhibit ATP synthesis in mitochondrial suspensions, where they also abolish the phosphorylation-linked O_2 consumption (Slater and Ter Welle, 1969).

In most of the trinucleate Compositae species, oligomycin caused a considerable reduction both in the rate of O_2 uptake and in the ATP content, demonstrating that turnover of ATP is required for anabolic processes. The oligomycin-inhibited part of the O_2 consumption allows for an estimation of the ATP synthesis on the basis of a P/O ratio of 3. This synthesis amounted to at least $0.7 \times 400 \times 3 \times 2 = 1680$ nmol ATP per hour per mg Compositae pollen, that showed an inhibition of about 70% of the average O_2 consumption of 400 nmol h^{-1} mg^{-1} (derived from Table 1). For *Tradescantia* pollen, having an oligomycin inhibition of about 55%, a net synthesis of at least 840 nmol ATP h^{-1} mg^{-1} was calculated.

Contrary to the expectations, stimulation of O_2 uptake was observed in *Aster tripolium*, *Nicotiana* and *Alnus* pollen, coupled with decrease of their ATP contents. This offers evidence for the active turnover of ATP in these pollen species but their rate of ATP synthesis could not be estimated properly. The rapid adjustment to a higher ATP level in the controls of *Tradescantia* and *Nicotiana* (Figure 3), resulted from the establishment of a new EC due to transfer of the pollen from $4^{\circ}C$ to $30^{\circ}C$, and is also an indication of ATP turnover.

It remains obscure whether the absence of effects on O_2 uptake and ATP content in *Typha* is due to the slow penetration of oligomycin or to the absence of oxidative phosphorylation. Because of the low respiratory activity in this pollen, a constant ATP level could very well have been maintained by, e.g., glycolysis-linked phosphorylations, leaving a limited ATP-requiring anabolism undetected. The slight reduction in ATP level, sometimes observed after 8 h of incubation with oligomycin, and the somewhat more rapid effect of DCCD indicate that slow penetration of the compounds may also be involved to some extent.

The absence of direct oligomycin effects on respiration and ATP content of

Typha and *Pinus* pollen drew our attention to a possible role of the less efficient alternative pathway. Moreover, its involvement in the stimulated rate of oligomycin-mediated O_2 uptake could well have been explained by an indirect effect of AMP, that was reported to have induced the cyanide-insensitive pathway in the yeast *Moniliella* (Hanssens and Verachtert, 1976). However, only the main respiratory chain was involved when oligomycin stimulated the O_2 uptake (Table 3).

The alternative pathway is routinely demonstrated by the use of specific inhibitors, e.g. SHAM (Schönbaum et al., 1971; Henry and Nyns, 1975). A way to calculate the percentage of CN^--insensitive O_2 consumption is to take the SHAM-sensitive respiration as the proportion of the total inhibition by a combination of HCN and SHAM (Rissler and Millar, 1977). In intact cells, however, hydroxamic acids are reported to stimulate O_2 consumption (Harley et al., 1977). We observed a SHAM-mediated stimulation in pollen of *Nicotiana*. Such effects make the use of SHAM alone unfit for demonstration of the alternative oxidase in intact cells. Therefore, we calculated the contribution of this pathway from the difference in inhibition of O_2 consumption between antimycin A and SHAM plus antimycin A. This difference represents the maximum activity, since inhibition of the cytochrome pathway may result in stimulation of the alternative pathway (Bahr and Bonner, 1973). Recently, Lambers and Smakman (1978) showed *in vivo* activity of the alternative oxidase in young *Senecio* roots. On the other hand, using another method, Theologis and Laties (1978) found the *in vivo* contribution of the cyanide-insensitive pathway to be zero in aged potato slices which were demonstrated to contain the alternative oxidase. We found the presence of antimycin A-insensitive respiration only in the trinucleate pollen of *Aster tripolium* and *Cosmos bipinnatus*. The maximum contribution to the overall O_2 consumption was 8 and 28%, respectively. Because in the slowly respiring pollen species the alternative pathway was insignificant, it cannot have interfered with the energy conservation.

Although ATP synthesis of slowly respiring pollen species could not be estimated properly, we assume that it occurs in conformity with their rates of respiration. The large divergence in longevity among pollen species can then be ascribed to highly advanced anabolic syntheses, requiring the ATP. A rapid loss of vitality which occurs under humid conditions, probably results from inadequate syntheses rather than from loss of substrate. This is strongly suggested by the observation that respiration continues steadily in Compositae pollen for a considerable period after the germination capacity is lost (Hoek-

stra and Bruinsma, 1975 b). In nature, this imminent danger of loss of vitality may have led to the daily release of fresh pollen from newly opened florets (Hoekstra and Bruinsma, 1978).

Establishing ATP turnover in combination with EC values offers the opportunity to discern whether activities in respiring pollen are controlled by respiratory or anabolic sequences. *Nicotiana* and *Tradescantia* pollen were restrained in their respiratory activity, which point to either a poor differentiation of mitochondria or the presence of endogenous inhibitors of the respiratory chain, which are reported to be widely distributed in the plant kingdom (Rapoport and Schewe, 1977). Although this remains uncertain in *Nicotiana*, such inhibitors in *Tradescantia* pollen can be anticipated since the mitochondria are fully developed and the EC rises immediately upon immersion in the germination medium (Hoekstra, 1979). We suggest that they allow for active maintenance of vitality in pollen prepared for a rapid anabolism. This may be considered as another approach to accelerate fertilization and to shorten the independent life of the male gametophyte, probably without the necessity for daily release of fresh pollen as in Compositae.

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REFERENCES

Bahr, J.T., Bonner, W.D.: Cyanide-insensitive respiration. I. The steady states of skunk cabbage spadix and bean hypocotyl mitochondria. J. Biol. Chem. *248*, 3441-3445 (1973)

Brewbaker, J.L.: Pollen cytology and self-incompatibility systems in plants. J. Hered. *48*, 271-277 (1957)

Brewbaker, J.L.: Biology of the angiosperm pollen grain. Ind. J. Gen. Pl. Breed. *19*, 121-133 (1959)

Brewbaker, J.L.: The distribution and significance of binucleate and trinucleate pollen grains in the angiosperms. Amer. J. Bot. *54*, 1069-1083 (1967)

Day, D.A., Hanson, J.B.: Effect of phosphate and uncouplers on substrate transport and oxidation by isolated corn mitochondria. Plant Physiol. *59*, 139-144 (1977 a)

Day, D.A., Hanson, J.B.: Pyruvate and malate transport and oxidation in corn mitochondria. Plant Physiol. *59*, 630-635 (1977 b)

Gilissen, L.J.W.: The influence of relative humidity on the swelling of pollen

grains in vitro. Planta *137*, 299-301 (1977)
Hanssens, L., Verachtert, H.: Adenosine 5'-monophosphate-stimulated cyanide-insensitive respiration in mitochondria of *Moniliella tomentosa*. J. Bacteriol. *125*, 829-836 (1976)
Harley, J.L., McCready, C.C., Wedding, R.T.: Control of respiration of beech mycorrhizas during ageing. New Phytol. *78*, 147-159 (1977)
Henry, M.F., Nyns, E.J.: Cyanide-insensitive respiration. An alternative mitochondrial pathway. Sub-Cell. Biochem. *4*, 1-65 (1975)
Heslop-Harrison, Y., Shivanna, K.R.: The receptive surface of the angiosperm stigma. Ann. Bot. *41*, 1233-1258 (1977)
Hoekstra, F.A.: Mitochondrial development and activity of binucleate and trinucleate pollen during germination in vitro. Planta *145*, 25-36 (1979)
Hoekstra, F.A., Bruinsma, J.: Viability of Compositae pollen: Germination *in vitro* and influences of climatic conditions during dehiscence. Z. Pflanzenphysiol. *76*, 36-43 (1975 a)
Hoekstra, F.A., Bruinsma, J.: Respiration and vitality of binucleate and trinucleate pollen. Physiol. Plant. *34*, 221-225 (1975 b)
Hoekstra, F.A., Bruinsma, J.: Reduced independence of the male gametophyte in angiosperm evolution. Ann. Bot. *42*, 759-762 (1978)
Iwanami, Y.: Acceleration of the growth of *Camellia sasanqua* pollen by soaking in organic solvent. Plant Physiol. *52*, 508-509 (1973)
Iwanami, Y., Nakamura, N.: Storage in an organic solvent as a means for preserving viability of pollen grains. Stain Technol. *47*, 137-139 (1972)
Lambers, H., Smakman, G.: Respiration of the roots of flood-tolerant and flood-intolerant *Senecio* species: Affinity for oxygen and resistance to cyanide. Physiol. Plant. *42*, 163-166 (1978)
Okunuki, K.: Über den Gaswechsel der Pollen II. Acta Phytochim. *11*, 27-64 (1939)
Rapoport, S., Schewe, T.: Endogenous inhibitors of the respiratory chain. Trends Biochem. Sci. *2*, 186-189 (1977)
Rissler, J.F., Millar, R.L.: Contribution of a cyanide-insensitive alternate respiratory system to increases in formamide hydrolyase activity and to growth in *Stemphylium loti in vitro*. Plant Physiol. *60*, 857-861 (1977)
Schönbaum, G.R., Bonner, W.D., Storey, B.T., Bahr, J.T.: Specific inhibition of the cyanide-insensitive respiratory pathway in plant mitochondria by hydroxamic acids. Plant Physiol. *47*, 124-128 (1971)
Slater, E.C., Ter Welle, H.F.: Applications of oligomycin and related inhibitors in bioenergetics. In: Inhibitors tools in cell research, pp 258-278, Bücher, Th., Sies, H. eds. Berlin-Heidelberg-New York: Springer 1969
Snedecor, G.W., Cochran, W.G.: Statistical methods. Ames, Iowa, USA: The Iowa State Univ. Press, 1967
Theologis, A., Laties, G.G.: Relative contribution of cytochrome-mediated and cyanide-resistant electron transport in fresh and aged potato slices. Plant Physiol. *62*, 232-237 (1978)

Hoofdstuk V

Protein Synthesis of Binucleate and Trinucleate Pollen and its Relationship to Tube Emergence and Growth

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ABSTRACT

Under humid conditions both bi- and trinucleate pollen species incorporate very low amounts of leucine, 0.4 pmol min^{-1} mg $pollen^{-1}$ on an average. During germination *in vitro*, however, the two types of pollen greatly differ in their capacity for protein synthesis.

