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A New Concept in Assessing Cotton Pollen Germinability[1]

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ABSTRACT

Contrary to current concepts of pollen germination and tube growth, intact pollen tube-like structures up to 4,000 μm in length were forecfully ejected, instantly, from pollen grains of cotton, *Gossypium barbadense* L. and *G. hirsutum* L., suspended in a hanging droplet, indicating a unique mechanism for pollen tube formation. Tubes disintegrated instantly after "germination" unless the germinating medium was supplemented with certain calcium, manganese, zinc, chlorine, or magnesium salts. The optimal combination was $Ca[NO_3]_2$ and $MnSO_4$, each at 0.14 g, in 100 ml of distilled water. As sucrose concentration increased, the tubes became shorter and smaller in diameter and were ejected at a slower rate. The frequency of germination was unaffected until the sucrose concentration reached 1.7 *M*.

Germination dropped to about 50% in concentrations from 1.7 to 2.0 *M*. Cotton pollen did not germinate until after anthesis. From 30 min to 8 hours after anthesis, germination exceeded 98%. It dropped to 30% after 24 hours and to 1% in 32 hours. Cotton pollen viability was sensitive to temperature, in that full viability was observed in the range of 32 to 40 C with no germination above 42 C. This technique is a simple and effective method for inducing more than 98% germination in both *G. hirsutum* and *G. barbadense* and accurately measures cotton pollen viability.

Additional index words: *Gossypium barbadense* L., *Gossypium hirsutum* L., Tissue culture, Osmotic pressure, Pollen tube, Pollen viability.

A rapid and relible method of germinating cotton pollen to measure viability is needed to evaluate the presence of cytoplasmic male sterility and fertility-restoring genes in parents selected for hybrid cotton production. This method would also be useful in assessing pollen viability as related to environmental stress.

Pollen viability has been measured by several means. The most useful methods have been vital staining and in vitro germination. The use of vital stains, however, is not reliable for most species. Hence, in vitro germination is more commonly used to determine pollen viability (7). A problem is that in vitro germination is lower and pollen tube length and growth rates do not equal those observed with in vivo germination (8). Stanley (8, 9) reasoned that in vivo germination of pollen is enhanced by natural secretions not duplicated in artificial media. Some principal components that have been used in successful pollen germinating media are sucrose, boron, calcium, and agar (3, 5, 7, 10).

The nature of the pollen grain wall and cytoplasm of *Gossypium spp.* has made vital staining difficult. The in vitro germination of cotton pollen has also been difficult. Kearney and Harrison (2) unsuccessfully attempted in vitro methods of cotton pollen germination and measured pollen viability by the percentage of pollen grains which burst when placed in weak sugar solutions and distilled water. Miravalle (4) reported a modification of a technique developed by Bronkers (1) in which he obtained an average of 56% germination in *G. hirsutum* L. 'Acala 4-42.' Taylor (10) reported an average of 30% germination of *G. hirsutum* pollen seeded on a dense agar plate supplemented with calcium nitrate ($Ca[NO_3]_2$, manganous sulfate ($MnSO_4$), boric acid (H_3BO_3) and sucrose. He found that only an occasional pollen grain of *G. barbadense* L. germinated on the above medium. Taylor's method was more rapid and produced longer tubes than previous methods used to germinate cotton pollen.

The objectives of the study reported in this paper were to use tissue culture techniques to determine the effects of several components of pollen germinating media on cotton pollen germination, and to establish optimal conditions that would provide a reliable technique to ascertain pollen viability.

MATERIALS AND METHODS

A microdroplet assay technique (6) was modified to determine the effect of various concentrations of sucrose and several mineral salts on cotton pollen germination. Specific combinations of sucrose and mineral salts were pipetted on to a 4 × 4, 65 mm Rodac plate in 20 μl droplets. Pollen was dispersed in each droplet by touching the drop with a dehisced anther. Each anther could inoculate about four drops; an average of 80 anthers per flower made possible approximately 320 treatments per flower. Fifty to 200 pollen grains were dispersed in each drop. The dish was inverted, suspending the pollen in a "hanging drop." The pollen was observed at 60× magnification with a stereo binocular microscope to evaluate "germination." The four experiments described below probably best represent the findings of this study.

