Environmental Health Perspectives
Vol. 37, pp. 125-132, 1981

In Vitro Germination Characteristics of Maize Pollen to Detect Biological Activity of Environmental Pollutants

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In vitro pollen germination was examined as a method to determine the mutagenic and physiological effects of environmental pollutants on higher organisms. Results were presented indicating that mutations could be distinguished by their *in vitro* germination characteristics if sporophytes homozygous for the mutated allele were tested. The addition of agents directly into the *in vitro* medium was shown to be an effective method to assess their physiological effects. The exposure of pollen grains during the *in vitro* germination process to ultraviolet radiation in the B range (280-320 nm) was found to produce little or no change in the germination or ruptured percentage but a sharp decrease in pollen tube growth after 1 hr.

In vitro pollen germination appears to be a valuable method to examine the mutation types and physiological effects produced by a broad range of environmental pollutants. In fact, since *in vitro* germination is related to reproducion and gene transmission,the biological activity of these agents on *in vitro* germination should be tested routinely to determine their possible effects on food producion and gene frequency changes in future generations.

Introduction

The rapid increase in industrial and agricultural technology has been accompanied by a marked increase in the number and types of biologically active agents released into the biosphere. These agents can produce either mutagenic or physiological effects or a combination of both and their effects can occur in any cell at any stage of life cycle. Most of these agents act directly but in some cases, some have unpredictable indirect effects. As an example, chlorofluorocarbons which are widely used as aerosol propellants and refrigerants, catalytically reduce the ozone layer which in turn, allows an increase in the ultraviolet radiation in the B range (UVB) (280-300 nm) to reach the biosphere (*1-3*). UVB is known to produce diverse biological effects including the reduction of photosynthetic activity and efficiency (*4*) the induction of genetic alterations (*5,6*) and an increase in the incidence of skin cancer in humans (*2*). However, UVB has low penetration capacity and in most higher organisms, only the exposed surface cells of the sporophyte rather than the germinal cells producing gametes are affected. With such species. the mutagenic effects of UVB would be of little or no importance in a genetic or evolutionary sense since no UVB-induced alterations would be transmitted to future generations. In some species with maize the outstanding example, pollen would be normally exposed to the UVB in the biosphere and any UVB-induced chromosomal changes would be transmitted to the next generation or could cause sterility. From the above, it is apparent that biologically active agents released into the biosphere as a result of technology can be of many diverse forms which can generate numerous immediate and permanent effects.

In vitro pollen germination in maize represents a complex interaction involving the morphology and physiology of the pollen grain and the medium components. The morphological and physiological differences are the result of the haploid genotype of the pollen grain (pollen genotype or haploid effect), the genotype of the diploid sporophyte on which the pollen grains are produced (pollen source genotype or diploid effect) and their interaction. The haploid

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effect is most clearly shown at the waxy locus. In this case, the *Wx*- and *wx*-bearing grains from a heterozygous *Wxwx* plant can be readily distinguished by a simple starch test (*7,8*). Significant differences in *in vitro* germination characteristics were reported between pollen grains containing the dominant and recessive alleles at various loci including the waxy locus (*9*). The diploid effect is more difficult to isolate since the diploid condition of the sporophyte and the confounding effects of dominance, epistasis etc. interferes with determining the specific genetic constitution of the haploid grains the sporophyte produces. However, significant differences in the *in vitro* germination characteristics of pollen grains from various genotypes were found (*10-12*). The initial *in vitro* germination medium suggested for maize pollen germination was developed using pollen grains from only one genotype and was an aqueous, semisolid medium containing only sucrose and bacto-agar (*13*). Later, but again with the use of only one pollen source genotype, the addition of calcium nitrate and boric acid to this basal medium was reported to markedly increase the germination percentage and pollen tube growth (*14, 15*). More recently, studies using more than one pollen source genotype or pollen genotype indicated that a significant interaction between pollen source genotypes or pollen genotypes and various calcium nitrate-boric acid combinations was present for both germination percentage and pollen tube growth (*10,11, 16*). With this background information, it is apparent that the structure and physiology of the pollen grain is under genetic control and the composition of the medium is critical for germination and tube growth.

