STUDIES OF OPTIMUM COMPONENTS AND pH RANGES FOR POLLEN GERMINATION AND POLLEN TUBE GROWTH IN THE CULTURE MEDIA.

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RESUMO

Seis sucessivos experimentos foram conduzidos com o objetivo de desenvolver um meio ideal de cultura para a germinação e determinação da viabilidade do pólen de algodão (Gossypium L.). A fonte fornecedora do pólen foi Arkugo 4, uma cultivar que amadurece normalmente na cidade de Fayetteville, Estado do Arkansas nos Estados Unidos da América.

Como primeiro experimento o nível de agar foi estabelecido e posteriormente sacarose, ácido bórico, nitrato de cálcio, sulfato de manganês e pH.

Baseado nas observações obtidas o meio de cultura que mais se aproximou do ideal continha 1,0% de agar, 15,0% de sacarose, 0,03% de ácido bórico, 0,06% de nitrato de cálcio, 0,09% de sulfato de manganês dissolvidos em água destilada e um pH ajustado para 7,0.

SUMMARY

This study was conducted to identify an adequate artifial medium to induce in vitro germination of cotton pollen (Gossypium hirsutum L.)

The source of the pollen was Arkugo 4, a cultivar of cotton that matures normally at Fayetteville, Arkansas.

Taylor's medium for pollen germination was used with the first modification tested being the amount of agar in the medium. Sucrose concentration was altered next, and then concentrations were varied for boric acid, calcium nitrate, manganese, and pH.

A medium considered close to optimum, was derived to be used as the best medium for in vitro germination of pollen from the Arkugo 4 cultivar. The best medium included 1.0% agar, 15.0% sucrose, 0.02% boric acid, 0.06% calcium nitrate, and 0.09% maganous sulfate dissolved in distilled water and held at a pH of 7.0.

KEY-WORDS: Cotton pollen germination in vitro, pollen tube growth, pollen viability.

INTRODUCTION

For many years the test of viability of cotton pollenhas attracted the interest of experimental cotton breeders.

Usually the vitality of the cotton pollen is expressed by percentage of germination in vitro and the use of agar medium remains the most accurate and routine method of determining viability of cotton pollen.

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Cotton pollen has been found to be very difficult to germinate on artificial media (LERTMONGKOL[12]). Usually tubes desintegrate instantly after germination. BARROW[2] demonstrated that these problems did not occur when the germination medium was suplemented with certain calcium, mangenese, zinc, chloride, or magnesium salts. The optimal combination was $Ca(NO_3)_2$ and $MnSO_4$, each at 0.14% in distilled water. Germination was unaffected until the sucrose concentration reached 1.7M, but it dropped to about 50% in concentration from 1.7 to 2.0M. TAYLOR[15] observed that cotton Pollen showed 64% germination when the medium consisted of 100 ml of distilled water, 3,5% agar, 25% sucrose, 0.07% $MnSO_4$, 0.04% $Ca(NO_3)_2$, calcium and 0.04% H_3BO_3. For better results, it was found necessary to age the agar plates for at least 24 hours. If this was not done, he demonstrated that the pollen grain tends either to sink into the surface of the agar where it will not germinate, or to take up excessive moisture and rupture.

The need for boron by higher plants was demonstrated by AGULHON[1] and extensively verified in the three succeding decades. GAUCH & DUGGER[8] and PRAHLER[14] hypothesized and supported with experimental evidence one essential role of boron in plants. They conclude from their data that one role involves an interaction of boron with sugar to form a sugar-borate complex (ionizable), which moves through cellular membranes more readily than non-borated, non-ionized sugar molecules. DICKINSON[7] found that pollen tubes were quite short (100 um) after 3 hours incubation without added borate and progessive increases in tube lenght occured when borate was increased from 0.5 to 20 ug. 100 ml^{-1}.

Some researchers have concluded that sucrose only controls the osmotic pressure and does not supply nutrients for pollen tube growth (VISSER et alii[19]). A positive corelation between sugar concentration and percent germination was reported by VASIL[16]. Calcium and boron were the most important ions for germination of "Spathiphyllum" pollen, while magnesium and potassium apparently helped germination (HENNY[9]).