In the first experiment, a sucrose gradient was used at 20 concentrations in 0.1 *M* intervals ranging from 0.1 to 2.0 *M* sucrose; distilled water was used as a control. Superimposed on each sucrose concentration were $Ca[NO_3]_2$ and $MnSO_4$, each at concentrations of 1.40, 0.70, 0.35, and 0 g per liter; H_3BO_3 at 0.8, 0.4, 0.2, and 0 g per liter; and a combination of all three in their respective concentrations listed above. This procedure resulted in three concentrations of each compound separtely and in combination (12 treatments) and four controls in each specific con-

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centration of sucrose or distilled water (a total of 336 treatments and controls). The entire experiment was repeated twice on the three pollen sources listed below. Various parts of the experiment were repeated from 12 to more than 100 times to evaluate the effects of minerals and sucrose concentrations on the pollen tube.

Fresh cotton flowers at anthesis were harvested from greenhouse-grown P-32, (*G. barbadense*); 5643, a selection from 'Rex' (*G. hirsutum*) × 'Sea Brook Sea Island' (*G. barbadense*); and DHNE, a doubled haploid nectariless breeding stock of *G. hirsutum*. Pollen from each source was used to inoculate each series of treatments. An explosive extension of a pollen tube was evidence of germination. The percentage of germination and tube condition was noted. The time requirement for this experiment was 1 day.

The second experiment contained two replications of 11 chemicals and two controls, each in three concentrations: 1.40, 0.70, and 0.35 g per liter and a 0 g control. The chemicals were potassium nitrate (KNO_3), ammonium nitrate (NH_4NO_3), $Ca[NO_3]_2$, magnesium sulfate ($MgSO_4 \cdot 7H_2O$), potassium phosphate (KH_2PO_4), calcium chloride ($CaCl_2 \cdot 2H_2O$), ammonium sulfate NH_4PO_4), calcium phosphate ($Ca[H_2PO_4]_2$), zinc sulfate ($ZnSO_4$), potassium chloride (KCl), and calcium sulfate ($CaSO_4 \cdot 2H_2O$). Two controls were $Ca[NO_3]_2$ + $MnSO_4 \cdot 2H_2O$ and $CaSO_4 \cdot 2H_2O$ + $MnSO_4 \cdot 2H_2O$. The droplets of three concentrations and a zero control for each chemical and two controls, 52 total, were inoculated with pollen from P-32 and DHNE. Tube condition was observed in each treatment.

A standard pollen germinating medium (SPGM) was made with $Ca[NO_3]_2$ and $MnSO_4$, each at 0.014 g in 10 ml of distilled water for additional experiments. In a third experiment, DHNE flowers were collected 10 days, 72, 48, and 24 hours, and 30 min prior to anthesis and 30 min, 2, 4, 6, 8, 24, and 32 hours after anthesis. Percentage of germination was recorded after seeding pollen on SPGM droplets. The experiment was repeated three times.

In the fourth experiment, flowers were collected 15 hours before anthesis and placed in a humidity chamber overnight at constant temperatures of 32, 38, 39, 40, 41, 42, 43, and 48 C. After an apparent normal anthesis, pollen was dispersed in SPGM droplets and germination percentage was recorded. This experiment was repeated twice.