Binucleate pollen species such as *Typha*, which are characterized by slow respiration in humid air and prolonged lag periods during germination *in vitro*, contain large amounts of monoribosomes at dehiscence. Polyribosomes are formed soon after the pollen is wetted in the germination medium and a considerable incorporation of leucine is initiated after 10-15 min.

More rapidly respiring, binucleate pollen, such as *Tradescantia*, showing a short lag period, may contain many polysomes at dehiscence already and incorporates leucine within 2 min of incubation.

On the contrary, rapidly respiring, trinucleate Compositae pollen contains very limited amounts of ribosomal material and never attains any substantial level of incorporation.

Cycloheximide completely inhibited both protein synthesis and tube emergence and growth in the slowly respiring binucleate pollen species. The more rapidly respiring types are less dependent on protein synthesis, while germination of the phylogenetically advanced, trinucleate Compositae pollen proceeds completely independently.

It is concluded that the level of phylogenetic advancement of the male gametophyte is characterized by its overall state of metabolic development at dehiscence rather than by the number of its generative cells.

Abbreviations: BSA, bovine serum albumin; CHI, cycloheximide; EGTA, ethyleneglycol-bis(β-aminoethyl ether) N,N'-tetra-acetic acid; RH, relative humidity; TCA, trichloroacetic acid.

INTRODUCTION

Incubation in humid air allows pollen to absorb water vapour and to exert metabolic activities. In general, under these conditions the phylogenetically advanced, trinucleate pollen species respire at much higher rates than binucleate ones, a phenomenon associated with rapid loss of vitality (Hoekstra and Bruinsma, 1975). In trinucleate Compositae pollen, this rapid respiration is connected with a high turnover of ATP (Hoekstra and Bruinsma, 1979).

Trinucleate pollen contains fully developed mitochondria at dehiscence, allowing for a rapid germination (Hoekstra, 1979). In contrast, many binucleate pollen species have far less developed mitochondria; these are further assembled during a lag phase prior to emergence of the pollen tube. Intermediate types also occur which are binucleate, but equipped with fully developed mitochondria, e.g. *Tradescantia*. Their respiration in humid conditions is restrained, but when immersed in the germination medium they produce pollen tubes within a few minutes (Hoekstra and Bruinsma, 1979).

It is interesting to know in how far the stage of development of mitochondria at pollen dehiscence is accompanied by an analogous condition of the protein-synthesizing apparatus. Protein synthesis during the lag period on the stigma might be particularly essential for tube growth in pollen species having less developed mitochondria. Literature data on the presence of polysomes in fresh pollen and the effect of CHI on tube growth, as reviewed by Mascarenhas (1975), are rather confusing and indicate that, indeed, many differences exist among pollen species.

In this paper we describe protein synthesis and the occurrence and assembly of polysomes in pollen species with widely differing respiratory capacities. We also studied to what extent protein synthesis requires the energy generated during respiration in humid air.

MATERIALS AND METHODS

Pollen sources, collection, storage, pretreatments, and germination in vitro

Pollen species were selected on the basis of widely differing stages of mitochondrial development at dehiscence and rates of respiration in humid air (Hoekstra, 1979; Hoekstra and Bruinsma, 1975): the binucleate species *Typha*

latifolia L., *Nicotiana alata* Link et Otto, and *Tradescantia paludosa* Anders. et Woods., and the trinucleate species *Aster tripolium* L., *Chrysanthemum leucanthemum* L., and *Cosmos bipinnatus* Cav.. Pollen collection, storage, pretreatments, and germination *in vitro* have been described elsewhere (Hoekstra, 1979), *Chrysanthemum* and *Cosmos* pollen were germinated in the same way as *Aster*. Only highly viable pollen samples were used.

Extraction and determination of free leucine pools

Lots of 200 mg, originally dry pollen were homogenized in a teflon-glass Potter homogenizer for 2 min in 4 ml icecold extraction medium consisting of 70% (v/v) ethanol, 1% (v/v) thiodiglycol and 0.7 mg citric acid ml^{-1} (Linskens and Schrauwen, 1969). After centrifugation of the homogenates for 10 min at 7000 *g* ($2^{o}C$), the pellets were re-extracted with 2 ml of the extraction medium, and the washings centrifuged. The combined supernatants were shaken with three volumes of chloroform, and after spinning at 1000 *g* for 10 min the aqueous layers containing the amino acids were pipetted from above the interphase and frozen at $-20^{o}C$ until analysis. Prior to the extraction, 0.57 µmol norleucine was added as an internal standard.

Pollen, incubated in the germination medium at a density of 20 mg ml^{-1}, was homogenized in such amounts of extraction medium that the final concentration of ethanol reached 70% (v/v). Sugars were subsequently removed by passing the extract over a Dowex 50X8, 20-50 mesh column according to Stein (1953). Eluates were evaporated in a rotating vacuum evaporator at $35^{o}C$.

The concentrated fractions were then passed through an automatic amino acid analyzer and the free leucine determined directly from the recorder tracings. From each pollen species duplicate extractions and determinations were performed at intervals during incubation in humid air and in the germination medium.

Amino acid composition of soluble proteins

Aliquots of pollen were suspended in a buffer containing 0.15 M NaCl and 0.1 M NaEDTA, pH 8.0, (Hess et al., 1974), immediately frozen and completely disrupted in a precooled, X-press cell desintegrator (LKB, Bromma, Sweden). After thawing, the homogenate was centrifuged at 40,000 *g* for 30 min, and the supernatant dialyzed overnight against 0.01 M phosphate buffer, pH 6.9. The soluble proteins were lyophilized and submitted to hydrolysis for 24 h at

105°C in 6 N HCl prior to analysis in the amino acid analyzer. Operations were performed at 2-4°C.

Leucine incorporation into protein

Suitable amounts of (4,5-^{3}H)L-leucine in a 2% ethanolic solution (The Radiochemical Centre, Amersham, U.K.), with a specific activity of about 55 Ci mmol^{-1}, were frozen in a precooled scintillation vial and subsequently lyophilized to near dryness. Immediately, anhydrous acetone was added, thoroughly mixed, and the homogeneous distribution of radioactivity assessed by combustion of samples in a Packard sample oxidizer and subsequent counting in a Nuclear Chicago, Mark I, liquid scintillation spectrometer. The acetone containing the radioactive leucine was added to diethyl ether-washed, dry pollen in the ratio 3 acetone / 2 pollen (v/w). After 10 min, the acetone was removed by vacuum evaporation, with the vial only slightly opened to prevent pollen from being blown away. The dry pollen was thoroughly mixed in the closed vial, and tested for both homogeneous distribution of radioactivity and germination capacity. Samples showing heterogeneous distribution of radioactivity, or loss of vitality caused by traces of water in the acetone (Iwanami and Nakamura, 1972), were discarded. On the basis of an average pool size of about 1 nmol per mg pollen, the specific radioactivity of the (^{3}H)-leucine was calculated to be reduced to 30 mCi per mmol as a result of the mixing with the endogenous pool of free leucine. This means that about 66,000 dpm (^{3}H)-leucine was infiltrated per mg pollen without substantially contributing to the leucine content.

Triplicate samples of 30 mg of radioactive pollen were incubated on slides, either directly in an atmosphere of 97% RH at 30°C, or for 12 h in RH = 97% at 4°C prior to germination *in vitro* (24°C) at a density of 20 mg ml^{-1}. Alternatively, comparable amounts of labeled leucine were pipetted directly into the germination medium. Interference with the incorporation by possible microbial contamination could be ruled out since germination media were filter sterilized, and the previous ether washings practically freed pollen from microorganisms.

Incorporation of labeled leucine was terminated by adding 15 ml of an ice-cold 10% (w/v) TCA solution containing 40 mM cold leucine. Small amounts of BSA solution (0.3%) were added to promote coagulation. After 30 min at 4°C, the precipitated protein and pollen grains were collected by centrifugation, washed once with icecold 5% (w/v) TCA washing solution containing 10 mM leucine,

heated for 15 min at 90°C, chilled on ice, and collected on 0.6 µm, pore-size membrane filters (Sartorius, Göttingen, West Germany, no. SM 11605). After three more washings with 5% TCA and one with 80% ethanol, the filters were dried at 70°C. Filters and contents were combusted in a Packard sample oxidizer, and the $^{3}H_2O$ recovered was mixed with Packard Monophase 40 scintillation cocktail. Tritium was counted with an efficiency of 31-36%, using the channels ratio method of quench correction. The radioactivity in non-incubated controls was entirely washed out by the procedure mentioned above. Application of the method of combustion circumvented previous homogenization of the pollen.

CHI was infiltrated into the dry pollen together with the labeled leucine, at a concentration of 250 µg per ml acetone; in the germination medium, the concentration was 100 µg ml^{-1}.