In a majority of the species studied by KWACK[10] a relatively high pH was more favorable for growth when calcium was supplied in the cultural medium. BRINK[4] had shown that pollen germination and tube growth were considerably influenced by changes in pH of the medium. The optimal pH for pollen growth varied with different species The pH range for satisfactory growth of pollen seems rather narrow for all species tested.

The purpouse of this note is to report the development of optinum cotton pollen germination test media including agar, sucrose, boric acid, calcium nitrate, manganous sulfate, water and pH which will give us an efficient method to measure the viability of cotton pollen.

MATERIALS AND METHODS

This study was conducted using cotton Arkugo 4 pollen, a cultivar of cotton that matures normally at Fayetteville, Arkansas. (36° 07' 30"N, 47m elevation).

The average of 30% in vitro germination reported by TAYLOR[15] was considered low. Experiments were carried out to study the level for each of these components (agar, sucrose, boric acid, calcium nitrate, manganous sulfate and pH) which could result in an improved percent germination. For each of these components three levels except pH were compared. The preparation of the media involved heating the solution to 100 C. Immediately after boiling, two drops of the solution were poured onto concave slides which were allowed to cool to room temperature, and were stored in a refrigerator at 5 C for at least 24 hours, as suggested by TAYLOR[15], to prevent

pollen grain from sinking into the surface of the agar where they would not germinate.

In these tests only fresh pollen was used. Pollen was collected from each processed sample with a number 4 camel hair brush then was gently brushed onto the agar surface of the concaved slides. A coverslip was placed on the slide, sealed with vaseline and then placed on moistured filter paper in a petri-dish which was then covered and kept at 25°C. After 24 hours germination and pollen tube growth was stopped by adding 1 ou 2 drops of acetocarmine, and storing at 5 C, for later counting. Subsequently 25 pollen grains were examined in each quadrant of each of four slide fields for each test and recording were made of the number of germination in each quadrant. A grain was classified as germinated if at least one recognizable pollen tube was present. For pollen tube length, 25 randomly pollen were measured and their lengths were recorded. The percent germination and length measurements were made at a magnification of X 100.

In this way three levels each of agar, sucrose, H_3BO_3, $Ca(NO_3)_2$ $MnSO_4$, and eigth levels of pH were compared. In the agar experiment, the concentrations 0.5, 1.0, and 2.0% were compared. Minimal amounts of sucrose and H_3BO_3 were added as a basic medium to assure germination differentials for the three agar levels.

In the next experiment sucrose concentrations (15.0, 25.0, and 35.0% in diestilled water) were compared using the same basic medium as above plus 0.07% $MnSO_4$. In the third experiment three concentrations of H_3BO_3 (0.02, 0.03, and 0.04% in distilled water) were compared in order to improve germination of fresh pollen from cotton. The basic medium was 1.0% agar, 15.0% sucrose, 0.05% $Ca(NO_3)_2$, and 0.07% $MnSO_4$. In the fourth experiment, three levels of $Ca(NO_3)_2$ (0.04, 0.05, and 0.06% in distilled water) were compared as modified of TAYLOR'S[15] medium. In the fifth experiment, three levels of $MnSO_4$ (0.05, 0.07, and 0.09% in distilled water) were studied to see the effect of manganese level on cotton pollen germination. The basic medium was TAYLOR'S[15] modified to have 1.0% agar, 15.0% sucrose, 0.03% H_3BO_3, and 0.06% $Ca(NO_3)_2$. In each experiment above the pH was adjusted for 7.0. After the best level of agar, sucrose, H_3BO_3, $Ca(NO_3)_2$, and $MnSO_4$ were obtained, then the last test was to compare germination percentage of fresh cotton pollen at different pH levels from 4.0 to 8.0. The basic medium was 1.0% agar, 15.0% sucrose, 0.03% H_3BO_3, 0.06% $Ca(NO_3)_2$, and 0.09% $MnSO_4$ dissolved in distilled water.

RESULTS AND DISCUSSION

EXPERIMENT 1 – Agar

Pollen germination can be achieved on agar in most species but problems were encountered in preliminary trials with cotton pollen. These problems were media related. The concentration of agar and nutritional supplements were thought to be involed. In this experiment three concentrations of agar (0.5, 1.0, and 2.0% in distilled water) were compared. Agar is not a complete nutritional medium and adequate nutrients must be added for pollen germination and tube growth. However, when lower concentrations of agar are used (for example, when soft agar is used), the pollen tends to sink into the surface of the medium where it will not germinate. The agar content, by altering the physical characteristics of the medium, controls the degree of embedding of the grains into the surface which may affect the amount of oxygen absorbed by the grain VISSER[18].

