To the Graduate Council:

I am submitting herewith a thesis written by Samuel H. Wauford entitled "In Vitro Germination of Upland Cotton Pollen: An Analysis of Parameters Affecting Germination and Tube Length." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Botany.

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IN VITRO GERMINATION OF UPLAND COTTON POLLEN: AN ANALYSIS OF PARAMETERS AFFECTING GERMINATION AND TUBE LENGTH

A Thesis

Presented for the

Master of Science

Degree

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Samuel H. Wauford

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ABSTRACT

An improved culture medium was developed for the *in vitro* germination and tube growth of upland cotton (*Gossypium hirsutum* L. cv. Hancock) pollen. The pollen grains were dusted onto a 3.5% agar medium and then separated with a dissecting needle. The parameters tested to determine their effects on the percent germination and tube length of cotton pollen were pH, carbohydrates, magnesium, manganese, calcium, potassium, boron, gibberellic acid, indole-3-acetic acid, and succinic acid-2,2-dimethylhydrazide.

All of the data were analyzed by a Duncan's Multiple Range test to show significant differences among the treatments. The germination of cotton pollen was more sensitive to the variability of the greenhouse conditions and the *in vitro* culture conditions than was the pollen tube growth.

A high pH (7.6) significantly increased the percent germination and tube length of cotton pollen *in vitro*. Magnesium sulfate and gibberellic acid both significantly increased the mean pollen tube length; however, they had no significant effect on the percent germination. Calcium nitrate at all concentrations tested inhibited both germination and tube growth. Manganous sulfate and boric acid were vital for the growth of the cotton pollen tube *in vitro*. Potassium nitrate and indole-3-acetic acid did not

significantly affect either germination or tube growth, but a positive trend in the percent germination and tube length was associated with their presence in the medium.

The final medium improved by experimentation contained 100 ml distilled water, 25 g sucrose, 5.9 mM manganous sulfate, 1.0 mM potassium nitrate, 1.0 uM gibberellic acid, 0.1 indole-3-acetic acid, adjusted pH of 7.6, and 3.5 g agar. The mean percent germination and mean tube length of cotton pollen grown on the final medium were 47% and 2.6 mm respectively. Cotton pollen grown on the final culture medium had an increased percent germination and tube length of 74% and 68% respectively over the best previously published medium (Taylor, 1972).

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CHAPTER I

INTRODUCTION

The need to develop a dependable *in vitro* germination medium for cotton pollen has arisen due to the emphasis being placed on obtaining genetic traits through interspecific and intergeneric crosses. Many crosses have been attempted *in vivo*; however, incompatibility in the styles prevented fertilization of the ovules (Govila, 1972). The elimination of the style and the subsequent *in vitro* fertilization of interspecific and intergeneric crosses may be possible after a dependable *in vitro* germination medium is developed.

In vitro studies on cotton pollen germination behavior may be helpful to the cotton breeder in other ways. For example, the *in vitro* germination of cotton pollen could be used to test the viability of the pollen at different time intervals after anthesis to discover the optimum time for cross-pollination to occur. *In vitro* germination studies also could be used to examine the effects of different environmental conditions for the ultimate purpose of getting optimum pollen germination and thereby better boll set.

During pollen germination and pollen tube growth, a series of coordinated biochemical and physiological changes occur. The *in vitro* germination of cotton pollen in a

chemically defined medium may serve as a model system for analyzing the physiological requirements of the pollen tube as it grows. Cytochemical analysis of *in vitro* germinated cotton pollen along with the knowledge of some of the physiological requirements may help researchers to understand the factors involved in germination and tube growth. There is the possibility that clues to the function of stimulatory or inhibitory substances may be discovered by searching for structural changes in the growing pollen tube.

This study was conducted to develop a dependable method for germinating cotton pollen *in vitro*. Some physical and physiological requirements of the cotton pollen were analyzed in relation to their effects on the germination and the tube length of the cotton pollen grown *in vitro*.

CHAPTER II

REVIEW OF LITERATURE

Early History of Pollen Germination *In Vitro*

The method of germinating pollen *in vitro* was used very early in the history of plant physiology. Braconnet (1829) published the first detailed chemical analysis of pollen, but Van Tieghem (1869) was the first to experiment with pollen tube growth *in vitro*. He found that pollen germination required oxygen, and that the oxygen was accompanied by carbon dioxide production. He observed also that starch disappeared from the grain during the growth of the pollen tube. Van Tieghem is thought to have been the first to add a carbohydrate in the form of cane sugar to his culture medium.

History of Cotton Pollen Germination *In Vitro*

The initial reason for attempting an *in vitro* germination method for cotton pollen was to measure the viability of the pollen (Kearney and Harrison, 1932). Banerji (1929) and Kearney and Harrison (1932) were unsuccessful with other crops. Iyengar (1938) extensively studied the morphology of the developing cotton pollen tube, but he was resigned to *in situ* dissections after failing to develop a method for *in vitro* germination.

Bronckers (1961) reported the development of a successful germination method, and Miravalle (1965) repeated this method using pollen of Gossypium hirsutum cv. Acala 4-42. He found that the average pollen germination was 55%, but the tube cytoplasm was cloudy and granular, and the average tube length was only 0.25 mm. Govila and Rao (1969) studied the effects of calcium on the germination and tube length of G. hirsutum and G. arboreum pollen. They observed that the addition of calcium complex consisting of 1.27 mM calcium nitrate, 0.8 mM magnesium sulfate, and 1.0 mM potassium nitrate (Brewbaker and Kwack, 1963) considerably accelerated the germination and tube growth of G. hirsutum pollen. Maximum germination (58%) was obtained on a liquid medium containing 35% sucrose, 1.6 mM boric acid, and calcium complex; however, most of the pollen grains were polysiphonous, and the average tube length was only 0.182 mm. Taylor (1972) developed an improved technique for the germination of cotton pollen in vitro. He used a 3.5% agar medium for his study, and he found that dusting rather than brushing pollen onto the medium gave a high germination percentage. Taylor's technique and the medium he developed did not overcome the rupturing of the pollen tube tip.

pH of the Medium

Brink (1925) showed that the *in vitro* germination and tube growth of sweet pea were considerably influenced by the change in pH of the culture medium. Sisa (1930) found that cruciferous pollen had to be grown in a culture medium containing sugar and agar, and the rate of germination depended on the pH of the medium. A pH level below 7.0 was unsuitable for the germination of cruciferous pollen. Loo and Hwang (1944) confirmed Sisa's work when they found that a culture medium with a pH between 5.0 and 6.0 was unsuitable for germination of *Brassica juncea* pollen. They adjusted the pH of the medium to 7.5 to obtain maximum germination. Kwack (1965) studied the effects of pH on the *in vitro* germination and tube growth of pollen from a variety of crop plants. He tested pollen from twenty-five genera in fifteen families, and found that the pH range for germination and tube growth *in vitro* was rather narrow. A relatively high pH was favorable when calcium was available in the medium. One half of the species tested showed best pollen germination and tube growth at pH 8.3. The other half of the species tested showed similar results at pH 7.3. The species favoring pH 7.6 had their germination and tube lengths decreased sharply when the pH of the medium was raised to 8.3.

Proteins are among some of the constituents of pollen grains that diffuse from the germinating pollen (Stanley

and Linskens, 1965). Stanley and Linskens (1965) showed that the amount of protein lost by the pear pollen into the medium corresponded to the time interval that the pollen remained in the medium. Less protein was lost from pine and palm pollen in a medium with a pH between 6.0 and 7.0, and this range corresponded to the optimum pH for germination and tube growth in vitro.

Calcium Complex in the Medium

The essential role of calcium in a pollen germination medium was first discovered by Brink (1924c) when he showed that calcium alone stimulated pollen growth and antagonized the injurious effect of other metal ions. He also observed that a large number of pollen grains in a culture media gave a larger germination percentage than did a small number of pollen grains in the same volume of culture media. Savelli and Caruso (1940) convincingly demonstrated the pollen culture phenomenon which became known as the "population effect." Brewbaker and Majumber (1961) believed the population effect only occurred with pollen grown in vitro. They experimented with different growth factors that might be present in the pistil, but were lacking in the culture medium. Brewbaker and Kwack (1963) found that the population effect was overcome by a calcium complex consisting of calcium ions in the presence of potassium and magnesium.

They observed that the effects of increasing the calcium concentration in the culture medium were apparent in the rigidity and straightness of the pollen tubes, with little or no coiling at high concentrations of calcium. Without boric acid the calcium was unable to promote germination. Brewbaker and Kwack (1964) extended their studies using ^{45}Ca-labeled salts to show that calcium was distributed uniformly along the pollen tube wall, and essentially no label appeared in the cytoplasm of burst tubes.

As previously mentioned, calcium overcame the population effect best if potassium and magnesium were present in the culture medium (Brewbaker and Kwack, 1963). Weisenseel and Jaffe (1976) found that the in vitro growth of lily pollen required at least 10 to 100 uM potassium in the medium, and the added potassium affected the elongation and the diameter of the pollen tubes. They theorized that the growing tube pumped protons out, and the resulting rise in the membrane potential established an electrochemical gradient that stimulated the uptake of potassium.

Manganese in the Medium

Manganese ions have been shown to partially substitute for calcium ions in a number of physiological processes (Naitoh, Eckert, and Friedman, 1972; Terepka, Coleman, and Hunter, 1975). Weisenseel and Jaffe (1976) theorized that

manganese ions are absorbed by the pollen tubes, and they act within to speed the entry of potassium ions.

Manganese added to a culture medium stimulated both the percent germination and the tube growth of Brassica juncea pollen (Loo and Hwang, 1944). The germination and tube growth increased as the manganese concentration increased, and the stimulating effect of manganese was manifested in a wide range of concentrations which resulted in normal tube growth (Loo and Hwang, 1944). Zielinski and Olez (1963) found that prune and pear pollen tubes grown in vitro with 0.74 mM manganese added to the medium were three to four times longer than pollen tubes grown on media lacking manganese.

Boron in the Medium

Boron has long been known to be essential as a requirement for the growth of higher plants. Aghulon (1910) first suggested this after he found that corn, rape, and turnip plants fertilized with added boron increased their mean weights as the boron concentration increased. Aghulon's boron studies were confirmed by Maze (1915) and Warington (1923) who studied boron's effects on Zea mays and Vicia faba respectively.

Stigmatic fluid has been used in culture media to stimulate the growth of pollen (Manaresi, 1921). Schmucker

(1932) discovered that the stigmatic fluid of Nymphaea sp. contained high levels of boron. He substituted boron in the place of the stigmatic fluid and found that the pollen grains required almost the same concentration of boric acid as was present in the stigmatic fluid (Schmucker, 1935).

Boron's effects on the in vitro germinated pollen grain have been studied extensively on a wide variety of plants not only to find the optimum concentration for tube growth but also to discover boron's mode of action on a single growing cell. Vasil (1960a) studied the effects of boron on the pollen germination of a number of plants in the Cucurbitaceae, and found that 1.6 mM to 2.4 mM boron was favorable while higher concentrations inhibited germination. He found the favorable concentration of boron for germination and tube growth of pollen in the Gramineae to be similar to that of the Cucurbitaceae. For example, Pennisetum typhoideum cultured on media containing 150 mg of boric acid per liter gave 78% germination and good tube growth. The effects of boron on the elongation of the pollen tube was much more marked than on the percentage of germination (Vasil, 1960a).

The role of boron has been controversial since Shive (1936) first suggested that boron functioned in protein synthesis or in the synthesis of some component of the cell wall. Gaugh and Dugger (1953) postulated that borate ions formed sugar-borate complexes with sugar molecules, and these

complexes moved through the cellular membranes of the plant more readily than nonborated sugar molecules. O'Kelley (1957) observed that boron added to the germination medium stimulated oxygen uptake and sugar absorption by the growing pollen tubes. Dugger and Humphreys (1960) hypothesized that boron may be related to a specific enzyme involved in carbohydrate metabolism. Vasil (1960a) summarized the possible roles of boron on the germination and tube growth of pollen grains. He stated, "It promotes absorption and metabolism of sugars by forming sugar-borate complexes; it increases oxygen uptake; and it is involved in the synthesis of pectic materials for the wall of the actively growing pollen tube."