The incorporation of leucine was estimated considering each periodical increase in radioactivity of the acid-insoluble protein fraction to be the result of the labeling in the interval between two samplings, taking into account the specific activity of leucine at each interval. The method of calculation applied was mathematically elaborated by Swedes and Agnew (1975). Possible degradation of freshly labeled protein was not taken into account, since the amount of *de novo* synthesized proteins was negligible compared to that already present in the grains, and protein profiles of newly synthesized proteins and those already present did not differ (Frankis and Mascarenhas, 1978).

Polysome isolation and fractionation

The equivalent of one gram dry pollen was separated from the germination medium by filtration, mixed with 10 ml of icecold high salt isolation buffer which prevents RNase action (Davies et al., 1972) and immediately frozen in liquid N_2. The isolation buffer for both free and membrane-bound polysomes consisted of 0.15 M KCl, 50 mM $MgCl_2$, 0.2 M sucrose, 5 mM 2-mercaptoethanol, and 1% Triton X-100 (v/v), in 0.2 M Tris-HCl buffer, pH 9.0, adapted according to Jackson and Larkins (1976). Finally EGTA, previously adjusted to pH 9.0 with KOH, was added to a concentration of 25 mM. Non-germinated control samples were directly mixed with the isolation medium.

The frozen suspensions were passed through a precooled X-press cell desintegrator, and the disrupted mass was thawed. Recovery of membrane-bound polysomes was enhanced by magnetic stirring of the homogenate for 10 min at 0°C prior to centrifugation for 10 min at 30,000 *g*. The supernatant was immediately

layered on a 2 ml pad of 1.75 M sucrose in gradient buffer (see below) for a rapid separation of polysomes from the ribonuclease activity in the medium. After centrifugation at 280,000 *g* for 90 min at 2-4°C in the Ti-52 swing-out rotor of a Hereaus-Christ Omega II ultracentrifuge, the supernatant was removed by suction and the pellet resuspended in a minute amount of gradient buffer. Occasionally, a supplementary cycle of 10 min at 10,000 *g* was required to remove large particles contaminating the polysome preparation. The polysome suspension was either immediately layered upon sucrose gradients or stored at -196°C. The susceptibility of polysome preparations to pancreatic ribonuclease A was tested by incubation with 5 μg ml^{-1} for 10 min at 30°C.

Suitable amounts of polysomes were centrifuged at 230,000 *g* for 90 min at 2-4°C in a Ti-40 swing-out rotor through gradients formed by layering 7 portions of 1.8 ml buffer containing 0.20, 0.44, 0.67, 0.91, 1.14, 1.37, and 1.61 M sucrose in 14 ml cellulose nitrate tubes. The gradient buffer contained 0.1 M KCl and 30 mM $MgCl_2$ in 40 mM Tris-HCl buffer, pH 8.5, and 5 mM EGTA that was dissolved in buffer and adjusted to pH 8.5 prior to addition. Gradients were allowed to equilibrate for 40 h at 0°C before use. Profiles were scanned by passing the gradients through an LKB Uvicord III absorptiometer, with the absorbance monitored at 254 nm.

Since monosomes and subunits are easily lost in the sucrose pad (Larkins and Davies, 1975), ribosomal material was also collected by centrifugation without a pad at 280,000 *g* for 2 h which, however, resulted in an almost complete degradation of polysomes. After gradient centrifugation, the amounts of ribosomal material recovered were quantitatively evaluated by determining the weight of the paper cut from the area's under the peaks, and establishing a relationship between weight of paper and absorbance at 254 nm.

Sedimentation values of the various particles were determined with the Schlieren optics accessory to discern the positions of the small and large ribosomal subunits, and monosomes in the polysome profiles. After correction for the sucrose present in the solution (Clark, 1976), S-values were found to be 39.2, 60.5, and 80.9, respectively, which will be arbitrarily designated 40, 60, and 80, in the figures.

RESULTS

Changes in the pool size of free leucine

In nearly all pollen species tested, the pool size of free leucine during

Table 1. *Effects of incubation in humid air and in the germination medium on the pool size of free leucine in bi- and trinucleate pollen.*

Species	free leucine pool (nmol mg pollen^{-1})		
	non-germinated	incubated for 6 h in humid air	germination *in vitro* for 20 min at 24°C
binucleate			
Typha latifolia	1.07	1.26	1.06
Nicotiana alata	1.94	2.02	2.01
Tradescantia paludosa	0.64	1.32	2.34
trinucleate			
Aster tripolium	0.88	1.10	1.69

incubation in both humid air and germination medium increased (Table 1). For incorporation experiments, this implicated an accelerated decrease of the specific radioactivity of leucine, resulting in a gradual underestimation of the rate of protein synthesis. The correction method, mentioned in Materials and Methods compensated for possible discrepancies. In no case, however, would the interpretation of results have been different if the corrections for changes in specific activity had not been made.

Leucine incorporation during respiration in humid air

Figure 1 shows the extent of protein synthesis in both binucleate and trinucleate pollen species during respiration in humid air. Although the pollen had not previously been incubated in RH = 97% at 4°C, constant rates of incorporation were observed already after 2 h of incubation, strongly inhibited by CHI. In contrast to all the other pollen species, *Tradescantia* showed hardly any incorporation at all. Originally, this was thought to be the result of desiccation. However, fresh pollen, containing 21% water, which retained vitality despite washing and application of label in diethyl ether, also failed to incorporate (^{3}H)-leucine during respiration in humid air, as opposed to an immediate incorporation during germination *in vitro* (cf Fig. 2).

From the curves the rate of leucine incorporation was calculated to be approximately 0.44 pmol min^{-1} per mg *Aster* pollen; 0.22 pmol min^{-1} per mg *Typha* pollen; for *Nicotiana* initially 0.13 pmol min^{-1}mg^{-1} up to 4 h, whereafter the rate fur-

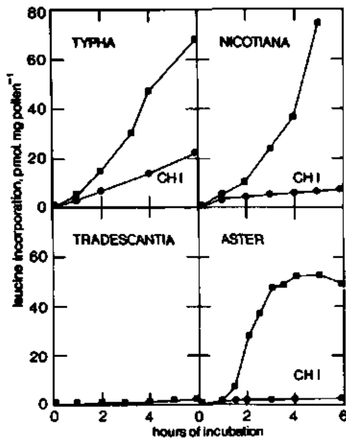

Fig. 1. *Incorporation of (^{3}H)leucine into hot TCA-insoluble material, of different pollen species during incubation in air of* 97% RH *at* 30°C, *with and without CHI. Tradescantia* pollen was incubated at 22°C, because of its low resistance against high temperatures. Data are the average from 3 experiments.

ther increased to 0.80 pmol min^{-1} mg^{-1}; and 0.01 pmol min^{-1} mg^{-1} for *Tradescantia*. Analyses of the amino acids in soluble proteins revealed leucine to participate in the total amino acid composition for 9, 7½, 6, and 7½ mole per cent in the cases of *Typha*, *Nicotiana*, *Tradescantia* and *Aster* pollen, respectively. Rates of leucine incorporation may thus be translated into moles of peptide bonds produced per unit of time, assuming that proteins are synthesized with a similar average composition as those already present in the grains at dehiscence (Mascarenhas et al., 1974). Since ultimately about 5 moles of nucleotide triphosphates are required per mole of amino acid bound (Penning de Vries, 1975), this means that $0.44 \times \frac{100}{7\frac{1}{2}} \times 5 = 29$ pmol ATP min^{-1} will sustain protein synthesis in *Aster* pollen. This is only one per thousand of the rate of ATP generation in Compositae (Hoekstra and Bruinsma, 1979).

Leucine incorporation during germination in vitro

Much higher rates of leucine incorporation occurred during pollen germination *in vitro*, except for the trinucleate *Aster* and *Chrysanthemum* (Fig. 2). The related rates are indicated in Table 2. CHI reduced incorporation more than 99% in each case. Application of labeled leucine straight into the medium led to rates very similar to those shown in Fig. 2. However, previous infiltration of (^{3}H)-leucine and subsequent equilibration in humid air for 12 h at 4°C, was essential for the detection of rapidly labeled protein in the early stages of germination *in vitro*. *Tradescantia* pollen showed incorporation already within 2 min of incubation in the medium (Fig. 2). *Nicotiana* and *Typha* started more

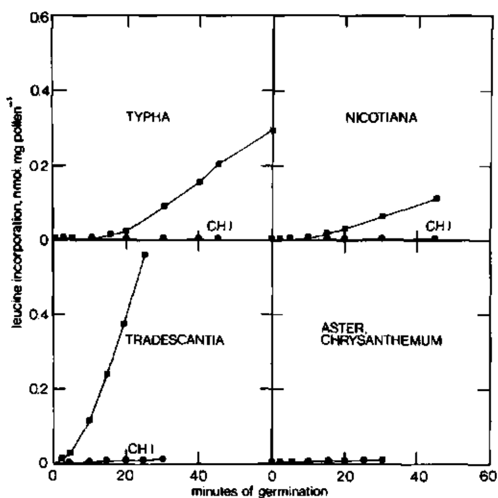

Fig. 2. *Incorporation of (^{3}H)leucine into hot TCA-insoluble material, of germinating bi- and trinucleate pollen species at 24°C, with and without CHI.* Prior to incubation in the germination medium, the label was infiltrated and redistribution was allowed for 12 h in humid air at 4°C. Values are the averages from 3 experiments.

slowly but ultimately incorporated more leucine prior to the outgrowth of their tubes because of longer lag phases (Table 2).

Since the time required for the incorporation to start might be indicative of the condition of the protein-synthesizing apparatus at dehiscence, the occurrence of polysomes was analyzed at intervals during germination *in vitro*.