RESULTS AND DISCUSSION

The effect was striking of all concentrations of $Ca[NO_3]_2$, $MnSO_4$, or their combination with H_3BO_3 in distilled water on the germination of cotton pollen. Pollen tubes were ejected explosively, and germination began less than 2 min after pollen had been dispersed in the droplets. Within 3 to 4 min, 98 to 100% of all grains had germinated (Fig. 1). The tubes were normal in appearance and ranged in length from 100 to 4,000 μm, about 40 times the pollen diameter. The average length was about 1,500 μm. These observations are not in agreement with current concepts of pollen tube growth. For tubes of this size to emerge instantly, there must be a special mechanism for tube development. Pollen dispersed in distilled water controls appeared to burst; however, closer observation showed that they did indeed eject a tube that disintegrated in seconds. Therefore, $Ca[NO_3]_2$ and $MnSO_4$ appear to have a stabilizing effect on the tube. Boric acid improved tube stability only slightly relative to the distilled water controls. Pollen tubes expelled in only H_3BO_3 and distilled water were very fragile and deteriorated rapidly. There were no visible adverse or beneficial effects of H_3BO_3 when used in combination with $Ca[NO_3]_2$ and $MnSO_4$. All selections of both *Gossypium spp.* and the interspecific cross germinated equally well. It appeared that *G. barbadense* pollen tubes were more favorably affected by $Ca[NO_3]_2$ and *G. hirsutum* tubes were favored by $MnSO_4$.

The germination frequency was unaffected by increasing the sucrose concentration to 1.6 *M* with $Ca[NO_3]_2$ + $MnSO_4$; however, from 1.7 to 2.0 *M* sucrose, germination dropped to 50%. Increasing the sucrose concentration from 0.0 to 1.0 *M* decreased the pollen tube length and also decreased the rate of tube ejection slightly. Cotton pollen became bouyant at 1.1 *M* sucrose, indicating an osmotic equilibrium at that point. Tubes ejected in concentrations above 1.1 *M* were similar in length to tubes ejected in distilled water. They differed by being smaller in diameter, lacked cytoplasm, and were highly con-

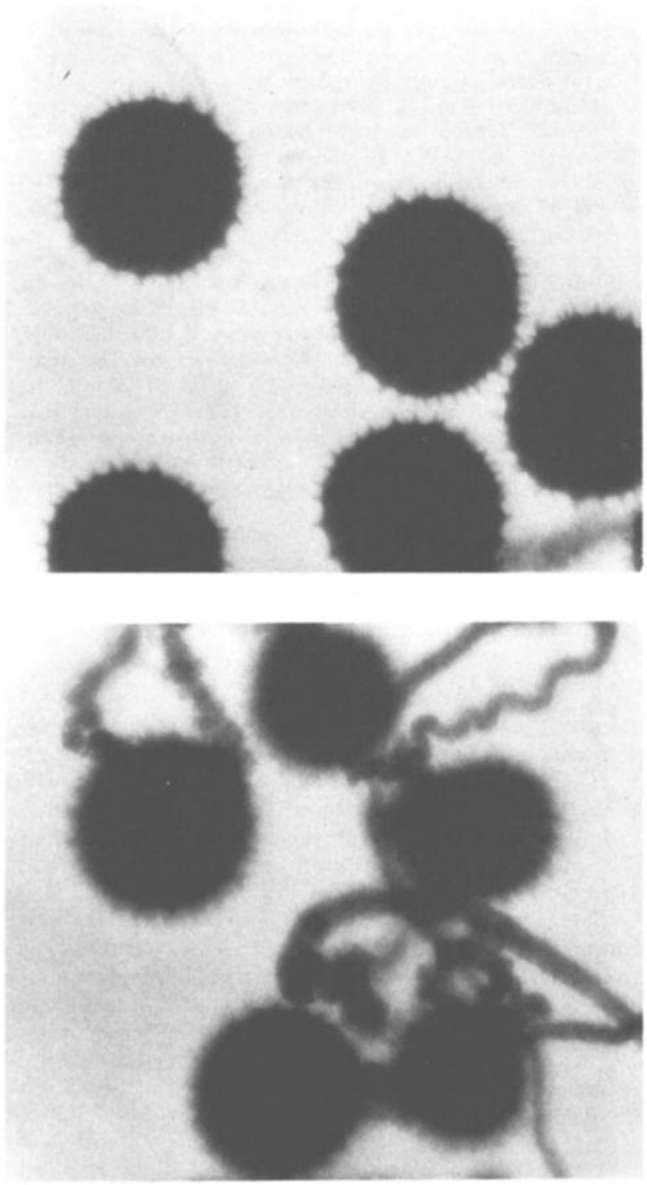

Fig. 1. Cotton pollen grains photographed 2 min apart before and after germination.

voluted. This result indicates the presence of a mechanism within the pollen grain that can eject tubes against a greater osmotic pressure than exists within the grain. In control solutions without $Ca[NO_3]_2$ or $MnSO_4$, the tubes deteriorated instantly in concentrations up to 1.0 *M* sucrose. From 1.1 to 2.0 *M* sucrose, no tubes were ejected and the pollen grains increased in bouyancy.