Sugars in the Medium

The role of sugars in the germination of pollen has been debated for many years. Although Van Tieghem (1869) was the first to add a carbohydrate (cane sugar) to his culture medium, Brink (1924a, 1924b) first suggested that sugars may regulate the osmotic pressure as well as supply nutrition to the growing tubes. Visser (1955) believed that sugars only created a favorable osmotic condition for germination, while all the nutrients necessary for germination and tube growth were endogenously supplied by the pollen grain. O'Kelley (1955) disproved Visser's theory by using ^{14}C-labeled sugars to show that pollen tubes respired during

their growth, and absorbed externally supplied sucrose, glucose, or fructose. Hellmers and Machlis (1953) found that pollen grains absorb and metabolize a variety of mono-, di-, and trisaccharides. The pollen tubes exogenously supplied with sugar continued to grow, and they remained healthy while the pollen grains placed on a medium without an exogenous sugar source died. Hrabetova and Tupy (1963) tested twenty oligosaccharides, monosaccharides, and sugar alcohols, and they observed that raffinose was the best substrate for growth and respiration of apple pollen tubes. Raffinose was hydrolyzed much more slowly than sucrose by apple pollen, and the growth effect was of longer duration.

Clues to the growth factors of pollen tubes will come only from an extensive analysis of the physical and chemical interactions between pistil and pollen during tube growth through the pistil. Portnoi, Horovitz, and Galil (1975) found sucrose, glucose, and fructose in the stigmatic exudate of Yucca aloifolia. Yucca pollen germinated well in a culture medium containing sucrose and/or glucose, but only slight germination occurred on fructose. Dynansagar and Thomas (1970) studied the effects of sucrose, glucose, raffinose, and mannose on the in vitro germination and tube growth of Phaseolus radiatus pollen. They observed that sucrose was the best carbohydrate for germination and longest pollen tubes. Raffinose was found to be the least suitable

sugar for germination, although the tubes grew as long as those on a sucrose medium. Nygaard (1977) used pine pollen in suspension culture to investigate the utilization of certain exogenous sugars by pollen. He found that D-ribose, L-arabinose, 2-deoxy-D-ribose, D-mannitol, and D-inositol were not metabolized by the pine pollen tubes. Fructose, glucose, galactose, xylose, and oligosaccharides which produced free fructose, glucose, or galactose were metabolized. All of the metabolizable sugars except xylose were converted to starch. Nygaard observed that exogenous sucrose was rapidly converted to fructose and glucose in the culture medium, and there was a preferential uptake of fructose by the pine pollen tubes. Nygaard summarized his studies with exogenously supplied sugars in suspension cultures of pine pollen by stating, "The cleavage of the oligosaccharides in the media indicates that uptake mechanisms exist only for fructose, glucose, galactose, and xylose."

Growth Hormones and Regulators in the Medium

The effects of growth hormones such as gibberellins and auxins on the germination of pollen *in vitro* have been studied extensively. Since gibberellic acid affects cell elongation (Stowe and Yamaki, 1957), it seemed pertinent for researchers to study the effects of gibberellic acid on the germination and elongation of pollen tubes.

Kato (1955) observed a stimulation of lily pollen tube growth *in vitro* with added gibberellic acid. At 50 mg per liter gibberellic acid the pollen tubes were five times longer than pollen tubes grown on media without gibberellic acid. High concentrations of gibberellic acid inhibited growth of the pollen tubes. Since Kato's studies with gibberellic acid, many researchers have obtained varying results depending on the plant species. Gibberellic acid added to the culture media increased the tube growth of *Pisum sativum* pollen, but had no significant effect on the percent germination (Bose, 1959). Bamzai and Randhawa (1969) tested the effects of gibberellic acid on three species of mango pollen and found that there was species specificity in the response to gibberellic acid. Taylor (1972) observed that gibberellin at all concentrations tested severely curtailed the *in vitro* germination of cotton pollen.

Barendse, Pereira, Berkers, Driessen, Van Eyden-Emons, and Linskens (1970) showed that the gibberellic acid level per gram of fresh weight of maturing pollen decreased as the pollen reached maturity, and that the gibberellic acid level continued to decrease during germination. They believed that the breakdown of gibberellic acid in germinating pollen and the subsequent lack of gibberellic acid in the grain as the pollen tube elongates may be a factor that causes the early cessation of growth *in vitro*.

Barendse et al. (1970) demonstrated the presence of auxins in pollen and styles of Petunia hybrida as well as of three Lilium species. Smith (1939) reported that auxin (IAA) had some stimulating effect on both the germination and tube length of in vitro cultured pollen of Tradescantia sp., Polygonatum sp., and Pinus sp. On the other hand, applied auxin (IAA) was found to inhibit both pollen germination and tube growth of Milla biflora and Tropaeolum majus (Addicott, 1943). McLeod (1975) studied IAA's effects on pollen germination, and he showed that high IAA concentrations inhibited germination of tomato pollen in vitro but promoted tube elongation. Taylor (1972) found that IAA at all concentrations tested either partially or severely curtailed germination of cotton pollen in vitro.

The mechanism of action of certain growth inhibitors is not well understood. Reed, Moore, and Anderson (1965) postulated that the mechanism of action of succinic acid-2, 2-dimethylhydrazide (SADH, B-995) may be based on the hydrolysis of the compound "UDMH" (unsymmetrical dimethylhydrazide), which inhibits diamine oxidase from converting tryptamine to IAA. On the other hand, Ryuge and Sachs (1969) concluded from their studies that the UDMH moiety is not the active part of SADH. They believed the primary effect of SADH is to inhibit IAA synthesis. No previous studies on the effects of SADH on pollen grown in vitro are reported.

CHAPTER III

MATERIALS AND METHODS

Experimental Material

Pollen of upland cotton (Gossypium hirsutum L. cv. Hancock) was the experimental material in this study. The pollen source plants were grown in a greenhouse in ten inch pots containing 1:1 sand and vermiculite. Throughout the year only natural lighting was used. The pots were irrigated once a week with a modified Hoagland's solution (Guinn, 1974) and with tap water as needed. The plants were pruned periodicall to initiate new vegetative and reproductive growth. All unused flowers were excised daily to prevent the plants from becoming boll bound. These procedures guaranteed that flowers would be available for experimentation throughout the year. Those flowers chosen for experiments had normal stigmas and an abundance of pollen clinging to their anthers. Generally, the material was collected at approximately 10:00 A.M., and then taken to the laboratory in a plastic container to prevent the loss of pollen from the anthers.

In Vitro Culture Conditions

Taylor's (1972) culture medium consisting of 100 ml. of distilled water, 3.5 g bacto-agar, 25 g sucrose, 4.1 mM

manganous sulfate, 1.8 mM calcium nitrate, and 6.5 mM boric acid was used initially as the control medium. Various treatments were analyzed for their effects on the in vitro germination and tube length of the pollen. Treatments that improved germination and/or tube length of the pollen were incorporated into the control medium to improve its effectiveness.

Culture conditions for all of the experiments were as follows. Each mineral concentration (treatment) in the experiment was weighed out and placed into a separate 125 ml. flask. The control medium was added to each flask to bring the volume to 50 ml. 1.75 g. of agar-agar (Sigma) equaling 3.5% was added to each flask. The flasks then were capped with aluminum foil and autoclaved for eight minutes at 121 degrees C. and 20 pounds psi. While the medium was still hot, the flasks were removed and thoroughly mixed. Plastic petri plates 5 centimeters in diameter were labeled with adhesive tape to distinguish treatments. The bottoms of the plates were sectioned into quarters with a wax pencil. The plates were individually placed on a top loading balance, and 15 grams of medium were poured into each plate. The plates were covered and placed in a refrigerator for up to four days.

The ranges of concentrations of the experimental treatments are presented in Table 1. The treatments were

TABLE 1

RANGE OF EXPERIMENTAL TREATMENTS

Treatment	Range
pH	5.0 ---- 8.5
$Ca(NO_3)2$	0 ---- 4.0 mM
$MgSO_4$	0 ---- 3.3 mM
$MnSO_4$	0 ---- 5.9 mM
H_3BO_3	trace ---- 16.2 mM
KNO_3	0 ---- 3.0 mM
Fructose	5% ---- 25%
Glucose	5% ---- 30%
Raffinose	5% ---- 25%
Sucrose	5% ---- 30%
GA_3	0 ---- 10 uM
IAA	0 ---- 10 uM
B-9 (SADH)	0 ---- 3.0 mM

chosen either for their possible role in improving the medium or for testing their physiological role in this model system.

Inoculation of the Culture Medium

One plate from each treatment in the experiment was removed from the refrigerator thirty minutes prior to excising the flowers. The covers were removed, and the condensation was blotted from the insides of the covers. The covers were immediately returned to the plates, and the agar medium was allowed to reach room temperature. A radian section was cut from a 5.5 centimeter disc of filter paper. The filter paper was placed in one of the petri plates in a position so that the open section was directly over the first of four marked quarters. Before dusting pollen of the first flower onto the agar, the flower's petals were bent backwards, and the free pollen collected in the petals was scraped away. The pollen was then dusted lightly onto the agar surface through the open section of filter paper. The procedure was repeated with the same flower on the remaining plates. A new piece of filter paper was used when dusting the second flower.

Due to the sticky nature of the pollen grains, a method had to be developed to separate the grains. Various methods of separating the grains were attempted, and these are mentioned in Chapter IV. The method chosen for separating

the pollen grains in all of the experiments were as follows: The plates were placed on a dissecting microscope, and the pollen was magnified twenty-five times. An overhead lamp at low light intensity was used to illuminate the pollen grains. A dissecting needle was used to separate the individual grains, and the grains were spread over the agar surface at least 0.8 mm. and not more than 1.5 mm. from each other. On the average, 100 to 150 pollen grains were separated in each replication. The clumps of grains that remained on the medium were pushed away from the grains that were to be examined in the experiment. The plates were immediately covered, and they were left at room temperature for four hours. As no additional growth occurred beyond this time, the data usually were taken at the end of this period, but occasionally the plates were refrigerated at 2 degrees C. to prevent the growth of contaminants, and the data were taken later.

Data from each experiment were accumulated from six flowers. Two flowers were used as pollen sources each day on three different days.

Collection of Data

Data were collected to evaluate the effects of a treatment on both the germination and the tube growth of cotton pollen. The procedure for measuring tube growth was

as follows. Each petri plate was placed on the dissecting microscope, and random pictures of the pollen tubes (magnified sixteen times) were taken with a 135 millimeter Olympus camera mounted on the microscope. An accurate record was kept of each treatment, flower, and day by projecting a code on the film when the picture was taken. After developing the film, the negatives were placed in order of day, flower, and treatment into a slide projector, and the images were projected onto a large piece of drawing paper. The projector was adjusted so that the pollen grains were magnified seventy to ninty times. The average diameter of a pollen grain of Hancock was 0.11 mm. (measured by ocular micrometer). The approximate magnification of the projected pollen grain images was obtained by measuring with a millimeter rule. To determine tube length ten pollen tubes were chosen at random and measured by running a map measurer (calibrated in centimeters) over the length of the tube. On the tubes that ruptured, the measurement was taken to the point of rupture. Some pollen grains were polysiphonous, and only the longest tube was measured. After ten measurements were taken for each replication of each treatment, the highest and lowest measurement were discarded. Therefore, forty-eight measurements for each treatment were used to analyze the results.

Germination percentages were calculated by first counting all of the grains that were separated on the medium.

Accuracy was assured by dividing the test area into quadrangles with a dissecting needle. A record of the total number of grains was kept on a push button counter. Next, the grains that germinated were counted. A germinated pollen grain was defined as a grain having a tube that was as long or longer than the diameter of the grain. The percent germination on a treatment was defined as the number of germinated pollen grains divided by the total number of pollen grains.

All of the experiments were designed as randomized complete blocks. The flowers were grouped within the treatments, and the pollen grains were grouped within the flowers. The data were analyzed using the Statistical Analysis System (SAS) program. An analysis of variance table was obtained for each experiment. The sources of variation were the following: day; treatment; day related to treatment; flowers within days; and treatment related to flowers within day. A Duncan's Multiple Range (DMR) test was used in each experiment to evaluate the effects of different days on the *in vitro* germination and tube growth of cotton pollen. The same test was used in each experiment to evaluate the effects of treatments on the *in vitro* germination and tube growth of cotton pollen.