Table 2. *Rates and total amounts of leucine incorporation in bi- and trinucleate pollen during the lag phase of germination in vitro.* Pollen species are arranged according to their respiratory activity in humid conditions. The effects of CHI on germination are also indicated.

Species	O_2 uptake (humid air), nmol h^{-1} mg^{-1}	Lag phase of germination (min)	Leucine incorporation during lag phase		Germination in CHI (100 µg ml^{-1})
			rate, pmol min^{-1} mg^{-1}	total pmol mg^{-1}	
Typha latifolia	65	70	5.5	375	-
Nicotiana alata	113	35	3.5	87	±
Tradescantia paludosa	256	6	27.0	38	+
Aster tripolium	340	3	0.2	0.8	++

During incubation of the pollen species in humid air an increase in the amount of polyribosomes was never noticed.

Fig. 3 shows polysome profiles from *Typha* pollen during germination *in vitro*. The germinating pollen contained polysomes as evidenced by the observation that RNase treatment removed the heavier particles from the profile in favour of the 80 S material. At the start of germination, no recognizable polysomes were observed, but assembly had begun already after 5 min. However, the presence of a few polysomes in non-germinated *Typha* pollen was demonstrated by the slight increase of the 80 S peak after treatment with RNase. These polysomes accounted for only 0.23% of the ribosomal material recovered from the gradient in Table 3.

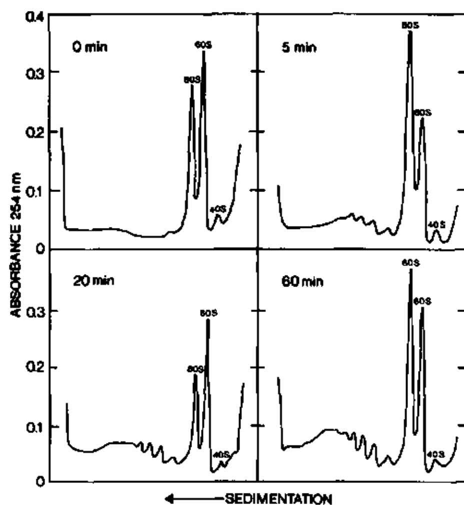

Fig. 3. *Polysome profiles from Typha pollen at intervals during germination in vitro at* 24°C. The amounts applied correspond to 350 mg, originally dry, pollen in each case.

The pollen of *Nicotiana* exhibited a slightly more rapid assembly of polysomes than that of *Typha*.

In contrast, *Tradescantia* pollen contained many polysomes already in the non-germinated condition (Fig. 4). This explains the extremely rapid incorporation of label upon incubation in the germination medium. Similarly, in other experiments it was observed that non-germinated, trinucleate maize pollen also contains a large amount of polysomes.

From non-germinated Compositae pollen, neither recognizable polysome profiles

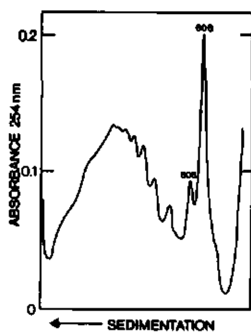

Fig. 4. *Polysome profile from* 220 mg *non-germinated Tradescantia pollen.*

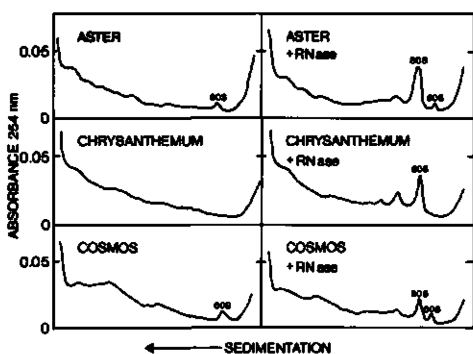

Fig. 5. *Polysome profiles from non-germinated, trinucleate Compositae pollen, with and without RNase treatment.* Upon the gradient, ribosomal material was layered from 320, 250, and 220 mg dry pollen of *Aster*, *Chrysanthemum*, and *Cosmos*, respectively.

nor monosomes could be recovered; also subunits were scarcely noticed (Fig. 5). RNase treatment, however, demonstrated that some polysomal material did occur. Scans of gradients from the germinated samples resembled those from non-germinated pollen, indicating that a detectable assembly of polysomes does not occur during germination of Compositae pollen. Freshly collected *Aster* and *Chrysanthemum* pollen samples were also analyzed to prevent possible errors due to desiccation and storage. However, the scans were similar as for the stored material. Therefore, Compositae are not expected to perform any appreciable protein synthesis during germination. The low values in Fig. 1 and 2 are in accord with this conclusion.

For the comparison of amounts of ribosomal material recovered from the different species of pollen, degraded polysomes, monosomes, and subunits were centrifuged over gradients, collected and measured spectrophotometrically as indicated in Table 3. In the two Compositae species, indeed, relatively small amounts of ribosomal material were present at a low A 260 / A 280 ratio. Com-

Table 3. *Comparison of the amounts of ribosomal material in 2 bi- and 2 trinucleate pollen species.* The material was recovered from gradients after previous centrifugation without a pad. Purity of the preparation is indicated by the ratio $A_{260} : A_{280}$.

Species	Amounts of ribosomes in absorbance units (260 nm) per gram dry pollen	$A_{260} : A_{280}$
Typha latifolia	20.2	1.92
Nicotiana alata	53.6	2.00
Aster tripolium	2.2	1.50
Chrysanthemum leucanthemum	2.0	1.62

parison with the weight of the paper cut from the areas under the peaks learned, that after subtraction of an underlying, slowly sedimenting peak of non-ribosomal origin, with an A_{260} / A_{280} ratio of 1.07, the remaining ribosomal peaks accounted for only 1.2 absorption units per gram of both *Aster* and *Chrysanthemum* pollen. This indicates that the trinucleate Compositae pollen contains only about 5% of the ribosomal material present in the binucleate *Typha*.

Importance of protein synthesis for tube emergence and growth

During germination of Compositae pollen, hardly any leucine incorporation was demonstrated. Since tubes emerge about 3 min after immersion in the medium, protein synthesis cannot play an important role in tube emergence and growth. Accordingly, CHI, even applied at 1 mg ml^{-1}, did not interfere with these processes (Table 2). However, the more protein was synthesized during the lag period in the different pollen species, the more severely CHI inhibited tube emergence and growth (Table 2). With the considerable incorporation of 375 pmol leucine per mg pollen during the lag period, *Typha* pollen did not even show short protrusions at 100 µg CHI per ml medium. In contrast, short tubes were formed by *Nicotiana* pollen, but growth was readily arrested. In *Tradescantia*, pollen emergence and early tube growth were observed to be rather insensitive to CHI, but at later stages the growth rate diminished.

DISCUSSION

The present paper attempts to relate the development of the mitochondrial respiratory system to the state of the protein-synthesizing apparatus in pollen before and during germination. For this purpose, the leucine incorporation and the presence of (poly)ribosomes were followed.

An increase in the free leucine pool, comparable with what we found after 6 h of incubation in humid air at 30°C, was described for maize pollen during storage for some days in humid air at 2°C (Linskens and Pfahler, 1973). Since in all pollen species tested protein synthesis was very limited during respiration in humid air, the pool size is not likely to be reduced under these conditions. During germination, however, the amount of free leucine will be determined by the rates of its synthesis or liberation, on the one hand, and incorporation, on the other hand.

An important assumption in the incorporation experiments is the homogeneous distribution of label after previous infiltration into the grain. Linskens and Schrauwen (1969) reported that a rapid release of free amino acids from germinating pollen occurs within 1 min after immersion in the germination medium. It is plausible, therefore, that the leucine if not yet homogeneously distributed rapidly diffuses during the absorption of water vapour.

Calculations indicate that in Compositae pollen the energy required for protein synthesis in humid air is negligible compared to the amount of ATP produced (Hoekstra and Bruinsma, 1979). Other biosyntheses must therefore be connected with this ATP production. Also in *Typha* and *Nicotiana* the amounts of ATP required for protein synthesis were low.

The extremely low incorporation in humid air of *Tradescantia* pollen is particularly surprising, since a large amount of polysomes is present. An explanation might be that protein synthesis is actively repressed by a similar inhibitor as suggested earlier for dormant *Artemia* cysts (Grosfeld and Littauer, 1975). Also, the mitochondrial respiratory system in *Tradescantia* pollen was shown to be partially repressed (Hoekstra and Bruinsma, 1979).

Linskens et al. (1970) demonstrated the gradual assembly of polysomes in germinating *Petunia* pollen. Polysomes, however, could not be found in the non-germinated control. In ungerminated *Nicotiana tabacum* pollen, Tupý (1977) showed 12% of the ribosomes to be present as polysomes, the assembly of which rapidly increased as germination proceeded. On electron micrographs, Cresti et al. (1975) could detect only monosomes in ungerminated *Lycopersicon peruv-*

ianum pollen. All these typically binucleate species behave like the *Typha latifolia* and *Nicotiana alata* pollen in the present paper. A minute amount of polysomes was detected in their non-germinated grains, only demonstrable upon treatment with pancreatic ribonuclease A. This treatment yielded 80 S monosomes that were resistant to dissociation due to the stabilizing effect of the nascent polypeptide (Martin, 1973; Yamamoto et al., 1975). These polysomes are likely to account for the scant protein synthesis during incubation under humid conditions (Fig. 1).

