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The conditions required to isolate and maintain viable cotton (*Gossypium hirsutum* L.) microspores*

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ABSTRACT

Cultural systems developed for the successful production of haploid plants from anther and microspore culture of a number of species are lethal to cotton microspores. Components of these systems were examined individually and in simple combinations to determine their specific effect on cotton microspore viability during isolation and culture. An organically buffered pH of 7.0 was critical for survival. The addition of potassium salts, near standard concentrations used for other species, enhanced survival and the cytoplasmic appearance of isolated microspores. Severe toxicity resulted from ammonium, calcium and magnesium salts, and these ions were tolerated only at very low concentrations. Iron, glutamine, serine, inositol, vitamins, and trace minerals were generally not detrimental to microspores at standard concentrations. An isolation and cultural maintenance system was developed that yields large quantities of healthy, viable cotton microspores. This initial step allows for further research in inducing cotton microspores to divide and undergo embryogenesis.

ABBREVIATIONS

ACES : (N-2-Acetamido-2-aminoethanesulfonic acid)
ADA : (N-2-Acetamidoiminodiacetic acid)
BES : [N,N-bis-(2-hydroxethyl)-2-aminoethanesulfonic acid]
HEPES: (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid)
MOPS : [3-(N- Morpholino) propanesulfonic acid]
TES : [2- tris-(hydroxymethyl) methyl-aminoethanesulfonic acid]
PIPES: (Piperazine-N,N'-bis(2-ethanesulfonic acid) dipotassium salt.

INTRODUCTION

The culture of haploid plants from increasing numbers of species promises to be a useful tool in plant improvement (Vasil 1980). Modifications of several commonly used nutrient preparations are successful in culturing haploids from anthers or pollen (Davis et al. 1974; Gamborg et al. 1968; Linsmaier and Skoog 1965; Murashige and Skoog 1962; Nitsch 1974). A semigametic method of producing specific cotton haploids was useful in the development of some breeding stocks (Barrow and Chaudhari 1976; Turcotte and Feaster 1967). However, for most breeding objectives, semigamy is too laborious to produce adequate numbers of cotton haploids. Therefore, an effective cultural method capable of producing large numbers of cotton haploids rapidly and economically would be valuable in developing breeding lines and stabililizing genetically heterozygous material.

Cotton tissue culture was limited to callus and cell suspensions and the initial stages of regeneration (Oswald et al. 1977; Price and Smith 1979; Reinert and Bajaj 1977) until Davidonis and Hamilton (1983) reported the regeneration of cotton plants derived from somatic embryos in callus. Haploid plants have not been cultivated from anther or pollen in cotton, except for haploid and diploid callus by Barrow et al. (1978). Androgenetic methods developed for other species do not work for cotton. After many modifications, these methods were still unsuccessful and microscopic examination revealed that cotton microspores did not survive in these culture media.

Techniques were developed in this study to systematically evaluate components of these cultural systems singly and in simple combinations to determine their specific effects on the survival of cotton microspores. Over a nine year period, thousands of experiments were conducted. Initially results were erratic and difficult to repeat, until specific factors were isolated and controlled. This paper summarizes those factors which significantly affect the isolation of viable cotton microspores, and reports the development of a method to isolate and maintain living cotton microspores.

MATERIALS AND METHODS

The first criterion of success in response to the treatments was to obtain living cells. The optimal stage of microspore development for culturing haploid plants in some species is during the mitotic division after meiosis. At this stage in cotton, the pollen wall is formed making it impossible to microscopically observe the cytoplasmic condition of the cell. Therefore, cotton flower buds (4 to 5 mm in length) in the late tetrad or early microspore stage were selected, and living microspores were distinguished microscopically by their light, translucent color, rounded shape, and by cytoplasmic streaming. (See Figure 1a). Dead cells were dark and distorted and are shown in

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Figure 1b. This method was used to evaluate the effects of all treatments on microspore survival. Two methods of microspore isolation and evaluation were developed and followed.

Single Anther Shortterm Method

Single anthers were placed in 20 µl wells of a 60 microwell culture plate arranged in 6 columns with 10 rows. A 20 µl aliquot of a specific treatment (nutrient additive) was added to each well. The microspores of a single flower bud could be subjected to 60 different treatments or combinations. The anthers were ruptured with a small glass rod releasing the microspores into the specific test solution and anther tissue was removed. The microspores were observed at 200X with an inverted microscope for viability.

To conduct long term microspore culture experiments, it was necessary to isolate large numbers of microspores aseptically and remove toxic substances from the isolating solution. This method is described below.