The optimal concentration of $Ca[NO_3]_2$ and $MnSO_4$ is about 1.4 g each per liter. The effective range of $Ca[NO_3]_2$ is aobut 0.1 to 2.8 g per liter and for $MnSO_4$, 0.1 to 1.4 g per liter. Chemicals comparable to $Ca[NO_3]_2$ and $MnSO_4$ in about the same concentrations are $MgSO_4 \cdot 7H_2O$, $CaCl_2$, $Ca(H_2PO_4)_2$, $ZnSO_4$, and KCl. In $ZnSO_4$, 4 to 5 min elapsed before germination occurred but the tube appearance and germination frequency were good. Chemicals that produced poor quality tubes were KNO_3, NH_4NO_3, and KH_2PO_4. Tubes in NH_4SO_4 and $CaSO_4 \cdot 2H_2O$ deteriorated in the same manner as those treated with distilled water. The best tubes were observed when $CaSO_4 \cdot 2H_2O$ and $MnSO_4$ were used in combination.

An effective standard pollen germinating medium for both species of cotton tested is 14 mg $Ca[NO_3]_2$ and 14 mg $MnSO_4$ dissolved in 10 ml of distilled water. This medium was found to be effective for about 3 days, before losing its capacity to promote good germination.

No germination occurred in any pollen collected before anthesis. Pollen taken from flowers just opening, on the day of anthesis, but about 30 min before the anthers dehisced, germinated about 10%. Pollen from anthers of the same flowers, after the anthers had dehisced, germinated over 98%. There seems to be a precise mechanism, possibly enzymatic in nature, to prepare the pollen for germination. Pollen continued to germinate at 98 to 100% up to 8 hours after anthesis under greenhouse or laboratory conditions. Germination dropped to about 30% after 24 hours and to 1%, 32 hours after anthesis.

Temperature also imposed an important effect on the germination of cotton pollen. Pollen germinated over 98% when flowers were removed from the plant and held 15 hours in a humidity chamber at constant 32 to 40 C. The anthers dehisced normally. Flowers handled similarly, but held 15 hours at 41 and 42 C, germinated about 10% with tube deterioration and bursting. At 43 and 46 C, flowers had normally appearing anthers and pollen, but only an occasional anther dehisced. No germination was observed in pollen taken from either dehisced or nondehisced pollen from flowers incubated at 43 and 46 C.

The exact nature of the tubes ejected from pollen grains in the hanging droplet method is not known. However, if there is a sufficient concentration of calcium (Ca), chlorine (Cl), manganese (Mn), zinc (Zn), or magnesium (Mg), the pollen grain can be moved through the droplet by manipulating the tube with a needle. Once ejected, the tubes cease to grow and are apparently no longer viable. Tubes from cotton pollen germinated on Taylor's medium (10), or on the stigma in vivo are much stronger and pollen grains can be hoisted by the tube, using a needle. It is reasonable to expect that, under natural conditions, the cellulose microfibrils are positioned and interlocked normally, adding considerable structure and strength to the tube wall. On the other hand, when tubes are forcefully ejected, the orderly arrangement of wall components must be affected, thus causing weaker tubes. It is evident from these observations that the pollen grain supplies a significant portion of the material used in tube growth. The ability of the pollen to eject tubes into solutions with higher osmotic pressure than exists within the pollen grain indicates a driving force capable of assisting the tube to penetrate the stigmatic tissue to affect fertilization.

Germination of pollen in the hanging droplet to which mineral salts have been added, should not be classed as normal germination because the tubes and pollen are no longer viable after the tube has been ejected. This hanging droplet method, however, provides an accurate measure of the germinabillty of pollen. It thus offers a rapid and simple means of measuring the effects of temperature, age, or other factors that may influence the viability of pollen.

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