In contrast, excellent polysome profiles were obtained from ungerminated *Tradescantia* pollen. The occurrence of polysomes was deduced earlier from the considerable increase of the 80 S peak after RNase treatment (Mascarenhas and Bell, 1969). This indicates that protein synthesis must have occurred in considerable amounts prior to dehiscence. Incorporation of leucine was immediately resumed within 2 min of germination. These results suggest that the binucleate *Tradescantia*, with its fully developed mitochondria at dehiscence, is better adapted to rapid tube initiation and growth than the binucleate pollen species with less developed mitochondria, which have to start with the assembling of polysomes.

Because of the apparent connection between rapid tube initiation and an advanced, though restrained, protein-synthesizing apparatus in *Tradescantia*, the rapidly germinating, trinucleate Compositae pollen were also expected to contain many polysomes. However, with these evolutionary advanced species, protein synthesis must have completely lost its importance at dehiscence already. The amount of ribosomal material present in the grains and the incorporation of leucine were very low. Only few polysomes turned out to be present (Fig. 5) which can account for the very limited incorporation. Since Compositae pollen tubes emerge rapidly, with *in vivo* growth rates of 1-2 cm h^{-1} and fertilization in about 30 min (Hoekstra and Bruinsma, 1978), protein synthesis must be completed already prior to dehiscence of the grains. Moreover, it is unlikely that *de novo* synthesis of mRNA and rRNA will be initiated within this short period, particularly since the vegetative nucleus is reported to have a degenerated appearance (Poddubnaja-Arnoldi, 1936). Accordingly, even high concentrations of CHI were unable to affect germination of Compositae pollen.

The reported inconsistencies in the inhibition by CHI of tube emergence and growth among pollen species (Mascarenhas, 1975), can now be explained in terms of differences in developmental stage. It is evident from our experiments that advanced pollen species which had a considerable protein synthesis prior to

dehiscence, are less sensitive to application of CHI than species that start assembling of polysomes only upon incubation in the medium. Advanced species are characterized either by the presence of many polysomes or, alternatively, by the paucity of any ribosomal material in their non-germinated grains.

Precocious protein synthesis in the different types of pollen may also be accompanied by an early synthesis of specific RNA species. In *Tradescantia* only mRNA's are transcribed during tube growth (Mascarenhas and Bell, 1970; Mascarenhas and Goralnick, 1971). The syntheses of other RNA species are terminated prior to dehiscence (Peddada and Mascarenhas, 1975). On the contrary, in *Nicotiana alata* pollen the syntheses of rRNA, tRNA and mRNA proceed during germination (Tupý et al., 1977; Süss and Tupý, 1978). These examples very well fit the hypothesis that fresh *Nicotiana* pollen represents a less developed gametophyte than *Tradescantia*, at all levels of syntheses involved.

The trinucleate nature, their highly developed mitochondrial respiratory system, and the extremely limited *de novo* protein synthesis suggest that tube formation is the only task left for the phylogenetically advanced Compositae pollen to be carried out as independent gametophyte. That this conclusion is not necessarily valid for other trinucleate families is demonstrated by the presence of a large amount of polysomes in fresh maize pollen. In this case, however, the functioning of a protein-synthesizing apparatus is understandable because pollen tubes of maize have to grow an extremely long distance, up to 30 cm. Exceptionally, the reverse also occurs: the short-styled *Impatiens walleriana* sheds binucleate pollen that initiates tube growth in 2-3 minutes (Linskens and Kroh, 1970). According to Hoekstra (1979), a short lag period is indicative of the presence of fully developed mitochondria. Respiration, however, was extremely repressed during incubation under humid conditions, as indicated by low EC-values (Hoekstra, unpublished results). Electron micrographs show mitochondria with numerous cristae, indeed, but also indicate the rare occurrence of ribosomes and the absence of polysomes (Van Went, 1974). Accordingly, CHI turned out not to interfere with tube emergence and elongation (Johri and Shivanna, 1977). The length of the style, therefore, certainly is of importance for whether additional protein synthesis is required or not.

We conclude that rapid tube initiation and growth are invariably connected with fully developed mitochondrial systems and with a completed or rapidly proceeding protein synthesis in the fresh pollen. This fully developed metabolic state occurs not only in trinucleate pollen but also in a number of advanced binucleate species that germinate and grow at comparable rates. We

therefore suggest that this stage is a prerequisite for the division of the generative cell. In the phylogenetic trend towards increased speed of tube initiation and growth for rapid fertilization (Hoekstra and Bruinsma, 1978), the attainment of the fully developed metabolic state is more essential than that of the trinucleate nature.

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REFERENCES

Clark, R.W.: Calculation of $S_{20,w}$ values using ultracentrifuge sedimentation data from linear sucrose gradients, an improved, simplified method. Biochim. Biophys. Acta *428*, 269-274 (1976)

Cresti, M., Pacini, E., Sarfatti, G., Simoncioli, C.: Ultrastructural features and storage function of *Lycopersicon peruvianum* pollen. In: Gamete competition in plants and animals, pp 19-28, Mulcahy, D.L., ed., Amsterdam : North-Holland Publ. Comp. 1975

Davies, E., Larkins, B.A., Knight, R.H.: Polyribosomes from peas. An improved method for their isolation in the absence of ribonuclease inhibitors. Plant Physiol. *50*, 581-584 (1972)

Frankis, R.C., Mascarenhas, J.P.: Direct evidence for the presence of stored messenger RNA's in the ungerminated pollen grain. Suppl. Plant Physiol. *61*, 195 (1978)

Grosfeld, H., Littauer, U.Z.: Cryptic form of mRNA in dormant *Artemia salina* cysts. Biochem. Biophys. Res. Comm. *67*, 176-181 (1975)

Hess, D., Gresshoff, P.M., Fielitz, U., Gleiss, D.: Uptake of protein and bacteriophage into swelling and germinating pollen of *Petunia hybrida*. Z. Pflanzenphysiol. *74*, 371-376 (1974)

Hoekstra, F.A.: Mitochondrial development and activity of binucleate and trinucleate pollen during germination in vitro. Planta *145*, 25-36 (1979)

Hoekstra, F.A., Bruinsma, J.: Respiration and vitality of binucleate and trinucleate pollen. Physiol. Plant. *34*, 221-225 (1975)

Hoekstra, F.A., Bruinsma, J.: Reduced independence of the male gametophyte in angiosperm evolution. Ann. Bot. *42*, 759-762 (1978)

Hoekstra, F.A., Bruinsma, J.: Control of respiration of binucleate and trinucleate pollen in humid conditions. Offered for publication.

Iwanami, Y., Nakamura, N.: Storage in an organic solvent as a means for preserving viability of pollen grains. Stain Techn. *47*, 137-139 (1972)

Jackson, O.A., Larkins, B.A.: Influence of ionic strength, pH, and chelation of divalent metals on isolation of polyribosomes from tobacco leaves. Plant Physiol. *57*, 5-10 (1976)

Johri, B.M., Shivanna, K.R.: Differential behaviour of 2 and 3-celled pollen. Phytomorphology *27*, 98-106 (1977)

Larkins, B.A., Davies, E.: Polyribosomes from peas. V An attempt to charac-

terize the total free and membrane-bound polysomal population. Plant Physiol. *56*, 749-756 (1975)

Linskens, H.F., Schrauwen, J.: The release of free amino acids from germinating pollen. Acta Bot. Neerl. *18*, 605-614 (1969)

Linskens, H.F., Schrauwen, J.A.M., Konings, R.N.H.: Cell-free protein synthesis with polysomes from germinating *Petunia* pollen grains. Planta *90*, 153-162 (1970)

Linskens, H.F., Kroh, M.: Regulation of pollen tube growth. In: Current topics in developmental biology, Vol. 5, pp 89-113, Moscona, A.A., Monroy, A. eds., New York: Academic Press 1970

Linskens, H.F., Pfahler, P.L.: Biochemical composition of maize (*Zea mays* L.) pollen. III Effects of allele x storage interactions at the waxy (wx), sugary (su_1) and shrunken (sh_2) loci on the amino acid content. Theoret. Appl. Genet. *43*, 49-53 (1973)

Martin, T.E.: A simple general method to determine the proportion of active ribosomes in eukaryotic cells. Exp. Cell. Res. *80*, 496-498 (1973)

Mascarenhas, J.P.: The biochemistry of angiosperm pollen development. Bot. Rev. *41*, 259-314 (1975)

Mascarenhas, J.P., Bell, E.: Protein synthesis during germination of pollen. Studies on polyribosome formation. Biochim.Biophys. Acta *179*, 199-203 (1969)

Mascarenhas, J.P., Bell, E.: RNA synthesis during development of the male gametophyte of *Tradescantia*. Develop. Biol. *21*, 475-490 (1970)

Mascarenhas, J.P., Goralnick, R.D.: Synthesis of small molecular weight RNA in the pollen tube of *Tradescantia paludosa*. Biochim. Biophys. Acta *240*, 56-61 (1971)

Mascarenhas, J.P., Terenna, B., Mascarenhas, A.F., Rueckert, L.: Protein synthesis during germination and pollen tube growth in *Tradescantia*. In: Fertilization in higher plants, pp 137-143, Linskens, H.F. ed., Amsterdam: North-Holland Publ. Comp. 1974

Peddada, L.B., Mascarenhas, J.P.: The synthesis of 5 S ribosomal RNA during pollen development. Develop. Growth Diff. *17*, 1-8 (1975)

Penning de Vries, F.W.T.: The cost of maintenance processes in plant cells. Ann. Bot. *39*, 77-92 (1975)

Poddubnaja-Arnoldi, V.: Beobachtungen über die Keimung des Pollens einiger Pflanzen auf künstlichem Nährboden. Planta *25*, 502-529 (1936)

Stein, W.H.: A chromatographic investigation of the amino acid constituents of normal urine. J. Biol. Chem. *201*, 45-58 (1953)