This paper presents the general method of pollen collection and medium preparation that will produce consistent and reproducible results; the results of selected *in vitro* pollen germination studies to illustrate the adaptability of this procedure; and a discussion of the advantages and limitations of *in vitro* pollen germination in studying the biological activity of environmental pollutants.

Materials and Methods

Only a general description of the pollen collection and medium preparation methods will be presented in this section, since a complete description of specific experiments is available in the publications cited. However, a detailed description of the UVB experiment which has not been previously published, is included here.

The pollen collection method involved cutting an actively-shedding tassel 25 to 50 cm below the tassel (*17*). The cut end was immediately submerged in water. When tassels are removed in this manner between 1500 and 1800 hours, after the daily cycle of pollen shedding is completed, no more pollen shedding occurs until 700 hours the next day. The pollen shed from the tassels between 700 and 900 hours on the day after cutting represented the pollen that would have been shed in the next daily cycle if the tassels had remained on the plant. Large sheets of paper were placed under the cut tassels and only pollen shed before 900 hours the day after cutting were used. Before inoculation, the collected pollen was mechanically screened to remove the empty anthers.

The basal medium required to produce *in vitro* germination and pollen tube growth consists of an aqueous solution containing 15% sucrose and 0.6% bacto-agar (*15*). The preparation involved heating this solution to 100°C and immediately thereafter pouring the solution into plastic Petri dishes (100 × 20 mm) to a depth of 4-6 mm. When the solution cooled to room temperature, the covers were placed on the Petri dishes. Any supplements to the medium were added before heating. Pollen inoculations were not made for about 12 hr after the dishes were covered and the relative humidity above the medium approached 100%. However, this time interval was not critical.

Pollen grains were scattered on the surface of the medium to ensure individual separation of the grains. The inoculated plates were incubated at room temperature. Activity on the plates was stopped at various periods after inoculation by flooding the surface of the medium with a killing and preservative solution (72 parts water, 5 parts formaldehyde, 3 parts glacial acetic acid, 20 parts glycerin). The Petri dishes were then stored at 2-5°C until classified and measured.

A microprojector (BL 42-63-59-10) was used to obtain both percentage and pollen tube length data (Fig. 1). The percentage data included: germination percentage; multiple germination percentage; intact percentage; and ruptured percentage. A grain was classified as germinated if at least one recognizable pollen tube was present. The magnification used to obtain the percentage data was 67.5×. Pollen tube length was obtained by measuring a large number of randomly-selected pollen tubes. No tubes were measured which arose from grains producing more than one pollen tube. The magnification used for measuring pollen tube length was 400×.

For the UVB study, pollen grains from five pollen source genotypes (Wf9×H55, Ky49×Ky27, K64×K55, H55, Oh43) were collected on each of two dates. On each date, 18 Petri dishes containing a 15% sucrose, 0.6% bacto-agar, 0.03% calcium