CHAPTER IV

RESULTS AND DISCUSSION

Selection of the Control Medium

Before the nutritional requirements for cotton pollen germination and tube growth could be evaluated, the physical state of the medium had to be selected. Govila and Rao (1969) developed a liquid culture medium which permitted cotton pollen germination; however, the tubes were only 0.182 mm long. Their medium was not suitable for the present study because any inhibition of tube growth by the treatment tested would be hard to analyze. Also, a treatment which affected the tube length after an initial growth period (growth from endogenous nutrients) would not be noticed. The high percent germination in their liquid medium did suggest that modifications of a liquid medium might be fruitful.

Varying mixtures of polyethylene-glycol (M.W. 380-420) with Taylor's (1972) medium (no agar) were tested: however, the cotton pollen ruptured in the liquid media containing low polyethylene-glycol concentrations, and plasmolyzed in high polyethylene-glycol concentrations.

Varying concentrations of nectary secretions from the subbracteal glands of cotton flowers were tested as germinating media. The cotton pollen plasmolyzed on media with

a high ratio of nectar to water, and ruptured on media with a low ratio of nectar to water. No germination occurred on any of the media tested containing nectary secretions.

Taylor's (1972) culture medium containing 3.5% bactoagar was used as the initial control medium in the present study because the cotton pollen tubes grown on this medium averaged 1.65 mm.; however, the germination was only 25%. Phytagar (GIBCO) was used initially in this study because of its higher quality; however, at the high concentration (3.5%), the agar boiled out of the flasks when autoclaved. Thereafter, agar-agar (Sigma) was used without any further problems in autoclaving.

Treatments that were found to increase the germination and/or tube length of the cotton pollen were incorporated into the revised medium. The decision to change the control medium as improvements were found was made because the major goal of this study was to develop a compatible medium for the germination and tube growth of cotton pollen. For example, a mineral that was found to have an optimal concentration in the initial control medium may not have had the same optimal concentration in the revised medium. Rather than combine all of the optimal mineral concentrations at the end of the study and possibly find inhibition of germination, improvements were incorporated in groups. These incorporations were made only after analyzing the results obtained by Duncan's Multiple Range (DMR) tests.

Technique for Pollen Inoculation

Taylor (1972) compared dusting the pollen with brushing the pollen onto the agar surface. He observed that when brushed the pollen had 15% germination, but when dusted it had 25% germination.

Various techniques were attempted in this study to separate the pollen grains. A burst of air from above the agar surface was unsuccessful. Different gauge screens were placed over the agar plates, and the pollen was dusted over them. This technique also was a failure. After dusting the pollen grains onto the agar surface, a piece of filter paper was used to drag the grains across the agar. This technique separated the grains; however, many of the grains ruptured in the process. The technique of probing the individual grains (Chapter III) was by far the most successful although very time consuming. Many times the germination was as high as 70% after probing the grains. Also, this technique prevented any "population effect" from influencing the data. In all subsequent experiments the pollen grains were approximately the same distance apart. This was one less variable with which to be concerned when evaluating treatments.

pH of the Medium

The data for the effects of pH on germination and tube length of cotton pollen on a culture medium are presented in

Figure 1. The treatments ranged from pH 5.0 to 8.5 in increments of 0.5 pH units. Taylor's (1972) medium (pH = 5.0) with 0.8 mM. magnesium sulfate added was used as the control (zero point).

Actual mean germination percentages ranged from 24% at pH 5.0 to 38% at pH 7.5. Although Figure 1 indicates that there is a trend in the number of pollen grains germinating with increasing pH up to 7.5, results from the Duncan's Multiple Range test revealed that 7.5 was the only pH at which germination was significantly different from the control.

There was an increasing trend in pollen tube length from pH 5.0 to 8.0; however, only pollen tubes growing on medium with pH 8.0 were significantly longer than pollen tubes growing on the control medium. Actual tube lengths ranged from a mean of 1.16 mm. at pH 5.0 to 1.82 mm. at pH 8.0. Because of the increase in percent germination at pH 7.5 and the increased length of the pollen tubes at pH 8.0, 7.6 was selected as the new pH to be incorporated into the revised medium.

The results of this study coincided with the results obtained by Loo and Hwang (1944) who found that a medium with pH 7.0 gave the highest percent germination of Brassica juncea pollen. They found that a medium with pH between 5.0 and 6.0 was unsuitable for germination. Cotton pollen

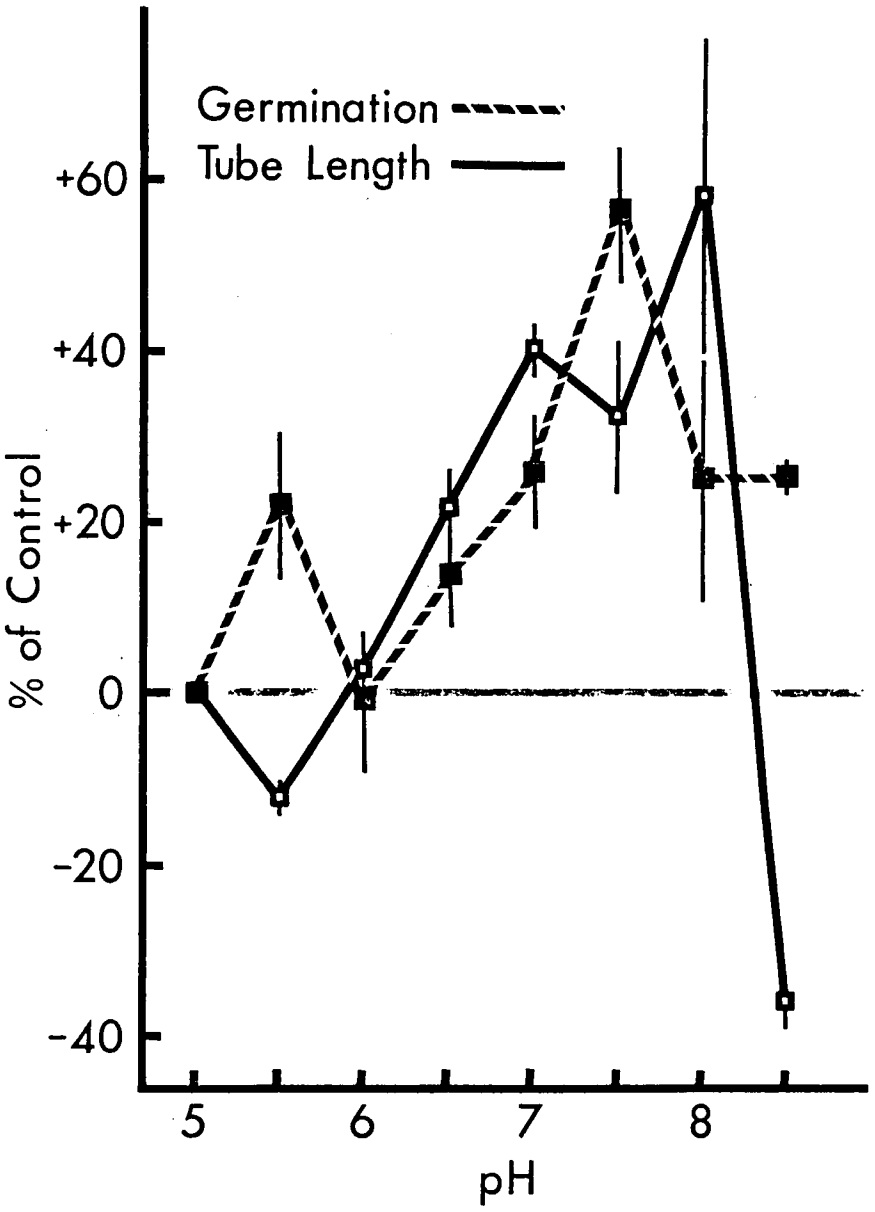


Figure 1. Effects of pH on percent germination and tube length of cotton pollen in vitro. The vertical line represents two standard errors.

germinated and grew in this range; however, going from pH 5.0 to 6.0 did not increase either germination or tube length. Kwack (1965) germinated Brassica pollen in vitro to study the effects of pH in the medium. He found the optimum pH range for germination and tube growth of that species to be rather narrow. In this study cotton pollen also was found to have a narrow optimum pH range between 7.5 and 8.0. The rapid decreases observed in percent germination and tube length of cotton pollen above pH 8.0 were observed in other plants by Kwack (1965).

Kwack (1965) postulated that the beneficial effects of high pH (above 7.0) resulted from an interaction between hydrogen and calcium ions at the surface of the pollen tube, especially at the tip. One of the major problems in germinating cotton pollen in vitro was the bursting of the pollen tube at the tip. The significant increase (58%) in tube length at pH 8.0 may be due to an increase in rigidity of the tube wall.

Another possible reason for the increases in percent germination and tube length of cotton pollen may be related to results obtained by Stanley and Linskens (1965). They observed that proteins diffused from germinating pear pollen into the medium. The amount of protein diffusing from the pollen into the medium corresponded to the time interval and the pH of the medium. Proteins from pine and palm pollen

diffused at a slower rate in a medium with pH between 6.0 and 7.0. This pH range corresponded to the highest germination and longest tubes in both species.

Observations from the present study may support the protein diffusion theory by Stanley and Linskens (1965). Soon after the cotton pollen grains were dusted onto the agar medium, a substance diffused from the grains and formed a "puddle" around the pollen grains. A few of the grains having puddles around them ruptured and exuded their cell contents; on the other hand, many pollen grains germinated and grew long tubes after the substance diffused into the medium.

The results and observations obtained from this study along with the results of Stanley and Linskens (1965) suggest the possibility that proteins diffuse from the exine layer of the cotton pollen grain. The proteins may diffuse at a slower rate when the grains are germinated on a medium having a high pH (above 7.0).

Calcium Complex in the Medium

The calcium complex (Brewbaker and Kwack, 1963) consists of calcium, magnesium, and potassium. The effects of these three cations on the percent germination and tube length of cotton pollen grown in vitro are examined in the present study.

The data for the effects of magnesium on the percent germination and tube length of cotton pollen grown in vitro are presented in Figure 2. Magnesium was added to the medium as magnesium sulfate, and treatments ranged from 0 mM to 3.3 mM. Taylor's (1972) medium (which contained no magnesium) with the revised pH of 7.6 being used for the control medium.

There was an inhibitory trend for percent germination at magnesium concentrations greater than 0.8 mM (Figure 2). The mean values, however, were not significantly different.

The mean tube length of the cotton pollen increased proportionately to the increase in magnesium in the medium up to 2.5 mM. Pollen tubes grown on a medium containing 2.5 mM magnesium were 60% longer than pollen tubes grown on a medium lacking magnesium. Magnesium at a concentration of 0.8 mM was chosen as the amount to be incorporated into the revised culture medium, because at this concentration percent germination did not decrease, while the tube length increased 40% over pollen grown on the control medium.

The data for the effects of calcium on the percent germination and tube length of cotton pollen grown in vitro are presented in Figure 3. Calcium was added to the medium as calcium nitrate, and the treatments ranged from 0 mM to 4.0 mM. The revised control medium consisted of 100 ml distilled water, 25 g sucrose, 4.1 mM manganous sulfate, 6.5 mM boric acid, 0.8 mM magnesium sulfate, adjusted pH of 7.6, and 3.5 g agar.