Süss, J., Tupý, J.: tRNA synthesis in germinating pollen. Biol. Plant. *20*, 70-72 (1978)

Swedes, J.S., Agnew, W.S.: Correction of apparent rates of protein synthesis for changes in the specific activity of an amino acid, supplied during growth. J. Biol. Chem. *250*, 6938 (1975)

Tupý, J.: RNA synthesis and polysome formation in pollen tubes. Biol. Plant. *19*, 300-308 (1977)

Tupý, J., Hrabětová, E., Balatková, V.: Evidence for ribosomal RNA synthesis in pollen tubes in culture. Biol. Plant. *19*, 226-230 (1977)

Van Went, J.L.: The ultrastructure of *Impatiens* pollen. In: Fertilization in higher plants, pp 81-88, Linskens, H.F. ed., Amsterdam: North-Holland Publ. Comp. 1974

Yamamoto, N., Hasegawa, M., Sasaki, S., Asakawa, S.: Red-far red reversible effect on polysome formation in the embryos of *Pinus thunbergii* seeds. Plant Physiol. *56*, 734-737 (1975)

Samenvatting

VITALITEIT EN METABOLISCHE EIGENSCHAPPEN VAN RIJP BINUCLEAAT EN TRINUCLEAAT STUIFMEEL

Dit proefschrift behandelt fysiologische en metabolische verschillen tussen binucleaat en trinucleaat stuifmeel. Alvorens deze verschillen te kunnen onderzoeken werd een studie verricht met betrekking tot de levensvatbaarheid van trinucleaat Compositae pollen, waarvan verslag is gedaan in Hoofdstuk I.

HOOFDSTUK I

Levensvatbaarheid van trinucleaat Compositae pollen: Kieming in vitro en invloeden van temperatuur en RV

Alhoewel *Chrysanthemum cinerariaefolium* pollen met succes kon worden gekiemd op vaste media die agar en/of gelatine bevatten, werd toch een vloeibaar medium ontwikkeld, met het doel de reproduceerbaarheid van de kieming te verbeteren.

- Het optimale vloeibare medium bevatte suiker (1,32 M), boorzuur (100 µg/ml) en calcium (2 mM), bij pH 6,8 (zie pag. 19 Fig. 1 en 2).
- Met geringe individuele aanpassingen in suikerconcentratie bleek dit medium geschikt voor pollenkieming van tenminste 34 soorten behorende tot 16 genera der Compositae (voor een opsomming zie pag. 18).
- Blootstelling aan vochtige lucht van 30°C gedurende 15 minuten, voorafgaand aan incubatie in het kiemmedium, verbeterde zowel het percentage kieming als de reproduceerbaarheid.
- De optimale temperatuur voor kieming *in vitro* bleek 25-30°C (pag. 20 Fig. 3).
- De pollenbuis bereikt *in vitro* slechts een lengte van 70-100 micron. Gebleken is dat ongeveer 10 minuten na het mengen van de korrels met het medium de groei stopt.
- Het percentage kieming *in vitro* bleek echter goed overeen te stemmen met het percentage korrels dat op de stempel kiemt.

Dankzij bovengenoemde technieken was het mogelijk de pollenvitaliteit nauw-

keurig te bepalen. Dit leidde tot een nader onderzoek van uitwendige factoren, die betrokken bleken te zijn bij processen die leiden tot verlies van vitaliteit.

- Hoge temperatuur en RV bekortten sterk de levensduur tijdens bewaring (zie pag. 21 Fig. 5, en pag. 22 Fig. 6) en verminderden ook de kwaliteit van pas vrijgekomen stuifmeel bij blootstelling van de bloeiende plant aan deze condities (zie pag. 21 Fig. 4). Gesuggereerd wordt dat fluctuaties in deze exogene factoren de achtergrond vormen van de dagelijkse schommeling in vitaliteit van vers stuifmeel.
- Gedroogd pollen bleek bij -20°C tot meer dan een jaar zonder verlies van vitaliteit te kunnen worden bewaard.

De zeer snelle daling in vitaliteit als gevolg van hoge temperatuur en RV suggereert een hoge metabolische activiteit in trinucleaat Compositae pollen. Hiervan werd een indruk verkregen via meting van de ademhaling (Hoofdstuk II).

HOOFDSTUK II

Vergelijkende studies tussen binucleate en trinucleate pollensoorten I: Ademhaling en vitaliteit in vochtige lucht

- De ademhaling in vochtige lucht bleek met een constante snelheid te verlopen (pag. 27 Fig. 1).
- Per gewichtseenheid ademde het trinucleate pollen van Compositae en Gramineae 2 à 3 maal intensiever dan 6 soorten binucleaat pollen (zie pag. 27 Tabel 1). Omgerekend per eenheid polleneiwit waren de verschillen nog groter. De verschillen bleken te gelden voor het gehele fysiologische temperatuurbereik (zie pag. 28 Fig. 3).
- Indien pollenmonsters voor een deel waren samengesteld uit dode individuen, werd de ademhaling gecorrigeerd voor ongekleurde korrels, nadat was vastgesteld dat een lineair verband bestond tussen de ademhalingssnelheid en het percentage korrels dat door tetrazoliumbromide (MTT) was gekleurd (zie pag. 27 Fig. 2).
- De verschillen in overleving tussen bi- en trinucleaat pollen bleken nog groter te zijn dan die in ademhalingssnelheid. Bovendien verloor trinucleaat Compositae pollen zijn vermogen tot kieming minstens 2 maal sneller dan zijn vermogen tot ademhalen (zie pag. 27 Tabel 1). De bruikbaarheid van MTT als "vital stain", welke berust op dehydrogenase activiteit is bij dit type stuifmeel dus twijfelachtig.

- Een vrij hoge RV bleek noodzakelijk om gaswisseling te kunnen registreren, met als ondergrens van detectie ongeveer 77%. Bij dalende RV nam de houdbaarheid van binucleate typen flink toe, maar die van trinucleate typen bleef toch gering (zie pag. 28 Fig. 4).
- Tijdelijke blootstelling van trinucleaat *Triticum* pollen aan droge laboratoriumlucht bleek niet alleen fataal voor de vitaliteit, maar leidde ook tot een sterke daling van de ademhaling na her-incubatie in vochtige lucht van RV = 97% (zie pag. 28 Fig. 5).

De waarneming, dat de metabolische activiteit van trinucleaat pollen op een hoger niveau ligt dan die van enkele binucleate typen, leidde tot de gedachte dat ontwikkelingsverschillen tussen de mitochondriën van beide typen stuifmeel een rol kunnen spelen. Dit werd in Hoofdstuk III onderzocht.

HOOFDSTUK III

Vergelijkende studies tussen binucleate en trinucleate pollensoorten II: Ontwikkeling en activiteit van mitochondriën tijdens kieming in vitro

Isoleringsproblemen waren ten dele de oorzaak van de zeer geringe activiteit van mitochondriën uit ongekiemd stuifmeel. In de volgende studies werd daarom de ontwikkeling van mitochondriën vervolgd tijdens kieming *in vitro*. Uit de kinetiek van deze ontwikkeling kan worden afgeleid hoe de situatie is in ongekiemd pollen.

- Media werden samengesteld waarmee actieve mitochondriën kunnen worden geïsoleerd uit de binucleate soorten: *Typha latifolia*, *Nicotiana alata* en *Tradescantia paludosa*, en uit de trinucleate *Aster tripolium*.
- De oxydatie van verschillende substraten suggereert de werking van 3 fosforylatieplaatsen in pollenmitochondriën (zie pag. 34 Fig. 1).
- Trinucleaat *Aster* pollen bereikte de maximale capaciteit van elektronentransport binnen 2 minuten na mengen met het kiemmedium. Binucleaat *Typha* en *Nicotiana* pollen bereikten deze maximumsnelheid pas veel later, na 75 respectievelijk 30 minuten; binucleaat *Tradescantia* stuifmeel daarentegen even snel als het trinucleate type (zie pag. 35 Fig. 2, en pag. 36 Fig. 3).
- De periode tot het bereiken van de maximale capaciteit van het elektronentransport bleek opvallend overeen te komen met die tot het verschijnen van de eerste kiembuizen (vergelijk pag. 36 Fig. 3 met pag. 34 Tabel 1).
- Binucleaat *Typha* pollen bleek *in vitro* een aanlooptijd van 12 minuten nodig te hebben voor het bereiken van een constante ademhalingssnelheid.

Gedurende die periode was de "energy charge" (EC) laag. Trinucleaat *Aster* pollen ademde direct op een constante snelheid en de EC bleef hoog. De overige twee onderzochte soorten gedroegen zich intermediair (zie pag. 34 Tabel 1, en pag. 37 Tabel 5).

- Elektronenmicroscopische opnamen geven aan dat mitochondriën uit ongekiemd trinucleaat *Aster* en binucleaat *Tradescantia* pollen goed ontwikkeld zijn, en dat die uit binucleaat *Typha* en *Nicotiana* pollen nog een verdere ontwikkeling behoeven (zie pag. 38, Fig. 6).
- Uit integriteitsmetingen aan mitochondriën uit *Typha* pollen gedurende het eerste halve uur in het kiemmedium bleek, dat de geringe capaciteit tot oxydatieve phosphorylering tenminste ten dele moet berusten op intrinsieke eigenschappen (zie pag. 36 Tabel 2). De ontwikkeling van de mitochondriële eigenschappen werd niet gestoord door remmers van de eiwitsynthese, terwijl het gehalte aan cytochromen constant bleef (zie pag. 37, Tabel 6 en Fig. 4).