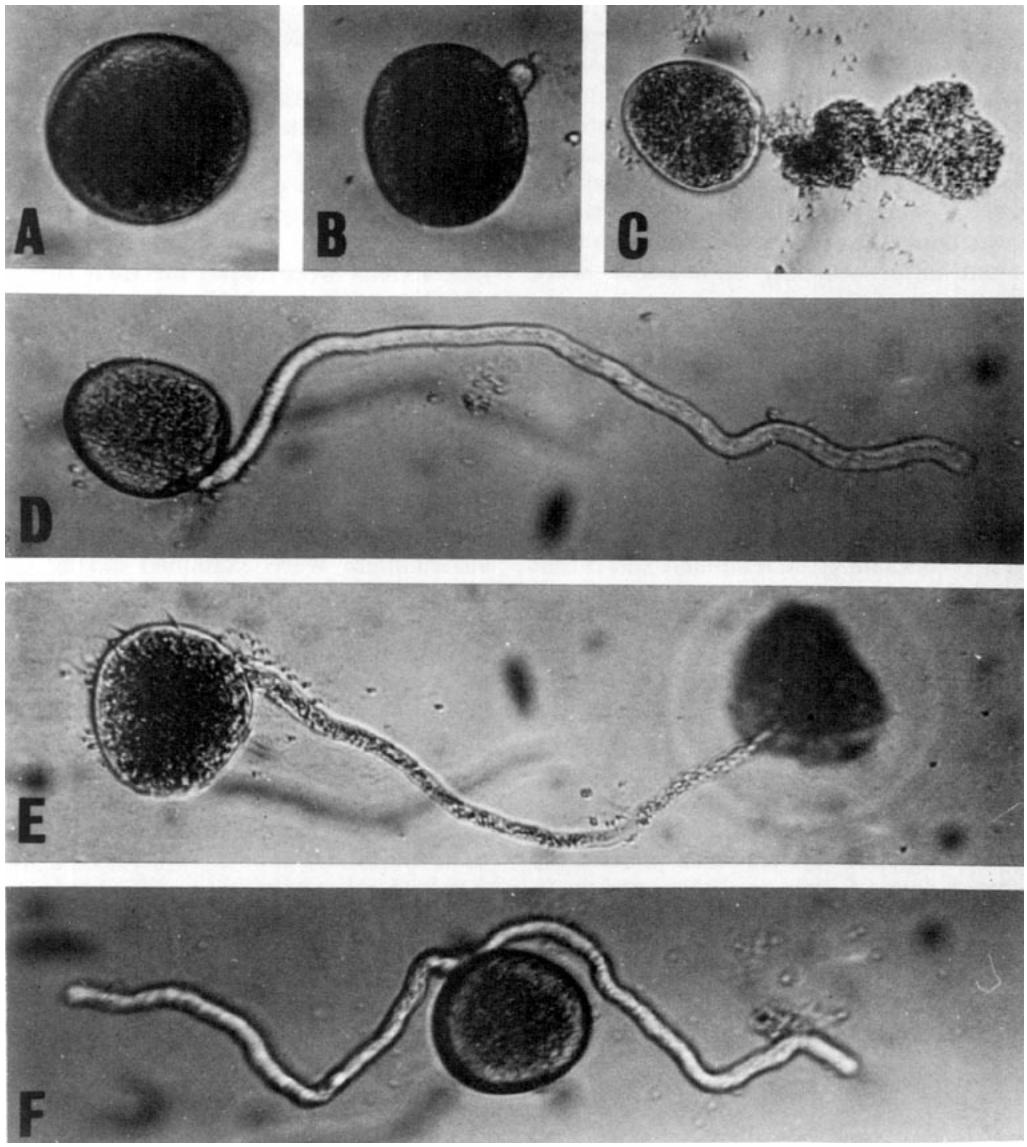

FIGURE 1. Reaction of pollen grains when placed on *in vitro* germination medium: *(A)* ungerminated grain; *(B)* pollen tube emerging from the single pore of the pollen grain; *(C)* grain ruptured through pore with contents exuded on surface of the medium; *(D)* grain germinated with one tube; *(E)* grain with tube end ruptured after considerable tube growth; *(F)* grain germinated with two tubes.

nitrate, 0.01% boric acid medium were inoculated with the pollen grains from each genotype. Immediately after inoculation, six of the dishes (without covers) were exposed to 3278 mW/m^2 (level 2) of UVB(280 nm+), six were exposed to 1688 nW/m^2 (level 1) and the remaining six represented the control (level 0 = 0 mW/m^2). A FS40 sunlamp was the source of the UVB radiation with the level of exposure controlled by the distance from the lamp. Level 1 was 9 cm from the lamp while level 2 was 5 cm. To remove radiation below 280 nm, a 5-mil cellulose acetate filter was placed between the lamp and the dishes. At 1, 2, and 3 hr after inoculation, two of the six dishes from each exposure level and pollen source genotype were flooded with the killing and preservative solution to stop further activity. Percentage data were taken for germination and ruptured percentage with two counts of 100 each taken on each dish. Therefore, for each pollen source genotype at each level for each hour after inoculation at each date, four percentages for each category were obtained. Twenty randomly