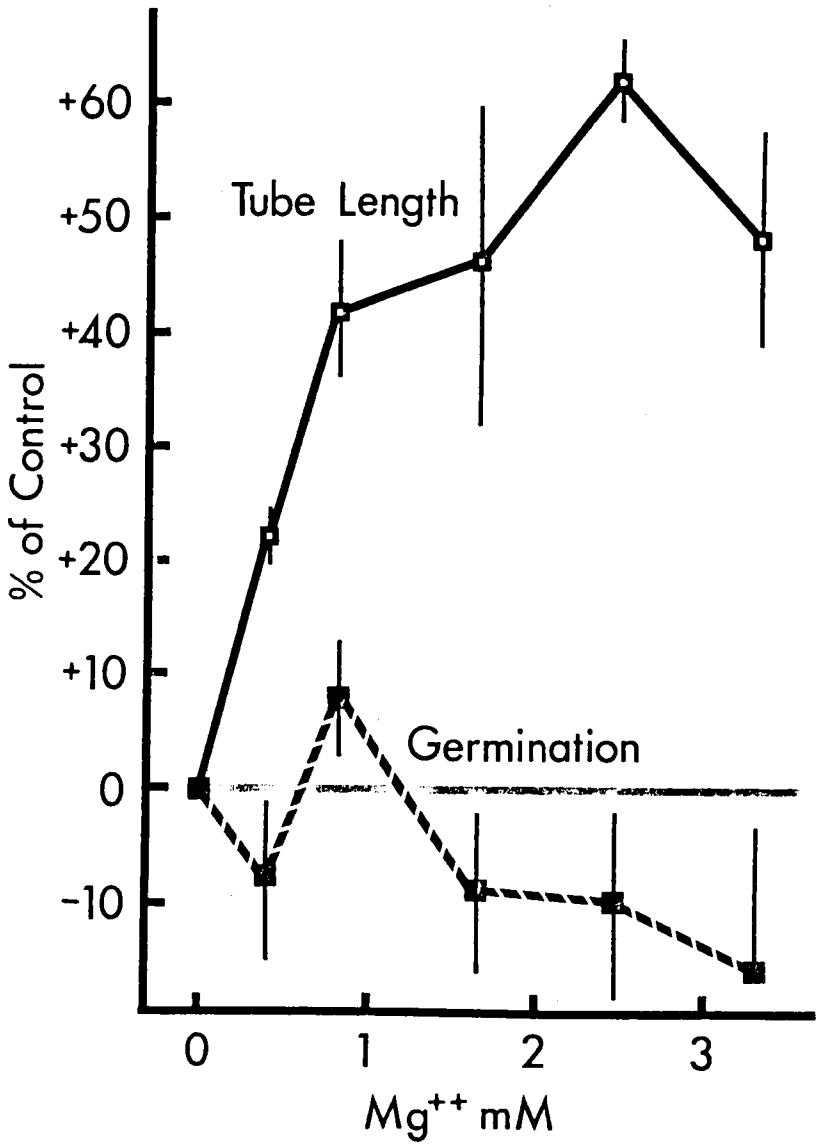


Figure 2. Effects of magnesium on percent germination and tube length of cotton pollen in vitro. The vertical line represents two standard errors.

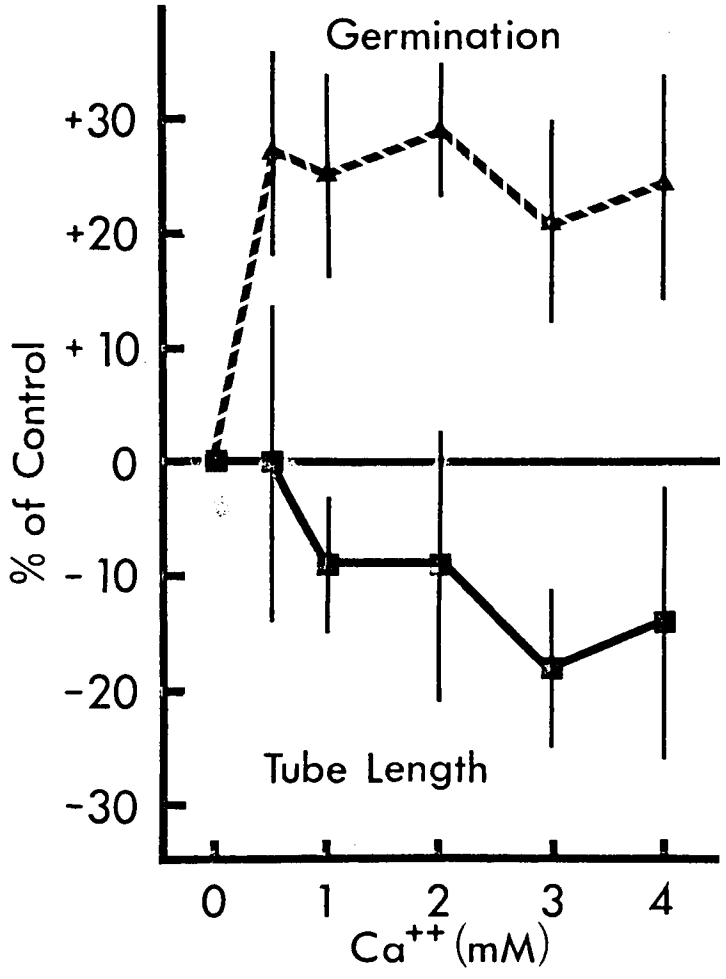


Figure 3. Effects of calcium on percent germination and tube length of cotton pollen in vitro. The vertical line represents two standard errors.

There was a positive trend for percent germination of the cotton pollen when calcium was added to the medium. The increase in percent germination was significantly different at all concentrations tested when compared to the control.

On the other hand, there were no significant differences in the mean tube lengths on any of the treatments tested when compared to the control. However, a trend toward inhibtion was associated with increases in calcium.

The data for the effects of potassium on the percent germination and tube length of cotton pollen grown in vitro are presented in Figure 4. Potassium was added to the medium as potassium nitrate, and treatments ranged from 0 mM to 3.0 mM. The revised control medium consisted of 100 ml distilled water, 25 g sucrose, 0.5 mM calcium nitrate, 4.1 mM manganous sulfate, 6.5 mM boric acid, 0.8 mM magnesium sulfate, 1.0 uM gibberellic acid, adjusted pH of 7.6, and 3.5 g agar.

The addition of potassium to the culture medium did not have any significant effect on the percent germination of the cotton pollen. The variability of the results obtained from this experiment may have been caused by a spider mite infestation in the greenhouse before and during the experiment. Due to the large variability, there were no significant differences among the mean tube lengths on any of the potassium treatments; however, there was a positive trend with increases in potassium up to 1.0 mM. At this concentration

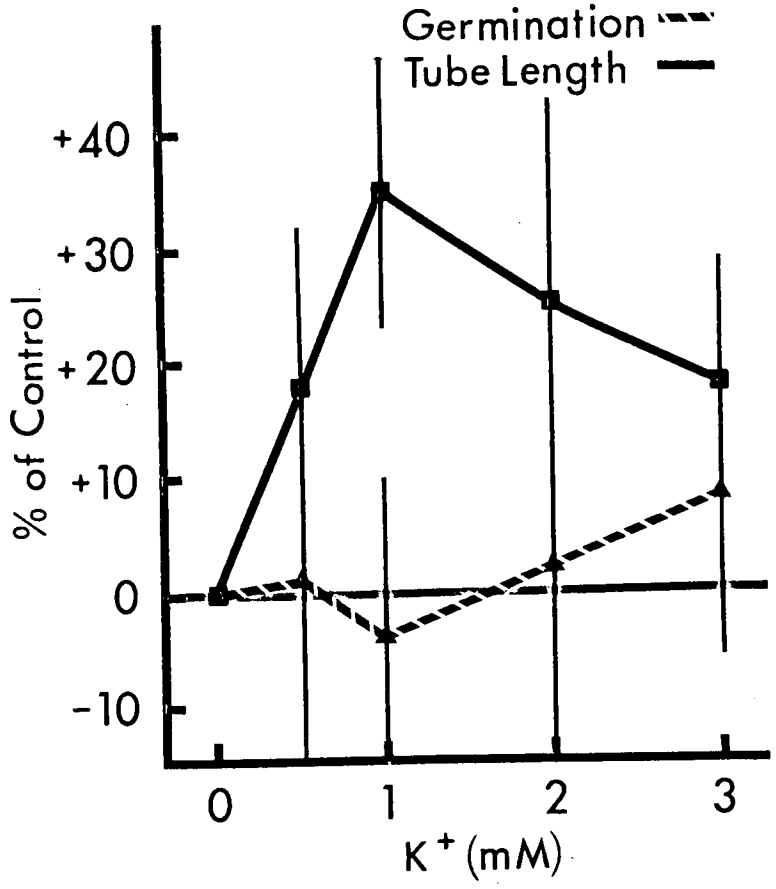


Figure 4. Effects of potassium on percent germination and tube length of cotton pollen in vitro. The vertical line represents two standard errors.

the mean tube length of the cotton pollen was 32% longer than the mean tube length on the control medium.

Brink (1924c) was the first to observe that a large number of pollen grains in a culture medium showed a larger germination percentage than a small number of pollen grains in the same volume of culture medium. Savelli and Caruso (1940) convincingly demonstrated the pollen culture phenomenon which became known as the "population effect." Brewbaker and Kwack (1963) found that the population effect was overcome by a "calcium complex" consisting of calcium ions in the presence of magnesium and potassium ions.

In the present study the initial control medium (Taylor, 1972) contained no magnesium, nor did Taylor (1972) test for its effects on the percent germination and tube length of cotton pollen grown in vitro. Besides playing a vital role in overcoming the population effect (Brewbaker and Kwack, 1963), magnesium is involved in energy transformations involving ATP. Therefore, one would expect magnesium to have an effect on the germination and/or tube growth of cotton pollen in vitro. These two factors may help explain the significant increase in the mean tube length of cotton pollen grown on media containing magnesium (Figure 2).

Brewbaker and Kwack (1963) observed that calcium played a vital role in pollen germination and pollen tube growth, and they found that exogenous calcium had to be added to the

medium to increase germination and tube growth. However, results from the present study show that exogenous calcium is inhibitory to the growing pollen tube. Although Taylor (1972) found that 1.7 mM calcium was optimal for his cotton culture medium, he was not dealing with the possible interactions between a high pH (7.6) and magnesium. The significant effect of magnesium on the mean pollen tube length of cotton (Figure 2, page 30) along with the benefical effects of the calcium complex (Brewbaker and Kwack, 1963) suggest that cotton pollen contains the necessary endogenous level of calcium for germination and optimum tube growth. The inhibitory trend in the mean tube length with increasing concentrations of calcium possibly results from the absorption of exogenous calcium by the growing pollen tube wall. Subsequently, the wall becomes more rigid and is less able to stretch.

Due to the stimulatory effect of calcium on the percent germination (Figure 3), 2.0 mM calcium was incorporated into the revised medium. After additional improvements in the medium, the effects of calcium were retested. The data for the effects of calcium on the percent germination and mean tube length of cotton pollen grown on the revised medium are presented in Figure 5. The calcium treatments ranged from 0 mM to 2.0 mM. The revised control medium consisted of 100 ml distilled water, 25 g sucrose, 5.9 mM manganous sulfate,

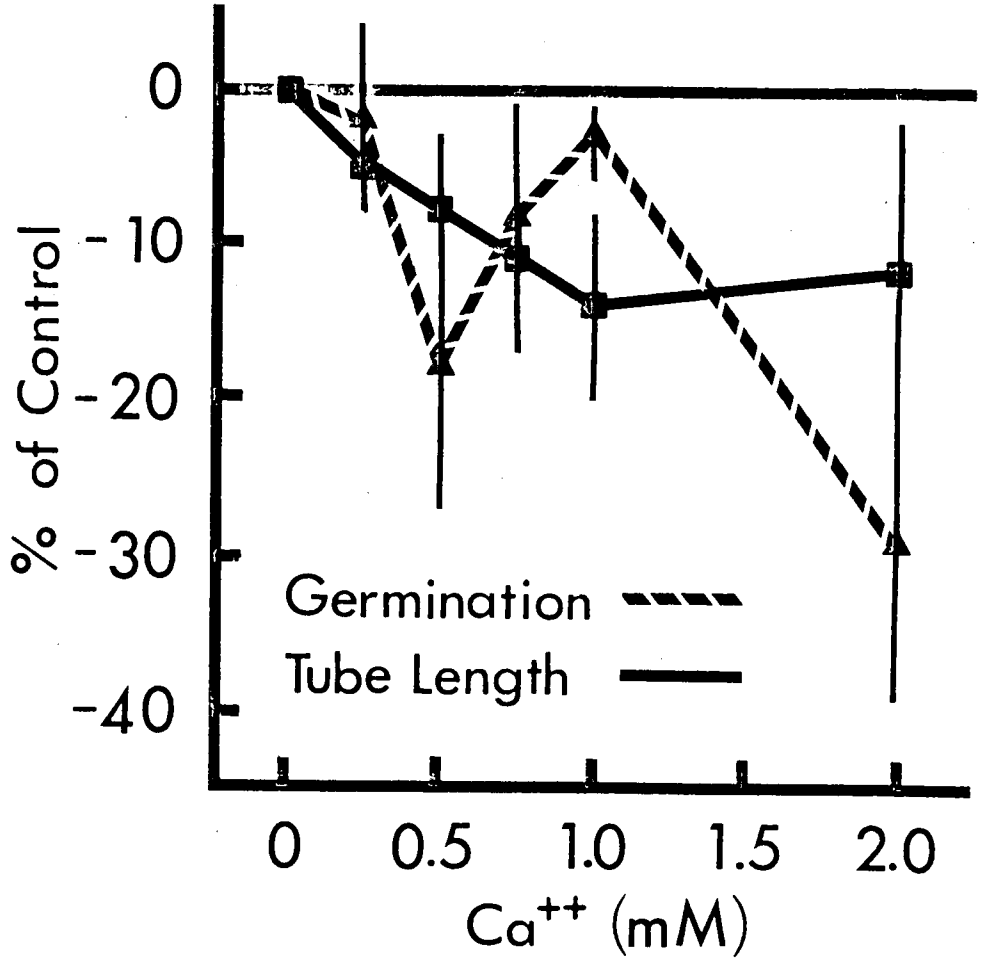


Figure 5. Effects of calcium on percent germination and tube length of cotton pollen on medium containing magnesium and potassium. The vertical line represents two standard errors.