The analyses of data on cotton pollen germination (Tables 1 and 2) supporte the need to have higher concentrations than 0.5% agar. The same did not

hold for tube growth. For pollen tube length 84% of treatment was atributable to the negative linear response of tube growth to agar concentration. The best agar concentration for both germination and tube growth, however, under these experimental conditions, was 1.0% in distilled water at this concentration the germination was 12.% and the tube length of pollen tube was 156.7 μm. The 2.0% agar gave a significantly lower germination percentage.

Table

Comparison of fresh Cotton Pollen Germination and Tube Growth at Different Concentrations of Agar [1, 2, 3]

Agar Concentration (%)	Percent Germination	Pollen Tube Length (μm)
0.5	9.0[4]	170.0
1.0	12.0	156.7
2.0	5.5	69.3

[1] Agar solutions in distilled water

[2] Each medium contained 15.0% sucrose and 0.03% H_3BO_3. The pH was adjusted to 7.0 with IN HCl or 1N KOH.

[3] The plants from which flowers were taken were grown in greenhouse between fall of 1980 and winter of 1981. The pollen was collected 10 November, 1980.

[4] Agar concentration effects differed significantly according to an appropriate Chi-square test.

Table 2

Analysis of Variance for Tube Length of Cotton Pollen Germinated on Three Concentrations of Agar, 1980

Source of Variation	df	Mean Square
Agar	2	125,597.50**
Slides Fields in Agar Concentrations (Error)	9	3,123.05

**Significant at $P \leq 0.01$

EXPERIMENT 2 – Sucrose

The most common nutritional supplement given pollen is sucrose. Sugar maintains an osmotic equilibrium in the pollen tube without which the cells become inflated and may burst LEOPOLD & KRIEDEMANN[11]. In many instances sugar also serves as a nutritive substrate for the metabolism of the cell (VISSER[18]). Therefore, an adequate sucrose level should provide a reliable media to ascertain pollen viability using in vitro germination.

Attempts were made to germinate cotton pollen in artificial media with varying concentrations of sucrose (15.0, 25.0, and 35.0% in distilled water).

Results were interpreted to mean that both percent germination and tube length of cotton pollen were highly affected by sucrose concentrations (Tables 3 and 4). Both germination percentage and tube growth decreased as sucrose concentrations increased. There was a highly significant negative linear response of pollen tube growth to sucrose concentrations. Best germination (32.2%) and tube growth (582.5 um) of cotton pollen was at 15.0%. At 35,0% the germination and tube growth were very poor. BARROW[2] concluded that cotton germination frequency was unaffected by increasing the sucrose concentration to 1.6 M: however, from 1.7 to 2.0 M sucrose, germination dropped to 50.0%. The concentrations used in this study were 0.44, 0.73, and 1.02 M respectively for 15.0, 25.0, and 35.0% sucrose.

The strong negative linear relationship encountered suggests even better germination and tube growth may have obtained with still lower concentrations of sucrose than used here.

EXPERIMENT 3 – Boric Acd

The need for boron by higher plants has been studied extensively by several investigators (2, 7, 8, 15, 17). GAUCH & DUGGER[8] believed that boron

combines with sugar to form a sugar-borate compelx (ionizable) which is translocated with greater facility than non-borated non-ionized sugar molecules.

In this experiment cotton pollen germination and tube growth were compared in media containing 0.02, 0.03, and 0.04% boric acid in distilled water.

Under the conditions described for this experiment, boric acid concentrations significantly affected cotton pollen germination and tube length (Tables 5 and 6). A negative but significant quadratic response of pollen tube growth to boren concentration was recorded. Thus the results indicated that the best concentration and tube growth was 0.03%. Increases in percent germination and tube length occured when borate was increased from 0.02 to 0.03%. However, decreases were apparent when borate was further increased to 0.04%.