selected pollen tubes on each dish were measured, a total of 40 measurements for each pollen source genotype at each level for each hour after inoculation at each date. An analysis of variance in the form of a complete factorial with pollen source genotype, exposure level and hour after inoculation as the main effects was performed for each category. For statistical analysis, the inverse sine transformation was applied to the percentage data (*18*). The minimum differences for significance were obtained by means of the revised Duncan's range using for p only the maximum number of means to be compared (*19*).

Results

Pollen Genotype Study

Pollen grains containing the dominant and recessive alleles at the waxy, sugary-1, and shrunken-2 loci (within each locus, the average genetic background effects between alleles were equal) were stored at 0, 1, 2, 3, 4 and 5 days at 2°C (*9*). After each storage period, a portion of each genotype was cultured on a 15% sucrose, 0.6% bacto-agar, 0.03% calcium nitrate, and 0.01% boric acid medium. Regardless of the allele present in the pollen grain, 1 day of storage greatly increased the germination percentage and significantly increased pollen tube length. After 4 days of storage, there was no *in vitro* germination. Significant differences at each storage period and a differential response to storage were obtained between the dominant and recessive alleles at certain loci for germination percentage, ruptured percentage, and pollen tube length.

Medium Supplement Studies

Surfactants are used in biological systems to reduce surface tension and in the process are introduced into the biosphere. However, they are known to produce subtle biochemical effects. The effects of three nonionic surfactants on the germination characteristics of pollen grains from three pollen source genotypes were studied (*20*). Pollen grains from three single cross hybrids (Wf9×H55, Ky228×Ky226, K64×K55) were cultured on a medium (15% sucrose, 0.6% bacto-agar, 0.03% calcium nitrate, 0.01% boric acid) supplemented with all possible combinations of three concentrations (10, 100, 1000 ppm v/v) of the following surfactants: Tween 80 (polyoxyethylene sorbitan monooleate); X-114 (alkyl phenoxypolyethoxy ethanol; and commercial sticker spreader (alkyl olefin aromatic polymers). A control containing no supplement was included. Over all genotypes, increasing concentrations of Tween 80 had the least effect on the germination characteristics measured (germination percentage, ruptured percentage, pollen tube length at 1, 2, and 3 hr after inoculation) and X-114, the greatest with no germination found above 10 ppm. For most of the germination characteristics, highly significant pollen source genotype × concentration interactions were found for each surfactant. The direction and magnitude of the surfactant effects depended on the surfactant, its concentration, the germination characteristic involved and the pollen source genotype.

UVB Study

The effects of weakly penetrating UVB radiation which could conceivably become a feature of the environment were examined during the *in vitro* pollen germination process. The variance analyses are presented in Table 1. Of major interest are the significance levels of the main effect, exposure level, and the interactions, L×G, H×L and H×L×G. Of these, only exposure level was significant at the 5% level for germination percentage and at the 1% level for ruptured percentage. The germination percentages were 46, 48, and 43 at the 0, 1, and 2 levels, respectively. The ruptured percentages were 4, 4, and 3 at the 0, 1, and 2 levels, respectively. For both characters, these differences among levels were relatively small although statistically significant. For pollen tube length, all sources of variation of major interest were significant at the 1% level. At 1 hr after inoculation, little or no difference among the pollen source genotypes was found among exposure levels (Table 2). However, at 2 and 3 hr after inoculation, large differences between level 0 and both levels 1 and 2 were apparent. The magnitude of these differences was influenced by pollen source genotype. As an example, at 3 hr after inoculation, the lengths at 0 and 2 levels were 386 and 294, respectively, for Wf9×H55 and 450 and 243, respectively; for Ky49×Ky27. These differences of 92 for Wf9×H55 and 207 for Ky49×Ky27 suggest the magnitude of the interaction. The overall effect of increasing UVB on all genotypes is graphically presented in Figure 2. At 1 hr after inoculation, differences among exposure levels were relatively small. However, at 2 and 3 hr after inoculation, differences between the 0 level and both the 1 and 2 levels were very pronounced. At all times after inoculation, the differences between the 1 and 2 levels were negligible, indicating that an equal reduction in pollen tube growth occurred in both levels.