1.0 mM potassium nitrate, 1.0 uM gibberellic acid, 0.1 uM IAA, adj ted pH of 7.6, and 3.5 g agar.

The addition of calcium to this revised medium significantly inhibited the percent germination of the cotton pollen. There were no significant differences among the mean tube lengths on any of the calcium treatments tested. As in the first calcium experiment, there was an inhibitory trend with increasing calcium concentrations.

The presence of potassium along with magnesium in the revised medium may have caused the inhibitory effect of calcium. Brewbaker and Kwack (1963) found that calcium overcame the population effect best in the presence of magnesium and potassium. Weisenseel and Jaffe (1976) found that the growth of lily pollen required at least 10 to 100 uM potassium in the medium, and the added potassium affected the elongation of the pollen tubes. Potassium also affected the mean tube length in the present study (Figure 4). There may be a cation replacement effect in the pollen tubes, whereby some cations are taken up by the tube wall and utilized more efficiently than calcium. If there is an adequate amount of endogenous calcium in the cotton pollen grain, then any factors that increased the absorption of exogenous calcium might raise the level to an inhibitory concentration.

Manganese in the Medium

The data for the effects of manganese on the in vitro germination and tube growth of cotton pollen are presented in Figure 6. Manganese was added to the medium as manganous sulfate, and treatments ranged from 0 mM to 6.0 mM. The revised control medium consisted of 100 ml distilled water, 25 g sucrose, 0.5 mM calcium nitrate, 6.5 mM boric acid, 0.8 mM magnesium sulfate, 1.0 mM potassium nitrate, 1.0 uM gibberellic acid, adjusted pH of 7.6, and 3.5 g agar.

The actual germination percentages ranged from a low of 34% on the control medium to 42% on the medium containing 6.0 mM manganese. Although there was much variability within the results, there appeared to be a positive trend in germination at the lowest levels of manganese tested. However, results from the Duncan's Multiple Range test revealed that there was no significant difference in percent germination among any of the treatments.

The addition of manganese to the culture medium did significantly affect the length of the cotton pollen tubes. Duncan's Multiple Range test revealed that the mean pollen tube lengths on media containing 2.4 mM, 4.1 mM, and 5.9 mM manganese were all significantly different from pollen tubes grown on the control medium. An increase in tube length was positively correlated with an increase in manganese. The mean tube length at the highest manganese concentration

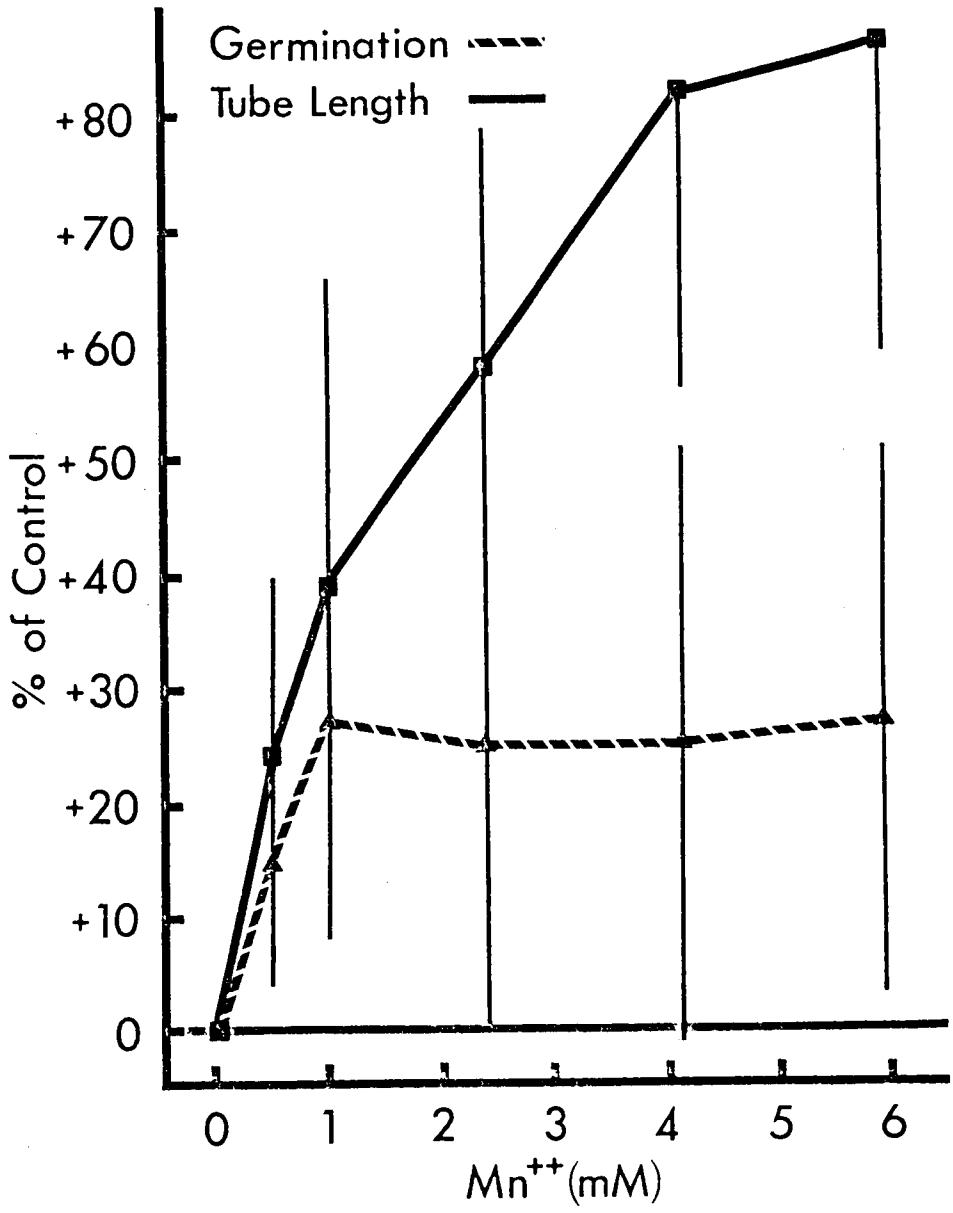


Figure 6. Effects of manganese on percent germination and tube length of cotton pollen in vitro. The vertical line represents two standard errors.

(5.9 mM) tested was 86% longer than the mean tube length on the control medium.

Loo and Hwang (1944) observed that manganese stimulated both the percent germination and the tube length of Brassica juncea pollen cultured in vitro. The germination percentage and tube length increased as the manganese concentration increased. Also the stimulating effect of manganese in a wide range of concentrations (Loo and Hwang, 1944). Although germination was not influenced strongly, the effects of manganese on the in vitro tube growth of cotton pollen were very similar to the effects of manganese on Brassica juncea pollen. The stimulating effect on tube growth was also manifested over a wide range of manganese concentrations.

Manganese ions have been shown to partially substitute for calcium ions in a number of physiological processes (Naitoh, Eckert, and Friedman, 1972; Terepka, Coleman, and Hunter, 1975). Weisenseel and Jaffe (1976) theorized that manganese is in some way absorbed by the pollen tubes and thereby speeds the entry of potassium. The large stimulating effect of manganese on the tube length of cotton pollen may help account for the fact that exogenous calcium does not stimulate the growth of cotton pollen tubes.

Boron in the Medium

The data for the effects of boron on the percent germination and tube length of cotton pollen grown in vitro

are presented in Figure 7. Boron was added to the medium as boric acid, and the treatments ranged from 0 mM to 16.5 mM boric acid. The revised control medium consisted of 100 ml distilled water, 25 g sucrose, 0.5 mM calcium nitrate, 0.8 mM magnesium sulfate, 4.1 mM manganous sulfate, 1.0 mM gibberellic acid, adjusted pH of 7.6, and 3.5 g agar.

The addition of boric acid to the culture medium did significantly affect the percent germination of the cotton pollen. The mean percent germination at 1.6 mM boric acid was 45% greater than the control; however, as the boric acid concentration increased, there was an inhibitory trend in the percent germination.

The addition of boric acid to the medium also significantly affected the mean tube length of the cotton pollen. At 1.6 mM boric acid the mean tube length of the pollen was 340% greater than the mean tube length of pollen grown on the control medium. As was observed in the percent germination, the mean tube length also decreased as the boric acid concentration increased (Figure 7). However, even at the high concentration (16.3 mM) the mean tube length was 120% greater than the control.

Schmucker (1932) discovered that the stigmatic fluid of Nymphaea contained high levels of boron. Vasil (1960a) studied the effects of boron on the pollen germination of a number of plants in the Cucurbitaceae, and found that 1.6 mM

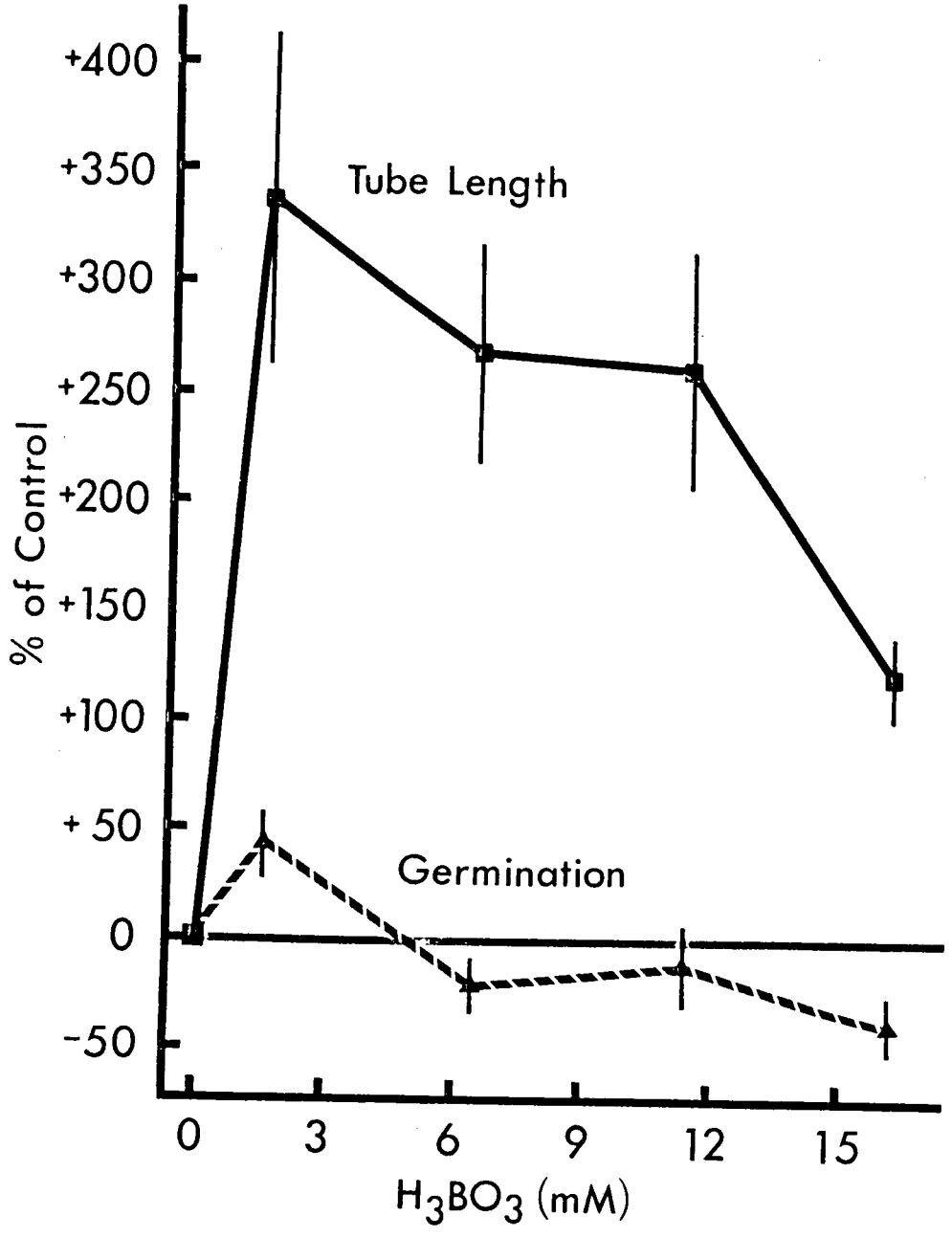


Figure 7. Effects of boron on percent germination and tube length of cotton pollen in vitro. The vertical line represents two standard errors.