Table 3

Comparison of Fresh Cotton Pollen Germination and Tube Growth at Different Concentrations of Sucrose[1, 2, 3]

Sucrose Concentration (%)	Percent Germination	Pollen Tube Length (μm)
15.0	32.2[4]	582.5
25.0	21.0	387.5
35.0	2.7	117.5

1 Sucrose solutions in distilled water

2 Each medium contained 1.0% agar, 0.03% H_3BO_3 and 0.07% $MnSO_4$. The pH was adjusted to 7.0 with IN HCl or 1N KOH.

3 The plants from which flowers were taken were grown in greenhouse between fall of 1980 and winter of 1981. The pollen was collected 10 November, 1980.

4 Sucrose concentration effects differed significantly according to an appropriate Chi-square test.

Table 4

Analysis of Variance for Tube Length of Cotton Pollen Germinated on Three Concentrations of Sucrose, 1980

Source of Variation	df	Mean Square
Sucrose	2	2,181,000.00 **
Slides within Sucrose Concentrations (Error)	9	30,250.00

**Significant at $P \leq 0.01$

Table 6

Analysis of Variance for Tube Length of Cotton Pollen Germinated on Three Concentrations of Boric Acide, 1980

Source of Variation	df	Mean Square
Boric Acid	2	1,041,083.33**
Slides Fields in Boric Acid Conc. (Error)	9	18,166.67

**Significant at $P \leq 0.01$

Table 5

Comparison of Fresh Cotton Pollen Germination and Tube Growth at Different Concentrations of Boric Acid[1, 2, 3]

Boric Acid Concentration (%)	Percent Germination	Pollen Tube Length (μm)
0.02	40.5[4]	662.5x
0.03	50.7	832.5
0.04	23.2	510.0

1 Boric Acid solutions in distilled water

2 Each medium contained 1.0% agar, 15.0% sucrose, 0.05% $Ca(NO_3)_2$, and 0.07% $MnSO_4$. The pH was adjusted to 7.0 with 1N HCl pr 1N KOH.

3 The plants from which flowers were taken were grown in greenhouse between fall of 1980 and winter of 1981. The pollen was collected on 13 November, 1981.

4 Boric acid concentration effects differed significantly according to an appropriate Chi-square test.

EXPERIMENT 4 — Calcium Nitrate

Calcium nitrate has been also been reported to enhance in vitro germination and pollen tube growth of a number of plant species (BREWBAKER & KWACK[3]). The action of calcium on the pollen grain is apparently associated with the pollen tube membrane (PFAHLER[14]) Studies have indicated that calcium binding takes places in the regions of the pollen tube wall, thus increasing wall rigidity and stability with a decrease in rupturing and a subsequent increase in the percent intact pollen tubes (BREWBAKER & KWACK[3]). COOK & WALDEN[6] demonstrated that adding calcium to sucrose-agar medium was very effective in increasing pollen germination of corn.

In this experiment the influence of calcium nitrate in increasing the percent germination was highly significant according to a Chi-square test (Table 7). The differences in tule growth associated with varying levels of calcium nitrate were not statistically significant (Table 8). Neither the linear nor the quadratic responses of the pollen tube growth to calcium nitrate concentrations were significant. Calcium nitrate at the concentration of 0.06% showed 55.7% germination and 990.0 μm of pollen tube length. At this concentration germination and tube growth have not peaked out. However, 0.06% $Ca(NO_3)_2$ was used as the best concentration for all other studies.

Table 7

Comparison of Fresh Cotton Pollen Germination and Pollen Tube Growth at Different Concentrations of Calcium Nitrate[1, 2, 3]

Calcium Nitrate Concentration (%)	Percent Germination	Pollen Tube Length (μm)
0.04	33.0[4]	850.0
0.05	36.2	802.5
0.06	55.7	990.0

1 Calcium nitrate in distilled water

2 Each medium contained 1.0% agar, 15.0% sucrose, 0.03% boric acid, and 0.07% manganous sulfate. The pH was adjusted to 7.0 with 1N HCl pr 1N KOH.

3 The plants from which flowers were taken were grown in greenhouse between fall of 1980 and winter of 1981. The pollen was collected on 13 November, 1981.

4 Calcium nitrate concentration effects differed significantly according to an appropriate Chi-square test.

Table 8

Analysis of Variance for Tube Length of Cotton Pollen Germinated on Three Concentrations of Calcium Nitrate, 1980

Source of Variation	df	Mean Square
Calcium Nitrate	2	378,520.00NS
Slides Fields in Calcium Nitrate Conc. (Error)	9	173,542.22

NS = Not Significant at $P \leq 0.05$.