Table 1. Mean squares and significance levels from the variance analysis of each category.

Source of variation	Degrees of freedom	Germination percentage	Ruptured percentage	Pollen tube length
Pollen source genotype (G)	4	10531.0[a]	2981.5[a]	53116[a]
Exposure level (L)	2	225.6[b]	138.2[a]	3607350[a]
L × G	8	79.0	15.7	22638[a]
Hour after inoculation (H)	2	463.8[a]	35.7	3606338[a]
H × G	8	46.0	38.1[b]	9609
H × L	4	11.0	23.2	664968[a]
H × L × G	16	13.8	10.5	32483[a]
Error	c	63.0	16.2	8198

[a]*F* values significant at the 1% level.
[b]*F* values significant at the 5% level.
[c]Error degrees of freedom were 315 and 3553 for the percentage and length data, respectively.

Table 2. Mean pollen tube length of the pollen source genotypes at each exposure level and hour after inoculation.[a]

Pollen source genotype	Exposure level	Mean pollen tube length, μm		
		1 hr after inoculation	2 hr after inoculation	3 hr after inoculation
Wf9×H55	0	227	364	386
	1	208	243	289
	2	213	261	294
Ky49×Ky27	0	216	323	450
	1	181	236	254
	2	219	230	243
K64×K55	0	223	372	411
	1	216	238	274
	2	218	248	286
H55	0	223	377	442
	1	203	244	292
	2	230	248	297
Oh43	0	235	391	406
	1	191	248	272
	2	220	216	258

[a]Minimum differences for significance were 36 and 47 at the 5 and 1% level, respectively.

Discussion

The use of pollen grains to detect the frequency and type of somatic mutations produced by exposure to environmental pollutants is limited. Firstly, a somatic mutation must occur in the pollen primordial cells to even be included in the pollen grain. If a mutation occurred in the zygote, 50% of the pollen grains would contain the mutation. However, it is most probable especially under chronic exposure conditions, that a mutation would be induced in one of many pollen primordial cells producing chimaeras of unknown size. As a result the frequency of mutation cannot be accurately estimated. Secondly, the mutation must alter either the morphology or physiology of the pollen grain in which it is contained (pollen genotype or haploid effect) so that recognition is possible. The waxy locus in maize is one of the very few loci in which the genotype of individual grains can be easily classified by a simple staining procedure. Therefore, pollen techniques in most if not all species of higher plants cannot accurately estimate mutation frequency and are limited to those types of mutations at a restricted number of loci which express distinct, easily-tested pollen genotype effects.

The results of the study presented here indicate that different alleles or mutant forms at various loci could be readily detected by *in vitro* germination characteristics. In this case, the comparison was between pollen grains obtained from sporophytes homozygous for each allele at each locus in the same average genetic background. At the present time, the specific physiological mechanisms involved in *in vitro* pollen germination and the influence exerted by the pollen genotype are not known. However, biochemical differences (*21-23*) between pollen grains containing the alleles at various endosperm loci and ash percentage and mineral content differences (*24*)