Bovengenoemde waarnemingen tonen aan dat het niet alleen het privilege van trinucleaat stuifmeel is volledig ontwikkelde mitochondriën te bezitten, maar dat ook sommige binucleate soorten ermee zijn uitgerust. Hoe de controle van de ademhaling verloopt bij pollen met verschillen in ontwikkeling van hun mitochondriën tijdens incubatie in vochtige lucht, werd beschreven in Hoofdstuk IV.

HOOFDSTUK IV

Vergelijkende studies tussen binucleate en trinucleate pollensoorten III: Controle van de ademhaling in vochtige lucht

- De gelijke snelheden van waterdampopname waren een aanwijzing dat de verschillen in ademhalingsactiviteit tussen bi- en trinucleaat pollen in vochtige lucht een intrinsieke, biochemische achtergrond moeten hebben (zie pag. 47 Fig. 1).
- De ontkoppelaar CCCP bleek een langzame ademhaling te versnellen en een snelle ademhaling te vertragen tot ongeveer gelijke waarden, die waarschijnlijk bepaald worden door de snelheid van actief substraattransport door de mitochondriële membranen (zie pag. 48 Tabel 1).
- De mate van remming van de zuurstofopname door oligomycine, DCCD, antimycine A en SHAM, alleen en in combinaties, bleek aan te geven dat nauwelijks oxydatieve fosforylering en anabolische activiteit optreedt bij langzaam ademende, binucleate pollensoorten,die worden gekarakteriseerd door

lange levensduur, hoge waarde van de EC en laag ontwikkelde mitochondriën (zie pag. 50 Tabel 2, en pag. 51 Tabel 3). De alternatieve oxydase bleek niet significant aantoonbaar.

- Andere binucleate typen zoals *Tradescantia*, die herkenbare mitochondriën bezitten, maar opvallen door lage EC-waarden, bleken slechts een gelimiteerde hoeveelheid ATP te produceren tijdens ademhaling in vochtige lucht (zie pag. 50 Tabel 2, en pag. 51 Tabel 3). De lage EC-waarde kan duiden op een geremd ademhalingssysteem.
- Bij snel ademend, trinucleaat pollen, dat goed ontwikkelde mitochondriën bezit, bleek de alternatieve oxydase significant aanwezig. De EC-waarden waren hoog, ondanks de grote vraag naar ATP van ongeveer 1,7 μmol h^{-1} mg $pollen^{-1}$ (zie pag. 50 Tabel 2, en pag. 51 Tabel 3).
- In gevallen waar oligomycine, tegen de verwachtingen in, de elektronenstroom via de cytochroomroute sterk stimuleerde, bleek toch een daling in ATP-gehalte op te treden, hetgeen oxydatieve fosforylering in deze soorten aannemelijk maakt. Een schatting van de ATP-productie was echter onmogelijk (zie pag. 49 Fig. 3, en pag. 51 Tabel 3).

Aldus werd ATP-synthese aangetoond in trinucleate en de meeste binucleate typen pollen tijdens incubatie in vochtige lucht. In Hoofdstuk V werd onderzocht in welke mate deze ATP-productie ten dienste stond van de eiwitsynthese en in hoeverre een geavanceerd mitochondrieel systeem in pollen samengaat met een actief, eiwitsynthetiserend apparaat.

HOOFDSTUK V

Vergelijkende studies tussen binucleate en trinucleate pollensoorten IV: Eiwitsynthese in relatie tot pollenkieming en -buisgroei.

- Zeer geringe hoeveelheden leucine bleken te worden geïncorporeerd tijdens pollenademhaling in vochtige lucht (zie pag. 64 Fig. 1). Deze incorporatie vergde niet meer dan een fractie van de hoeveelheid ATP die oxydatief werd gegenereerd.
- Tijdens de kieming *in vitro* verschilden de twee typen pollen aanzienlijk wat betreft het vermogen waarmee leucine werd geïncorporeerd (zie pag. 65 Fig. 2).
- Binucleate pollensoorten, die worden gekarakteriseerd door geringe ademhaling in vochtige lucht, lange levensduur, lange 'lag'periode bij kieming *in vitro* en laag ontwikkelde mitochondriën, zijn zeer gevoelig voor

de eiwitsyntheseremmer cycloheximide in het kiemmedium (zie pag. 65 Tabel 2). Polyribosomen worden eerst gevormd na mengen met het kiemmedium en de leucine-incorporatie begint na 10-15 minuten (zie pag. 66 Fig. 3, en pag. 65 Fig. 2).

- Sommige binucleate soorten en de trinucleate typen, gekarakteriseerd door een goed ontwikkeld mitochondrieel systeem en door een korte 'lag'periode bij kieming *in vitro*, bleken meer of minder ongevoelig voor cycloheximide. Dit wijst op het in aanzienlijke mate plaatsvinden van eiwitsynthese ten behoeve van buisgroei vóór het vrijkomen van het pollen uit de anthere. Verschillende capaciteiten van eiwitsynthese werden gevonden: van het voorkomen van een grote hoeveelheid polyribosomen in de ongekiemde korrel, met daaraan gekoppeld een zeer snelle hervatting van de leucine-incorporatie bij kieming *in vitro* in *Tradescantia*, tot een zeer geringe incorporatie bij trinucleaat Compositae pollen, waarin zelfs ribosomen zeer schaars bleken te zijn (vergelijk pag. 65 Fig. 2 met pag. 67 Fig. 4 en 5).

Slotbeschouwing

Deze studie toont voor het eerst aan, dat rijp stuifmeel van angiospermen onderling aanzienlijk verschilt in metabolische ontwikkeling en activiteit. De experimenten laten onder andere verschillen zien in de snelheid van pollenademhaling in vochtige lucht, met daaraan gekoppeld verschillen in overlevingsduur. Ze tonen ook verschillen aan in ontwikkelingsgraad van mitochondriën en in het verloop van de eiwitsynthese. De indruk wordt verkregen dat de verschillen betrekking hebben op het totaal van morfologische en biochemische processen in stuifmeel.

Wanneer we de ontwikkeling van de pollenkorrel beschouwen als te verlopen vanaf de betrekkelijke rust in de anthere tot aan de hoogste activiteit tijdens de buisgroei door de stijl, dan kunnen we de verschillen in metabolische ontwikkelingsgraad tussen de diverse soorten verklaren in relatie tot het moment waarop het pollen tijdens dit ontwikkelingsproces uit de anthere wordt vrijgemaakt. Een relatief laat vrijkomen leidt dan tot pollen met een hoge metabolische ontwikkelingsgraad, zoals bij de Compositae, waarbij het aantal zelfstandig uit te voeren processen sterk is gereduceerd: de tweede mitose tot de beide gameten heeft reeds plaats gehad, eiwit- en RNA-synthese spelen geen rol van betekenis meer tijdens kieming en buisgroei en de mitochondriën zijn reeds goed uitgerust voor de onmiddellijke levering van grote hoeveelheden ATP ten behoeve van een snelle buisgroei. Een relatief vroeg vrijkomen zal daarentegen resulteren in het pollentype zoals *Typha latifolia* bezit, waarbij tijdens kieming de mitochondriën nog een voortgezette ontwikkeling behoeven, de eiwitsynthese start met de assemblage van ribosomen, de kiembuis *in vitro* pas na een uur verschijnt, en de tweede mitose pas na vele uren van kieming in de stijl plaats vindt.

Vanzelfsprekend is het aantrekkelijk te trachten het voorafgaande te relateren aan de onderverdeling in bi- en trinucleaat pollen met hun specifieke eigenschappen. Echter, wat is er overgebleven van Brewbaker's (1957) correlatie tussen het driecellige type stuifmeel en de vermeend problematische eigenschappen, zoals slechte bewaarbaarheid en moeilijke kieming *in vitro* ? Veel van deze problemen konden worden teruggebracht tot een onvoldoende rekening houden met hetgeen in Hoofdstuk I is besproken, namelijk het belang van toepassing van equi-

libratie in vochtige lucht, voorafgaand aan kieming *in vitro*, alsmede de essentie van suiker, calcium en borium, voornamelijk met het doel de snelheid van de wateropname binnen bepaalde grenzen te houden. Heslop-Harrison en Shivanna (1977) suggereren dat de noodzaak tot voorzichtige waterdosering veeleer samenhangt met het type stempel, dan met het aantal cellen in het pollen. Ten opzichte van de soorten met binucleaat pollen echter is een proportioneel groot aantal van de trinucleate soorten in het bezit van droge stempels, hetgeen heeft geleid tot de correlatie tussen pollencytologie en kiembaarheid *in vitro*. De slechte reputatie op het gebied van houdbaarheid is ook in de hand gewerkt door het specifieke gedrag van Gramineae stuifmeel, dat niet bestand blijkt tegen uitdrogen. Trinucleaat Compositae pollen daarentegen kan, mits droog, bij -20°C minstens 1 jaar worden bewaard, en voor Cruciferae is een veilige periode van 2 jaar gemeld (Ockendon, 1974).

Door toepassing van het zeer natuurlijke medium waterdamp kunnen echter de moeilijkheden met kiemmedia grotendeels worden omgaan. Bovendien treedt ook geen ongewenste lekkage van materiaal uit het pollen naar het medium meer op. Als gevolg van de waterdampopname bleken trinucleate pollensoorten sneller te ademen dan binucleate typen. Dit duidt juist wel weer op het bestaan van fysiologische verschillen tussen de beide typen.