to 2.4 mM boric acid was favorable while high concentrations inhibited germination. Although only one boric acid concentration (1.6 mM) within that range was tested in the present study, this concentration was the best for germinating cotton pollen in vitro. Vasil (1960a) also observed that the effect of boron on the elongation of the tube was much more marked than on the percentage of pollen germination. Cotton pollen tubes grown on media containing boric acid were more than three times longer than pollen tubes grown on media that contained no boric acid.

The role of boron has been controversial since Shive (1936) first suggested that boron functioned in protein synthesis. Gauch and Dugger (1953) postulated that borate ions formed sugar-borate complexes with sugar molecules, and these complexes moved through the cellular membrances of the plant more readily than nonborated sugar molecules. Vasil (1960b) summarized the possible roles of boron on the germination and tube growth of pollen grains. He stated, "It promotes absorption and metabolism of sugars by forming sugar-borate complexes; it increases oxygen uptake; and it is involved in the synthesis of pectic substances for the wall of the actively elongating pollen tube." Whether boron is involved in one or more of these roles in cotton pollen germination and tube growth is not known; however, the results of this study show that boron is essential in the medium for obtaining optimum germination and long pollen tubes.

Sugars in the Medium

The effects of sugars on the percent germination and the tube length of cotton pollen grown *in vitro* are presented in Figures 8 and 9 respectively. Four sugars (fructose, glucose, raffinose, sucrose) were tested at concentrations that ranged from 5% to 30% in 5% increments. Each sugar was added to a revised medium that contained 100 ml distilled water, 0.5 mM calcium nitrate, 6.5 mM boric acid, 0.8 mM magnesium sulfate, 4.1 mM manganese sulfate, 1.0 uM gibberellic acid, adjusted pH of 7.6, and 3.5 g agar.

Figure 8 presents only those treatments that supported germination. Cotton pollen did not germinate on the media containing fructose at any of the concentrations tested; therefore, the data are not shown. Pollen did germinate on the media containing glucose at 15%, 20%, and 25% concentrations. The germination was 15% at both 15% and 20% glucose; however, the percent germination increased sharply to 33% on the medium containing 25% glucose. On the media containing raffinose, pollen grains germinated on the 20% and 25% concentrations. There was no germination in the media containing less than 15% sucrose. The germination percentage increased with increases of sucrose and obtained a maximum (33%) at 30% sugar.

Pollen tubes grown on a glucose medium attained a length (0.22 mm) approximately twice the diameter of the pollen

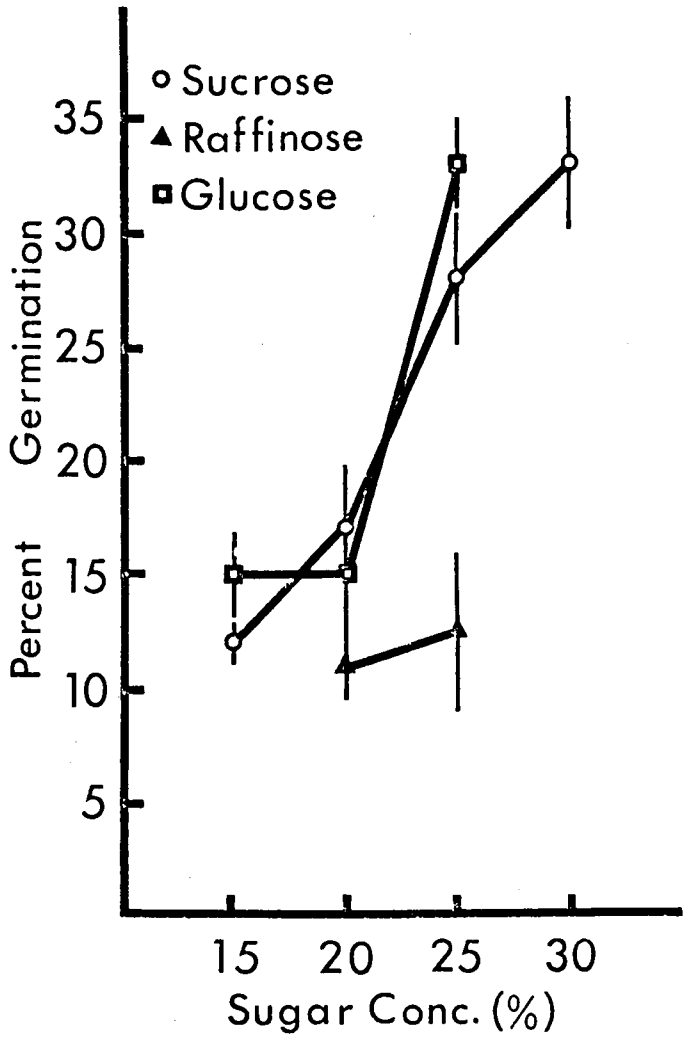


Figure 8. Effects of sugars on percent germination of cotton pollen in vitro. The vertical line represents two standard errors.

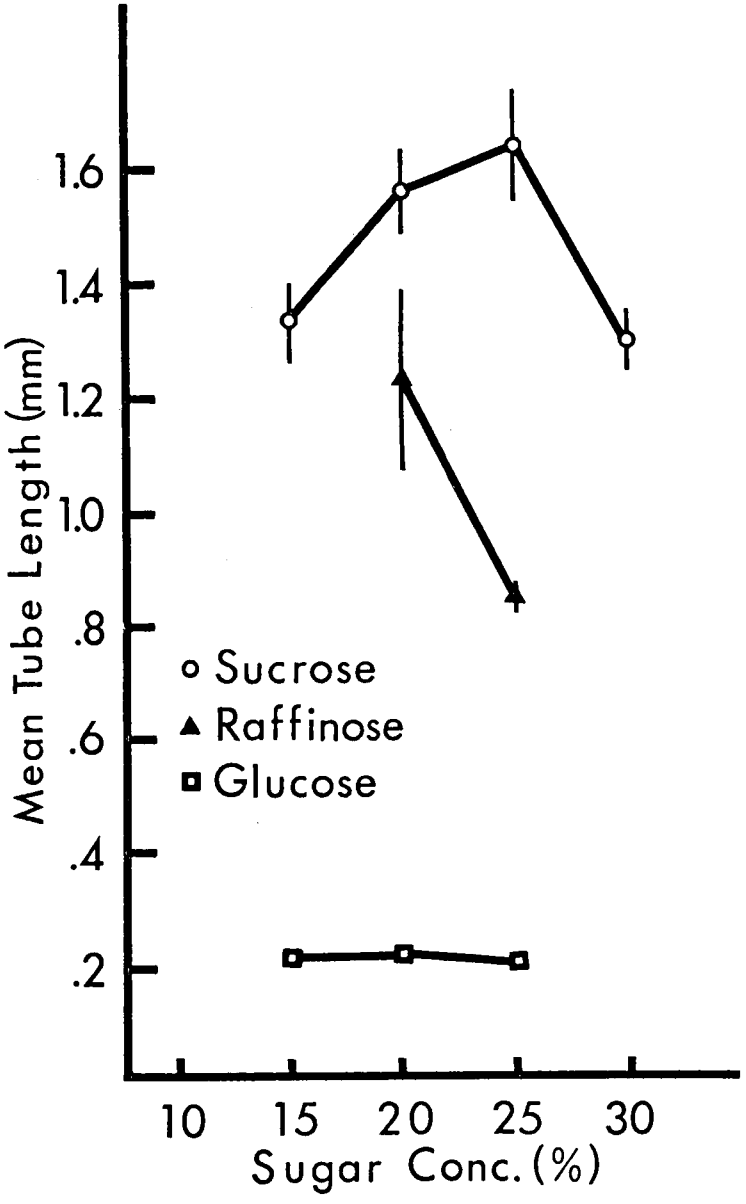


Figure 9. Effects of sugars on mean tube length of cotton pollen in vitro. The vertical line represents two standard errors.

grain. The tube length was not affected by increasing the glucose concentration from 15% to 25% (Figure 9). Pollen tubes growing on the raffinose media attained a maximum length of 1.20 mm at 20% sugar, whereas at 25% the tube length was only 0.85 mm. On the sucrose media the mean tube length increased from 1.33 mm at 15% sugar to 1.64 mm at 25% sugar. The mean tube length was slightly less (1.30 mm) at 30% sucrose.

A variety of mono-, di-, and trisaccharides have been tested for their effects on the in vitro germination and tube growth of numerous pollen species. Hrabetova and Tupy (1963) tested twenty oligosaccharides, monosaccharides, and sugar alcohols, and they found raffinose to be the best substrate for growth and respiration of apple pollen tubes. Portnoi, Horovitz, and Galil (1975) observed that yucca pollen germinated well in a medium containing sucrose and/or glucose, but only slight germination occurred on fructose. Dynansagar and Thomas (1970) observed that sucrose was the best medium for germination and tube growth of Phaseolus radiatus. They tested glucose, raffinose, sucrose, and mannose. Raffinose was the least suitable for germination; however, the pollen tubes grew almost as well on a raffinose medium as on a sucrose medium. The results from the present study closely agree with the observations of Portnoi et al. (1975) and Dynansagar and Thomas (1970). No germination of cotton pollen was observed on a medium containing fructose. On the other hand, the maximum germination of cotton pollen occurred

on both a glucose and a sucrose medium. Raffinose was unfavorable for cotton pollen germination, since only 13% of the grains germinated in the presence of this sugar. Also, raffinose was not as suitable as sucrose for tube growth. The maximum mean tube length on a sucrose medium was 0.41 mm longer than the maximum tube length on the best raffinose medium.

The role of sugars in the germination of pollen has been a controversial issue. Brink (1924a, b) suggested that sugars may help to regulate the osmotic pressure as well as supply nutrition to the growing tubes. Visser (1955) believed that sugars only create a favorable osmotic condition for germination, while all the nutrients necessary for germination and tube growth are endogenously supplied by the pollen grain. O'Kelley (1955) disproved Visser's theory by using ^{14}C-labeled sugars to show that pollen tubes respired during their growth, and absorbed externally supplied sucrose, glucose, or fructose. Hellmers and Machlis (1956) found that pollen grains absorb and metabolize a variety of mono-, di-, and trisaccharides.

The data presented in this study indicate a positive correlation between the concentration of sugars and the percentage of germination. One reason for the lack of germination in all the sugars at concentrations below 15% was the large number of grains that ruptured. The osmotic conditions created by the low sugar concentrations were not

favorable for the germination of cotton pollen. Although cotton pollen germinated on glucose as well as on sucrose media, sucrose was much more efficiently utilized by the growing pollen tubes than were glucose or raffinose. From the present study and the studies of O'Kelley (1955), and Hellmers and Machlis (1956), it is apparent that sugars have two functions. Firstly, they regulate the osmotic pressure for germination, and secondly, act as a source of food for the growing pollen tube.