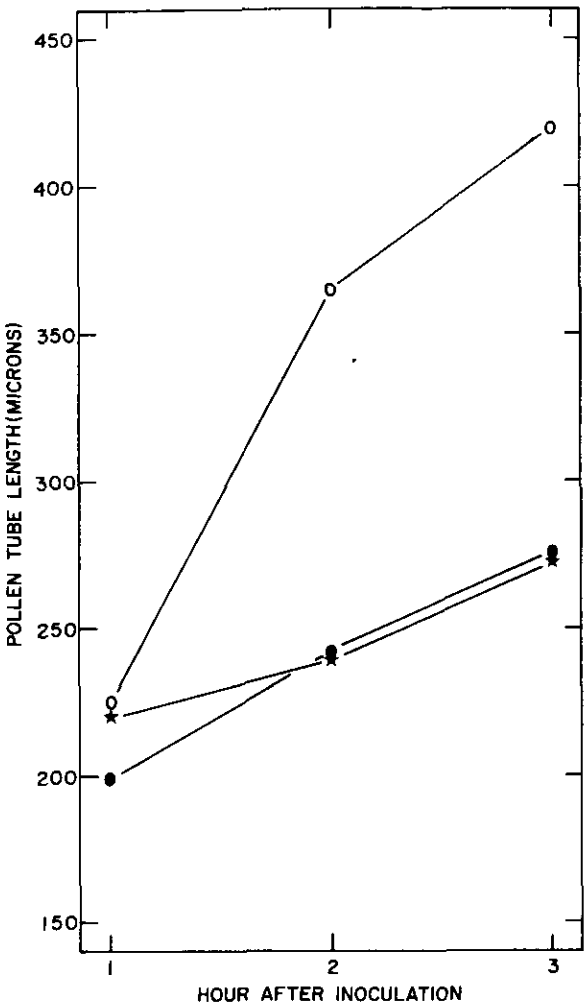

FIGURE 2. Effect of various UVB exposure levels on pollen tube length 1, 2, and 3 hr after inoculation. Mimimum differences for significance were 15 and 19 at the 5 and 1% level, respectively.

among various pollen source genotypes have been reported. These studies suggest that genetic alterations at many loci can be *in vitro* pollen germination. In many cases. the differences in *in vitro* germination characteristics resulting from genotypes can be amplified by altering the calcium-boron medium combinations (*10, 11, 16*). Therefore, *in vitro* germination characteriss could be used to provide information on the type of mutations induced by environmental pollutants if homozygous sporophytes containing the mutant alleles are available.

The physiological effects of various agents on *in vitro* pollen germination can be easily studied by adding these agents directly to the medium. For maize, the only limitations are that the agent be soluble or miscible with water and chemically stable in an aequeous medium for short periods. Gentile et al. (*25*) avoided these limitations with maize pollen by placing the agent at a given concentration on the surface of the prepared medium and then allowing the agent to dry. This modification introduces difficulties in comparing concentrations since the germination of maize pollen grains requires a delicate gas-liquid interface (*13*) around the pollen grain which will be altered by drying procedure. Using this modification, a large number of pesticides and adjuvants were found markedly to reduce *in vitro* pollen germination and tube growth with concentrations having small effects as expected (*25*). Pollen source genotypes were included as part of this study (*25*) but their individual response patterns were not reported. However, pollen source genotype interactions with various concentrations of nonionic surfactants added directly to the medium have been reported in maize (*20*). The effects of various pesticides on *in vitro* pollen germination of other species have been examined with similar depressions in germination and tube growth found (*26-28*). As indicated, only limited studies have been conducted using *in vitro* pollen germination as a biological assay to determine the physiological activity of some agents. However, these studies indicate the potential of this method.

The biological activities produced by radiation in the biosphere can be readily examined using *in vitro* pollen germination. Recently, an increase in the amount of UVB radiation in the biosphere was predicted as an indirect result of technology (*1-3, 29*). UVB has many known biological effects (*2, 4, 6*) but certainly the most significant in evolutionary terms is the induction of genetic changes (*5, 6*). In some species with maize the most obvious example, the genetic contents of the pollen grain is normally exposed and therefore, subject to the effects of UVB. This exposure could conceivably occur not only in the dormant mature pollen grain but also during *in vivo* germination and tube growth on the silk (Fig. 3). Exposure of mature pollen grains before germination to UVC (254 nm) was found to induce genetic changes which were transmitted to the next generation or caused sterility (*30,31*) and also altered the *in vitro* germination characteristics of one pollen source genotype (*32*). The results of the study described here indicate that UVB exposure during the *in vitro* germination process resulted in little or no tube growth after 1 hr. Differences between the two UVB exposure levels tested were negligible. Apparently. a threshold exists for UVB exposure so that increasing exposure over this threshold resulted in no decrease in tube growth. According to the results, the threshold level must