EXPERIMENT 5 – Manganous Sulfate

Quantitative data regarding pollen germination and tube growth in media containing different concentrations of manganous sulfate are very limited. Usually manganese studies are conducted in combination with other nutritional factors. BARROW[2] indicated that the optimal combination for cotton pollen germination and tube growth was manganous sulfate and calcium nitrate, each at 0.14 g, in 100 ml of distilled water. In experiment reported here an attempt was made to isolate the effect of manganous sulfate in cotton pollen germination and tube growth. Three concentrations (0.05, 0.07, and 0.09% in distilled water) were compared. The resulting data showed highly significant differences among the $MnSO_4$ concentrations used for cotton pollen percent germination and tube growth (Tables 9 and 10). A significant quadratic response of $MnSO_4$ for pollen tube growth was observed. The 0.09% concentrations gave 45.2% germination and an average of the pollen tube length of 467.5 = m Subsequent experiments used 0.09% $MnSO_4$.

EXPERIMENT 6 – Hydrogen-ion Concentration

The pH level of the cultural medium has been shown to affect pollen germination of several plant species (4, 6, 9, 13). Germination and tube growth (Tables 11 and 12) of cotton pollen were influenced by pH changes as measured under the conditions of this experiment. The positive linear response was highly significant and the significant quadratic response suggested that the response peaked at a pH of about 7.0 The highest germination obtained with pH values below 5.0 was 12.0%. Cotton pollen germination and tube growth were satisfactory only when the pH of the medium was between 5.4 to 8.0. The best pollen germination and

tube length was reached when the pH was 7.0. In most cases, when germination was high, pollen tubes were longer. In the COOK & WALDEN[6] studies on the germination of corn pollen a pH of 7.0 appeared to be optimum.

Table 9

Comparison of Fresh Cotton Pollen Germination and Tube Growth at Different Concentrations of Manganous Sulfate[1, 2, 3]

Manganous Sulfate Concentration (%)	Percent Germination	Pollen Tube Length (μm)
0.05	22.7[4]	455.0
0.07	41.0	215.0
0.09	55.7	476.5

1 Manganous sulfate in distilled water

2 Each medium contained 1.0% agar, 15.0% sucrose, 0.03% boric acid, and 0.06% calcium nitrate. The pH was adjusted to 7.0 with 1N HCl pr 1N KOH.

3 The plants from which flowers were taken were grown in greenhouse between fall of 1980 and winter of 1981. The pollen was collected on 16 November, 1980.

4 Manganous sulfate concentration effects differed significantly according to an appropriate Chi-square test.

Table 10

Analysis of Variance for Tube Length of Cotton Pollen Germinated on Three Concentrations of Manganous Sulfate, 1980

Source of Variation	df	Mean Square
Manganous Sulfate	2	810,083.33**
Slides Fields in Manganous Sulfate Conc. (Error)	9	102,305.55

**Significant at $P \leq 0.01$

Table 11

Comparison of Fresh Cotton Pollen Germination and Tube Growth at Different Levels of pH[1,2]

pH	Percent Germination	Pollen Tube Length (μ m)
4.0	6.5[3]	117.5
4.5	12.0	272.5
5.0	9.5	260.0
5.4 (unadjusted)	50.0	537.5
6.0	45.0	487.5
7.0	78.7	737.5
7.5	63.2	642.5
8.0	60.7	697.5

1 Each medium contained 1.0% agar, 15% sucrose, 0.03% boric acid, 0.06% calcium nitrate, and 0.09% manganous sulfate in distilled water. The pH's were adjusted with 1N HCl or 1N KOH.

2 The plants from which flowers were taken were grown in greenhouse between fall of 1980 and winter of 1981. The pollen was collected on 16 November, 1980.

3 pH level effects differed significantly according to an appriate Chi-square test.

Table 12

Analysis of Variance for Tube Length of Cotton Pollen Germinated on Different Levels of pH, 1980

Source of Variation	df	Mean Square
pH	8	1,746,361.11**
Slide Fields in pH Level (Error)	27	102,296.30

**Significant at $P \leq 0.01$

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