Growth Hormones and Regulators in the Medium

The data for the effects of gibberellin on percent germination and tube length of cotton pollen grown on a culture medium are presented in Figure 10. Gibberellic acid (GA_3) was used as the gibberellin source, and treatments ranged from 0 uM to 10 uM. The revised control medium consisted of 100 ml distilled water, 25 g sucrose, 0.5 mM calcium nitrate, 0.8 mM magnesium sulfate, 4.1 mM manganous sulfate, 6.5 mM boric acid, adjusted pH of 7.6, and 3.5 g agar.

Actual germination in this study ranged from a low of 36% at .01 uM gibberellic acid to 43% at 1.0 uM. The mean percent germination at 1.0 uM gibberellic acid was 13% greater than the control; however, there was no significant difference in percent germination among any of the treatments tested.

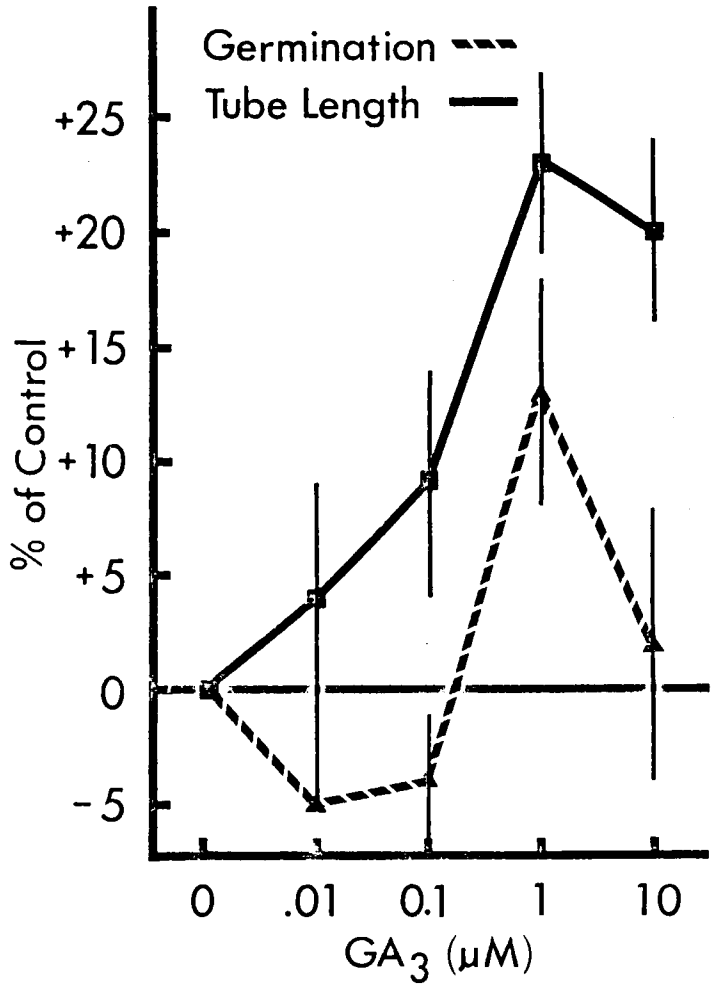


Figure 10. Effects of gibberellic acid on percent germination and tube length of cotton pollen in vitro. The vertical line represents two standard errors.

There was a positive correlation between tube length and the amount of gibberellic acid in the medium up to 1.0 uM. At this concentration there was a 23% increase in tube length over the control tube length. The highest concentration of gibberellic acid used (10 uM) was supraoptimal and gave only a 20% increase over the control tube length. Pollen grains grown on media containing 1.0 uM gibberellic acid or 10 uM gibberellic acid had tube lengths significantly longer than those grown on the control medium lacking gibberellic acid.

Researchers have examined extensively the effects of gibberellic acid on the *in vitro* germination and tube growth of pollen from various plant species. Kato (1955) observed that 50 mg per liter gibberellic acid in the medium stimulated lily pollen tube growth. The pollen tubes were five times longer than those grown on a medium without gibberellic acid. He observed also that high concentrations of gibberellic acid inhibited pollen tube growth. Bose (1959) showed that gibberellic acid increased the tube length of *Pisum sativa* pollen grown *in vitro*, but had no effect on germination. Their results are similar to the results obsevered in this study. Gibberellic acid affected cotton pollen tube length significantly, but had no significant effect on percent germination. Taylor (1972) observed that gibberellin at all concentrations tested severely curtailed the *in vitro* germination of cotton pollen.

The conflicting results obtained by different people when testing gibberellins in a pollen culture medium may be explained through species specificity. Bamzai and Randhawa (1969) tested the effects of gibberellic acid on pollen of three species of mango, and they observed different results for all three species.

Pollen apparently contains large amounts of gibberellic acid; however, Barendse et al. (1970) showed that the amount decreases as germination and tube growth proceed. They suggested that the lack of gibberellic acid needed by the growing tube caused cessation of the tube growth.

The significant increase in the tube length of cotton pollen on a medium containing gibberellic acid suggests that the quantity of gibberellic acid in the germinating cotton pollen grain is not sufficient for optimum growth. A second possibility is that additional gibberellic acid is not synthesized so that the endogenous concentration declines with growth.

The data for the effects of indole-3-acetic acid (IAA) on the percent germination and tube length of cotton pollen grown in vitro are presented in Figure 11. Treatments ranged from 0 mM to 10 mM IAA. The revised control medium consisted of 100 ml distilled water, 25 g sucrose, 0.5 mM calcium nitrate, 5.9 mM manganous sulfate, 1.6 mM boric acid, 0.8 mM magnesium sulfate, 1.0 mM potassium nitrate, 1.0 uM gibberellic acid, adjusted pH of 7.6, and 3.5 g agar.

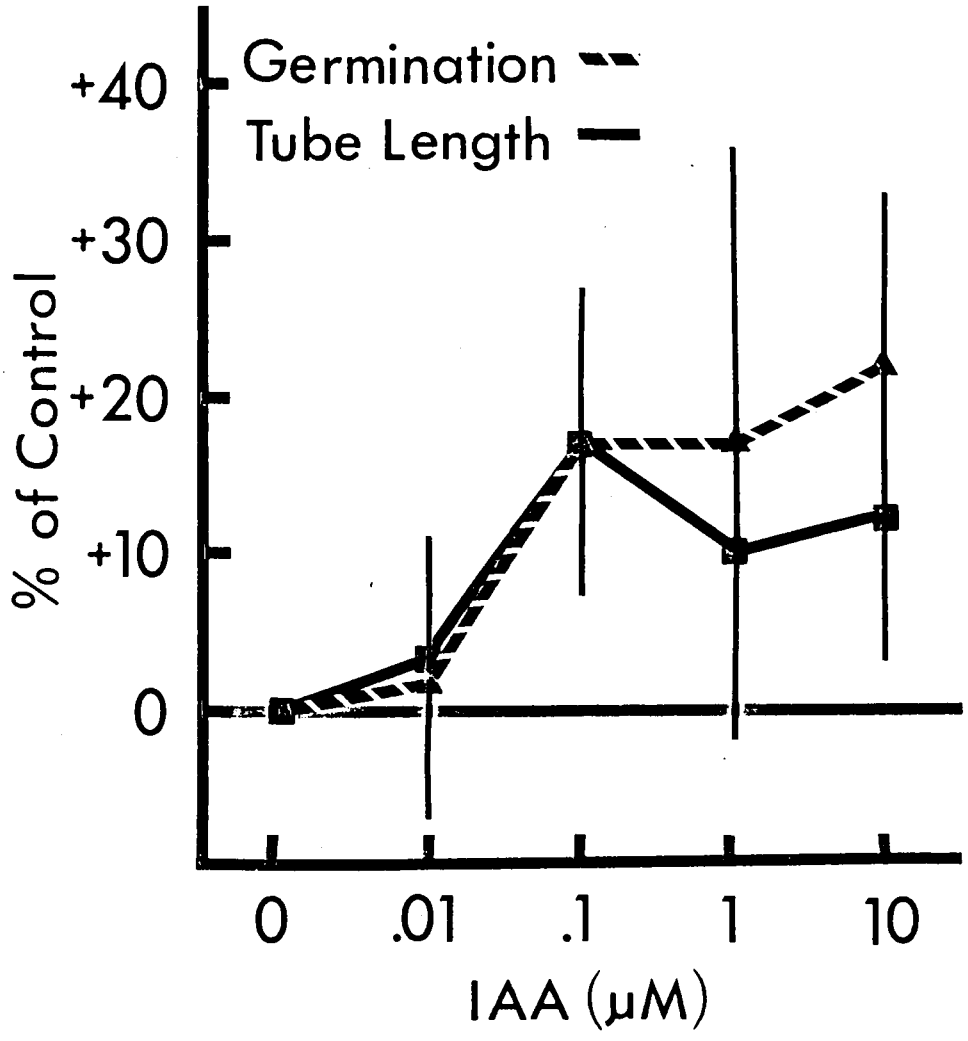


Figure 11. Effects of indole-3-acetic acid on percent germination and tube length of cotton pollen in vitro. The vertical line represents two standard errors.

The germination percentage appeared to increase with each increase in IAA concentration; however, Duncan's Multiple Range test revealed that the IAA treatments had no significant effect on the germination of cotton pollen *in vitro*.

Likewise, there were no significant differences in the length of the cotton pollen tubes on any of the treatments tested when compared to the control. Although not significantly different, there was a positive trend in the pollen tube length with increasing concentrations of IAA. Even a high IAA concentration (10 uM) was not inhibitory to the growth of the cotton pollen tube. Because of the positive trend observed in the germination and the tube length with IAA in the medium, 0.1 uM IAA was incorporated into the revised medium.

Muir (1947) observed that pollen contains substantial amounts of auxins. The stimulatory effect of IAA was shown to be specific for germinating pollen from a number of genera and species (Bamzai and Randhawa, 1969; Loo and Hwang, 1944; Stowe and Yamaki, 1957). Smith (1939) reported that IAA had some stimulating effect on both the germination and tube length of *in vitro* cultured pollen of *Tradescantia sp.*, *Polygonatum sp.*, and *Pinus sp.* High concentrations of IAA were reported to inhibit germination and tube growth. Taylor (1972) found that IAA at all concentrations tested either partially or severely curtailed germination of cotton pollen *in vitro*.

The conflicting results of IAA's effects on the in vitro germination and tube growth of pollen strengthens the theory of species specificity. The trend toward an increase in the percent germination and tube length of cotton pollen with increasing IAA concentrations may suggest that "Hancock" pollen in particular does not contain the optimum endogenous IAA concentration throughout germination and growth of the tube. A thorough experiment involving growth rates of pollen tubes on various concentrations of IAA may help researchers to to understand the positive trends in germination and tube length of cotton pollen on the high IAA concentrations.

The data for the effects of succinic acid-2,2-dimethyl-hydrazide (SADH; B-995) on the percent germination and tube length of cotton pollen grown in vitro are presented in Figure 12. The treatments ranged from 0 mM to 3.07 mM SADH. The revised control medium consisted of 100 ml distilled water, 25 g sucrose, 0.5 mM calcium nitrate, 5.9 mM manganous sulfate, 1.6 mM boric acid, 0.8 mM magnesium sulfate, 1.0 mM potassium nitrate, 1.0 uM gibberellic acid, adjusted pH of 7.6, and 3.5 g agar.

The addition of SADH to the culture media severely inhibited the percent germination of the cotton pollen at all concentrations except 0.67 mM. A similar inhibitory trend occurred when cotton pollen tubes were grown on media containing SADH.