De aanwezigheid van 3 kernen, reeds in het rijpe pollen, wijst op een reductie van het aantal als zelfstandige gametofyt uit te voeren processen. De vergevorderde mitochondriële ontwikkeling en eiwitsynthese dragen nog verder tot deze reductie bij. Het reeds beëindigd hebben van deze ontwikkelingsprocessen maakt een zeer snelle start van buisgroei mogelijk: bij Compositae op de stempel in ongeveer 10 minuten (Hoekstra en Bruinsma, 1978). Pollen komt dus in een vergevorderde staat van ontwikkeling vanuit de beschermende anthere op de stempel terecht en de pollenbuis kan vrijwel direct penetreren. Opmerkelijk is dat ook enkele binucleate soorten (*Tradescantia, Impatiens*) deze eigenschappen vertonen, de snelle start van buisgroei inbegrepen, uiteraard met uitzondering van de tweede mitose.

Nu wordt het interessant nogmaals de geclaimde fylogenetische progressie van de families met trinucleaat stuifmeel in beschouwing te nemen. Bij bestudering van de afstamming der angiospermen constateerde Brewbaker (1967) dat trinucleate taxa polyfyletisch afstammen van binucleate taxa, maar dat het omgekeerde niet voorkomt. Nu is het gevaarlijk af te gaan op dit soort stambomen omdat betrekkingen tussen families vaak moeilijk zijn vast te stellen en ideeën daaromtrent snel kunnen wisselen. Om meer zekerheid te krijgen over deze geclaimde fyloge-

netische nieuwheid van taxa met trinucleaat pollen, heb ik de cytologische waarnemingen van Brewbaker (1967) vergeleken met het model van Sporne (1969). In zijn omvangrijke werk heeft Sporne 202 dicotyle families een "index of advancement" gegeven op grond van correlaties van 21 specifieke eigenschappen. Families waartoe trinucleate taxa behoren blijken hierbij een gemiddelde te scoren van 65% tegen binucleate families 52%. Families met driecellig pollen hebben dus gemiddeld een hogere "index of advancement" dan die met binucleaat pollen, maar het interessante is, dat binucleate families soms ook een zeer hoge index is toegekend. Het is treffend, dat aan de Balsaminaceae (*Impatiens*), met metabolisch goed ontwikkeld, snel kiemend, binucleaat stuifmeel een index van 80% is toegekend, even hoog als aan de trinucleate Compositae.

Het voorafgaande leidt tot de gedachte, dat wellicht de gevorderde metabolische toestand van het pollen een typisch fylogenetisch recente eigenschap is, met een voltrokken tweede mitose als een weliswaar vaak voorkomend, maar niet noodzakelijk kenmerk. De gevorderde metabolische toestand kan echter wel worden beschouwd als voorwaarde voor de voltrekking van de tweede mitose.

Snel kiemend pollen bleek minder gevoelig te zijn voor remming van de eiwitsynthese in het kiemmedium door cycloheximide (CHI) dan hun langzaam kiemende tegenhangers. Het ligt voor de hand dat om deze snelle buisgroei te kunnen realiseren, reeds eerder, tijdens de ontwikkeling in de anthere, eiwit is gesynthetiseerd, uiteraard niet te beïnvloeden door CHI in het medium. In dit verband is de correlatie interessant die is gevonden tussen de relatieve gevoeligheid van pollen voor CHI en de levensduur van de bloem (Lin et al., 1971). Van de 9 onderzochte taxa, waarvan bloemen verleppen op de dag van opengaan, is het pollen relatief ongevoelig voor CHI, terwijl taxa waarvan de bloemen langer open blijven pollen bezitten dat door CHI sterk wordt geremd in kieming en buisgroei. Deze gegevens kunnen ook worden geïnterpreteerd in de zin van een snelle ethyleenvorming vanwege de zeer snelle penetratie en doorgroei van de pollenbuizen, welk ethyleen een snel verleppen van de bloem induceert. Dit zou betekenen dat ook het snelle verleppen van bloemen als gevolg van snelle penetratie en groei van stuifmeelbuizen, een relatief recent fenomeen in de evolutie van de angiospermen is.

De overleving van de soort wordt onder andere gewaarborgd door optimale overdracht en verspreiding van het genetisch materiaal, via zaad zowel als via pollen, onder voortdurende aanpassing aan veranderende externe omstandigheden. Wat het pollen betreft, dient binnen de levensduur van de eicellen een voldoende aantal compatibele pollenbuizen de stijl te doorgroeien om te komen tot het

maximale aantal vitale zaden. Om dit te realiseren zullen dus adaptieve veranderingen in de fysiologie van de bestuiving nodig zijn. Wat klaarblijkelijk polyfyletisch optreedt is een trend naar snelle ontwikkeling van de stuifmeelbuis. Anders geformuleerd, als er ook maar enige genetische basis is voor een sneller uitgroeien van de ene pollenbuis ten opzichte van een willekeurige andere, zoals wordt gesuggereerd door Mulcahy (1974), zal er in de loop van de evolutie een verhoging kunnen gaan optreden in de metabolische ontwikkelingsgraad van vers stuifmeel. Immers, spermacellen uit die pollenbuizen, die door sneller in te groeien eerder de embryozak weten te bereiken, zullen in proportioneel grotere aantallen met de eicellen versmelten.

Echter, een hoge metabolische ontwikkelingsgraad houdt ook een gevaar in, namelijk de snelle daling in vitaliteit als gevolg van de toegenomen kwetsbaarheid. Gezien de polyfyletische ontwikkeling van het trinucleate karakter, zullen ook vele wegen zijn gevolgd om selectieve nadelen van kwetsbaar stuimeel het hoofd te bieden. Hierbij kan gedacht worden aan een coëvolutie van de bloeiwijze, met als voorbeeld het hoofdje bij de Compositae, dat iedere dag een nieuwe rij bloempjes met vers stuifmeel levert (Hoekstra en Bruinsma, 1978), of de geleidelijk verbloeiende bloemtros zoals bij Cruciferae. Consequenties voor de stand van de bloem in verband met beïnvloeding van het stuifmeel door regen zijn reeds eerder bediscussieerd (Eisikowitch en Woodell, 1975). Het selectieve nadeel van een hoge ontwikkelingsgraad kan wellicht goed worden gecompenseerd via specifieke remming van de metabolische activiteit, zoals waarschijnlijk in binucleaat, snel kiemend stuifmeel van *Tradescantia* en *Impatiens* plaats vindt.

Het vermogen tot de snelle vorming van kiembuizen biedt ongetwijfeld grote voordelen, omdat het leidt tot een verlenging van de effectieve bestuivingsperiode, in het bijzonder van belang onder klimatologisch minder gunstige omstandigheden. Bovendien zal bij snelle penetratie van de stempel de kans verminderen op wegwaaien en afregenen van het pollen van, met name droge stempeloppervlakken. Wellicht is snelle kieming vooral gunstig in gematigde klimaten met een groot verschil in dag- en nachttemperatuur, om dezelfde redenen. Het optreden van trinucleaat stuifmeel bij het merendeel der waterplanten met ondergedoken bloemen (Brewbaker, 1957), doet vermoeden dat een snelle start van de pollenbuisgroei ook in water selectief voordelig is. De snelle kieming zal wegdrijven van het pollen kunnen beperken. Het is evenwel te verwachten dat zulk stuifmeel in een dergelijk nat milieu metabolisch geremd is, welke remming pas wordt opgeheven bij contact met de stempel.

LITERATUUR

Brewbaker, J.L.: Pollen cytology and self-incompatibility systems in plants. J. Hered. *48*, 271-277 (1957)

Brewbaker, J.L.: The distribution and significance of binucleate and trinucleate pollen grains in the angiosperms. Amer. J. Bot. *54*, 1069-1083 (1967)

Eisikowitch, D., Woodell, R.J.: The effect of water on pollen germination in two species of *Primula*. Evolution *28*, 692-694 (1975)

Hoekstra, F.A., Bruinsma, J.: Reduced independence of the male gametophyte in angiosperm evolution. Ann. Bot. *42*, 759-762 (1978)

Lin, Y., Chow, T., Lin, C.Y.: Nitrogen metabolism associated with pollen grain germination. Taiwania *16*, 67-84 (1971)

Mulcahy, D.L.: Adaptive significance of gamete competition. In: Fertilization in higher plants, pp. 27-30, Linskens, H.F. ed. Amsterdam, Oxford: North Holland Publ. Comp. 1974

Ockendon, D.J.: The value of stored pollen in incompatibility studies in *Brassica*. Incomp. Newsletter *4*, 17-19 (1974)

Sporne, K.R.: The ovule as an indicator of evolutionary status in angiosperms. New Phytol. *68*, 555-566 (1969)

Curriculum Vitae

Folkert Anne Hoekstra werd op 1 april 1947 geboren te Haarlem. Hij bezocht het Stedelijk Gymnasium te Haarlen en het Gemeentelijk Gymnasium te Hilversum, alwaar hij het einddiploma gymnasium β behaalde in 1965. Aansluitend begon hij met de studie aan de Landbouwhogeschool te Wageningen. Na in 1969 het kandidaatsexamen te hebben afgelegd in de richting Plantenveredeling, bracht hij een jaar door op het Pyrethrum Research Station te Molo, Kenya, alwaar reeds de basisgedachte van dit proefschrift werd ontwikkeld. In januari 1974 behaalde hij het ingenieursdiploma met lof (hoofdvak plantenveredeling; bijvakken plantenfysiologie, verzwaard, en virologie).

Vanaf juli 1973 was hij enige maanden werkzaam als studentassistent op het Instituut voor Toepassing van Atoomenergie in de Landbouw, ter voorbereiding van het promotieassistentschap aldaar, dat hij vervulde tot april 1977. Sindsdien is hij als wetenschappelijk medewerker verbonden aan de vakgroep Plantenfysiologie van de Landbouwhogeschool.