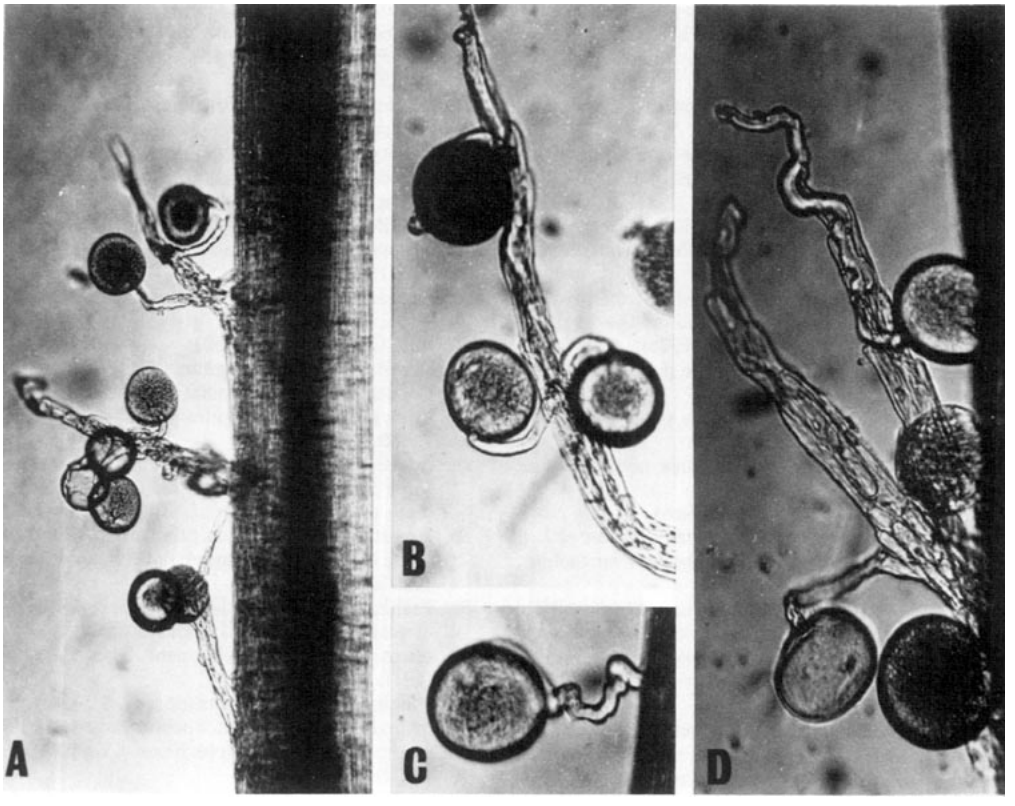

FIGURE 3. *Photomicrographs showing pollen grains germinating on the silk (taken 6 hr after pollination): (A, B)* (grains with tubes entering the silk hairs; the lighter-colored, empty grains indicate faster germination; *(C)* tube entering the silk body directly rather than through the silk hair; *(D)* pollen tube growing in wrong direction away from ovule.

be between level 0 (0 mW/m^2) and level 1 (1668 mW/m^2). The pollen genotype effects found in this study indicate UVB will produce different effects depending on the pollen source genotype. The relationship between *in vitro* germination and *in vivo* transmission has not been established but these results suggest that UVB will not only produce physiological effects but also genetic alterations which are differentially transmitted depending on the pollen source genotype. *In vitro* pollen germination studies of radiation effects has not been adequately explored but does offer an ideal method to examine all the effects of these weakly-penetrating agents on complex higher organisms.

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