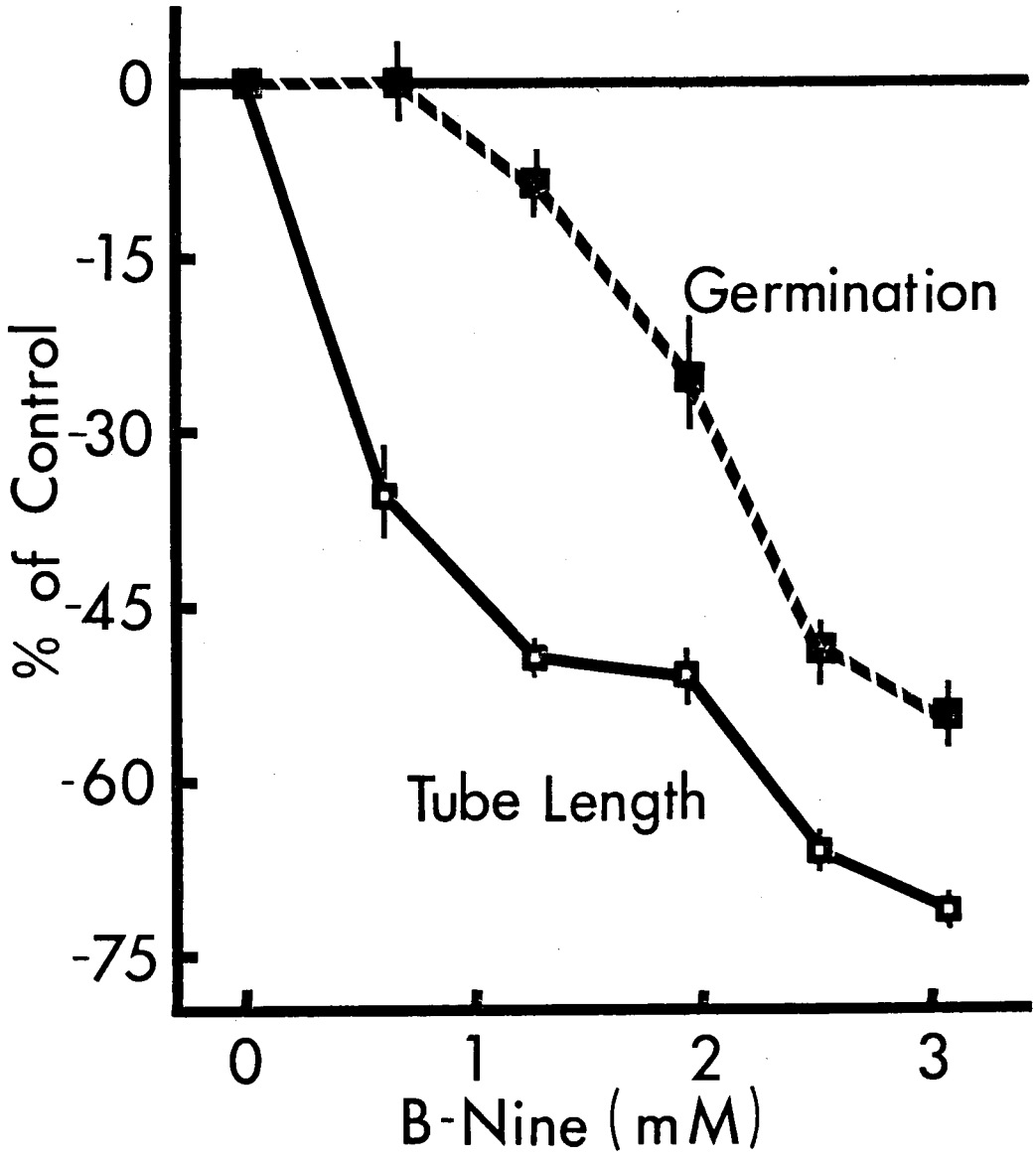


Figure 12. Effects of SADH on percent germination and tube length of cotton pollen in vitro. The vertical line represents two standard errors.

The mechanisms of action of certain growth inhibitors is not well understood. Reed, Moore, and Anderson (1965) postulated that the mechanism of action of SADH may be based on the hydrolysis of the compound "UDMH" (unsymmetrical dimethylhydrazide), which inhibits diamine oxidase from converting tryptamine to IAA. On the other hand, Ryugo and Sachs (1969) concluded from their studies that the UDMH moiety is not the active part of SADH. They believed the primary effect of SADH is to inhibit IAA synthesis. No previous studies on the effects of SADH on pollen grown in vitro are reported.

Although it is clear that SADH severely inhibits both percent germination and tube length of cotton pollen grown in vitro (Figure 12), no evidence is available from the data for understanding the mode of action of SADH. Since the inhibition of IAA synthsis is theorized as being the mechanism of action of SADH (Ryugo and Sachs, 1969), an experiment involving both SADH and IAA was developed to determine if there is an interaction between IAA and SADH.

The data for the effects of IAA and SADH on the percent germination and tube length of cotton pollen in vitro are presented in Figure 13. All of the treatments except the control contained 1.25 mM SADH, and the IAA treatments ranged from 0 uM to 10 uM. The revised control medium contained 100 ml distilled water, 25 g sucrose, 0.5 mM calcium nitrate,

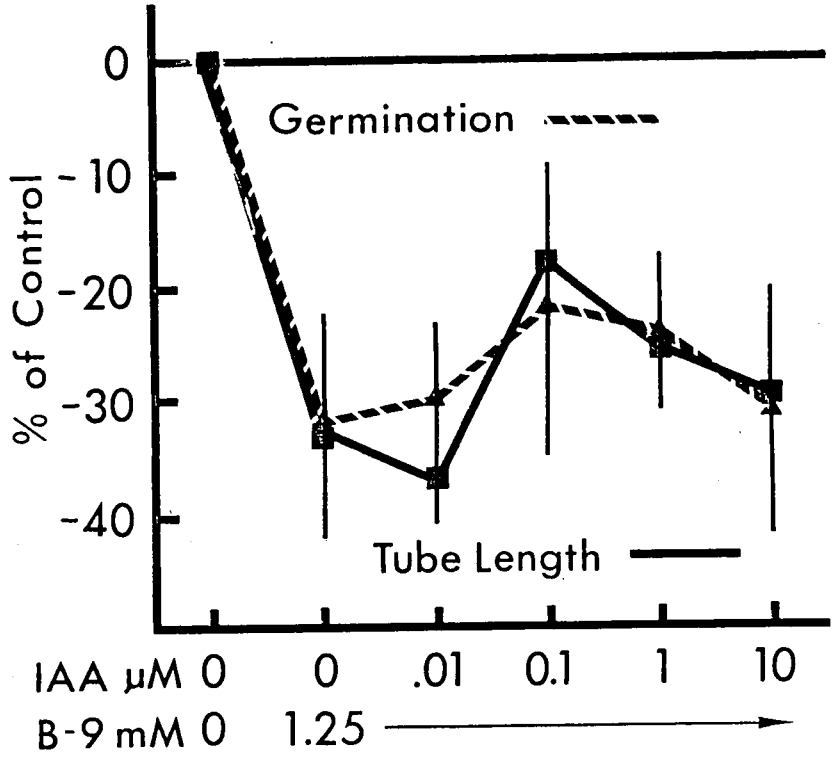


Figure 13. Effects of IAA and SADH on percent germination and tube length of cotton pollen in vitro. The vertical line represents two standard errors.

5.9 mM manganous sulfate, 1.6 mM boric acid, 0.8 mM magnesium sulfate, 1.0 mM potassium nitrate, 1.0 uM gibberellic acid, adjusted pH of 7.6, and 3.5 g agar.

The addition of SADH to the medium inhibited both the percent germination and the tube length of cotton pollen (Figures 12 and 13). The percent germination and tube length response on media containing both IAA and SADH (Figure 13) was closely correlated to the percent germination and tube length response on media containing IAA (Figure 11, page 53). IAA had no significant effect on increasing the tube length on either media with or without SADH, but the trends in each case were very similar.

Ryugo and Sachs (1969) theorized that SADH inhibits IAA synthesis; however, the present study shows that SADH either doe not inhibit IAA synthesis in the cotton pollen, or the pollen contains substantial amounts of IAA at maturity for normal germination and tube growth.

Summary Experiment

A summary experiment was developed to determine whether the parameters chosen for the final revised culture medium significantly increased the percent germination and/or tube length of the cotton pollen in vitro. The data for the effects of ten different media are presented in Table II and medium 1 is the initial control medium (Taylor, 1972).

TABLE II

SUMMARY OF EFFECTS OF DIFFERENCE PARAMETERS ON PERCENT GERMINATION AND TUBE LENGTH OF COTTON POLLEN IN VITRO

	H_2O (ml)	Sucrose (g)	pH	$Ca(NO_3)_2$ (mM)	$MnSO_4$ (mM)	H_3BO_3 (mM)	$MgSO_4$ (mM)	KNO_3 (mM)	GA_3 (uM)	IAA (uM)	Percent germination	Tube length (mm)
1.	100	25	5.0	1.7	4.1	6.5					27 ± 3a*	1.45a
2.	100	25	7.6	1.7	4.1	6.5					35 ± 4abc	11.89b
3.	100	25	7.6	1.7	4.1	6.5	0.8				37 ± 4abc	2.16bc
4.	100	25	7.6	2.0	4.1	6.5	0.8				32 ± 3ab	2.03b
5.	100	25	7.6	2.0	4.1	6.5	0.8		1.0		27 ± 1abc	2.06b
6.	100	25	7.6	2.0	4.1	6.5	0.8	1.0	1.0		38 ± 3bc	2.14b
7.	100	25	7.6	2.0	4.1	1.6	0.8	1.0	1.0		42 ± 4bc	22.14bc
8.	100	25	7.6	2.0	5.9	1.6	0.8	1.0	1.0		42 ± 4c	2.28cd
9.	100	25	7.6	2.0	5.9	1.6	0.8	1.0	1.0	0.1	44 ± 5c	2.32d
10.	100	25	7.6	0.0	5.9	1.6	0.8	1.0	1.0	0.1	47 ± 3cd	2.60de

*Means with the same letter are not significantly differenct at .05.

A high pH (7.6) significantly increased the percent germination and the tube length of cotton pollen *in vitro*. Magnesium sulfate and gibberellic acid both significantly increased the pollen tube length; however, they had no significant effect on the percent germination. Calcium nitrate at both concentrations tested inhibited germination and tube length. A positive response in the tube length was associated with a high concentration of manganous sulfate, while boric acid increased the tube length at a low concentration. Potassium nitrate and indole-3-acetic acid did not significantly affect either germination or tube growth, but a positive response in the percent germination and tube length was associated with their presence in the medium. Cotton pollen grown on the final culture medium had an increased percent germination and tube length of 74% and 68% respectively over the best previously published medium (Taylor, 1972).

CHAPTER V

SUMMARY

The objective of this study was to develop an improved culture medium for the *in vitro* germination and tube growth of cotton (*Gossypium hirsutum* L. c.v. Hancock) pollen. A cotton pollen culture medium proposed by Taylor (1972) was adopted as the initial control medium.

The parameters tested for their effects on the percent germination and tube length of cotton pollen included the following: pH, carbohydrates, magnesium, manganese, boron, calcium, potassium, gibberellic acid, indole-3-acetic acid, and SADH.

All of the data were analyzed by a Duncan's Multiple Range test to show significant differences among the treatments. There were significant differences in both the percent germination and the mean tube length of cotton pollen when comparing pollen from flowers of the same day and pollen from flowers of different days. The germination of cotton pollen was more sensitive to the variability of the greenhouse conditions and the *in vitro* culture conditions than was the pollen tube growth.

A high pH (7.6) significantly increased the percent germination and tube length of cotton pollen *in vitro*. Magnesium sulfate and gibberellic acid both significantly

increased the mean pollen tube length; however, they had no significant effect on the percent germination. Calcium nitrate at all concentrations tested inhibited both germination and tube growth. Manganous sulfate and boric acid were vital for the growth of the cotton pollen tube *in vitro*. Potassium nitrate and indole-3-acetic acid did not significantly affect either germination or tube growth, but a positive trend in the percent germination and tube length was associated with their presence in the medium.

The final revised medium contained 100 ml distilled water, 25 g sucrose, 5.9 mM manganous sulfate, 1.6 mM boric acid, 0.8 mM magnesium sulfate, 1.0 mM potassium nitrate, 1.0 uM gibberellic acid, 0.1 uM indole-3-acetic acid, adjusted pH of 7.6, and 3.5 g agar. The mean percent germination and mean tube length of cotton pollen grown on the final medium were 47% and 2.60 mm respectively. Cotton pollen grown on the final culture medium had an increased percent germination and tube length of 74% and 68% respectively over the best previously published medium (Taylor, 1972).

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