Aseptic Longterm Multiple Anther Method

1. Cotton buds 4 to 5 mm long were surface sterilized 30 seconds in 95% ETOH and rinsed with sterile distilled water.
2. Anthers were aseptically removed from all other bud tissue in a sterile isolation solution and ruptured in a tissue grinder with 0.25 mm clearance, which released uninjured microspores into the solution.
3. Debris larger than the tetrads or microspores was removed with a 100 µm screen.
4. Cells were washed 3 times by centrifuging in 15 ml conical centrifuge tubes momentarily at 100 X g and replacing the supernatant with 2 ml of fresh sterile solution.
5. The microspore pellet was resuspended twice in 2 ml of solution. The microspores settled to the bottom in 8 minutes, and the supernatant with the fine debris was removed by pipetting.
6. Eight to 10 buds yielded about 30,000 microspores essentially free of other tissue.

Pertinent Parameters Examined

The effects of the medium pH on microspores were studied by adjusting the pH at 0.5 intervals ranging from pH 5.0 to 8.0. The adjusted pH was unstable, so seven organic buffers were tested for their ability to stablize the pH during culture periods of 30 to 60 days. These buffers were ACES, ADA, BES, HEPES, MOPS, TES, and PIPES used at 10 mM in the medium and adjusted to the desired pH with 1N NaOH or 1N HCL.

The effects of sucrose, glucose, ribose and xylose on cotton microspore survival and viability were evaluated in 6 x 10 microwell plates, for convenience singly and in all two-way combinations at molarities ranging from 0 to 1 at 0.1 M intervals. To determine optimal levels of essential nutritional components, the mineral salts and amino acids of Nitsch's (1974) microspore medium, successful for culturing tobacco microspores, were tested individually beginning with her original concentration and by serially diluting each component by 1/2, resulting in dilutions of 1/2, 1/4, 1/8, 1/16, and 1/32. Ammonium nitrate [$(NH_4)_2NO_3$] potassium nitrate (KNO_3), magnesium sulfate ($MgSO_4.7H_2O$), potassium phosphate (KH_2PO_4), chelated iron (Na_4Fe_3EDTA), calcium chloride ($CaCl_2.2H_2O$), calcium nitrate [$Ca(NO_3)_2$], glutamine, serine, and inositol were tested. Nine of these components could be tested in the 6x10 microwell plates with 6 replications or dilutions and a control of 5.0% glucose, 5.0% ribose and a buffered pH of 7.0. No other components were included. Additional mineral salts, vitamins and trace minerals considered as essential in some culture media were tested in the same manner. The effects of these components on cell viability, were determined by microscopic observation of the cytoplasm.

RESULTS AND DISCUSSION

Cotton microspores were delicate and sensitive to mechanical pressure and changes during isolation. The tissue grinder described above prevented injury to the cells. However, they died in 15 to 30 minutes unless they were washed to remove toxic substances from the solution. A pH of 6.5, or lower, quickly killed the cells or distorted the cytoplasm. When the pH ranged from 6.7 to 7.3, excellent cell survival and cytoplasmic appearance was observed. Thus, pH 7.0 was selected for optimal cotton microspore survival and culture. However, the pH of culture solutions adjusted with NaOH was unstable and as the pH dropped, the condition of the microspores deteriorated rapidly. Several inorganic buffers were tried with limited success. Organic buffers were selected for their ability to maintain the pH of the isolation medium at 7.0 for extended periods of time. Two buffers, BES and HEPES, maintained the pH of the medium at 7.0 for two weeks or longer, but were cytotoxic. Four buffers, ADA, MOPS, ACES and TES were similar in their ability to maintain pH and to allow for the satisfactory isolation and culture of living microspores up to eight weeks. The PIPES buffer yielded the highest quality microspores and was used to buffer the pH of the medium in order to properly evaluate the effects of the other cultural components.

The best carbon source was glucose and ribose for long term cultures. This is consistent with reports by Davis et al. (1974), Price et al. (1977), and Barrow et al. (1978) that cotton cells in culture respond better to glucose than to sucrose. The optimal carbohydrate concentration proved to be 5% glucose and 5% ribose.

All concentrations of KNO_3 and KH_2PO_4 significantly improved the viability and cytoplasmic appearance of the cells. When evaluated in the buffered glucose, ribose solution and $(NH_4)_2NO_3$, $CaCl_2.2H_2O$, and $MgSO_4.7H_2O$ were very toxic at standard, 1/2, 1/4, and 1/8 dilutions. No differences were noted from the controls at lower concentrations. Iron, glutamine, serine and inositol had no observable beneficial or detrimental effect on cell survival, except at the highest concentrations where glutamine and sometimes iron were slightly toxic.

Because of the positive response of cells to KNO_3, the isolation medium became 5% glucose, 5% ribose, 10 mM PIPES and .038 M KNO_3 (3.8 g/l), and the other components were retested at the same concentrations used previously. The .038 M KNO_3 alone gave good results, but in combination with other minerals this proved to be too high, and was reduced to .019 M. Calcium nitrate was also tested in the basic isolation medium with .019 M KNO_3. Under these conditions, $(NH_4)_2NO_3$, $CaCl_2.2H_2O$, and $MgSO_4.7H_2O$ were still toxic at all concentrations and $Ca(NO_3)_2$ was highly toxic at all but the lowest concentration. Iron, glutamine, serine and inositol did not change the appearance of the cells from those in the control medium.

Inositol by itself did not adversely affect the microspores at concentrations up to 20 g/l, but in combination with 0.019M KNO_3, 1 g/l was optimal, and cells deteriorated at higher concentrations. This illustrated the need to orchestrate the final

concentrations, once optimal individual concentrations were established.

Each component of Nitsch's (1974) tobacco microspore medium was screened and selected first at its optimal concentration. It was then orchestrated to its best effect in combination with the other components to give the best cytoplasmic appearance and microspore viability during and after isolation. Calcium chloride was eliminated due to its severe toxicity. The modified medium enabled the successful isolation and maintenance of cotton microspores with more than 90% viability at the mature tetrad stage, and close to 100% viability of released microspores. Microspores survived eight weeks and more in this medium without cytoplasmic deterioration. Table 1 gives the current formulation of the cotton microspore medium, designated as CM-1, as it compares to Nitsch's (1974) medium.

The method of culture of cotton microspores in CM-1 is important. Culture vessels which restrict evaporation and or maintain solute concentrations should be used for longer term experiments.

Presently, microspores have not divided but pollen mother cells, isolated during metaphase II, have progressed to the tetrad stage, completing meiotic divisions in progress. This is encouraging for possibly initiating cell division in cotton microspores that may subsequently allow haploid cell-lines or plants to be established.

The development of CM-1 places cotton microspore culture at a new starting point to test growth regulator models and other cultural components to determine their capacity to stimulate growth and development. This type of research has not been possible using current haploid cultural systems because of their lethal effect.

If progress is made in initiating cell divisions in microspore cultures, further modifications would be expected. Presently, CM-1 is a survival medium. Other components such as growth regulators, trace minerals, and vitamins, have no visible detrimental effect, at standard concentrations on cotton microspores, but they likely will have positive or negative effects on any microspore development. When these effects are identified, further modifications in the medium will be necessary.

Table 1. Test Concentrations of Cultural Components for Cotton Microspore Culture.

Nitsch's Compounds Tested (8)	Concentration in grams per liter						
	Full	1/2	1/4	1/8	1/16	1/32	CM-1
KNO_3	3.800[a]	1.900	0.950	0.475	0.238	0.119	1.900
$(NH_4)_2NO_3$	2.900	1.450	0.725	0.363[a]	0.181	0.091	0.360
$MgSO_4.7H_2O$	0.740	0.370	0.185	0.093	0.046	0.023[a]	0.020
$CaCl_2.2H_2O$	0.660	0.330	0.165	0.083	0.041	0.021[b]	0
KH_2PO_4	0.280[a]	0.140	0.070	0.035	0.018	0.009	0.300
$Na_4FeEDTA$	0.038[a]	0.019	0.010	0.005	0.002	0.001	0.03
Glutamine	0.800	0.400	0.200	0.100[a]	0.050	0.025	0.100
Serine	0.100[a]	0.050	0.025	0.013	0.006	0.003	0.100
Inositol	20.000	10.000	5.000	2.500	1.250[a]	0.625	1.000
Sucrose	10.000	0	0	0	0	0	0
Glucose	0	NA[c]	NA	NA	NA	NA	50.000
Ribose	0	NA	NA	NA	NA	NA	50.000
PIPES	0	NA	NA	NA	NA	NA	3.780
pH	5.5	NA	NA	NA	NA	NA	7.0

[a] Individual optimal concentration for best appearing microspores.
[b] The lowest concentration expressed some toxicity to microspores and this salt was not added the final medium.
[c] Dillutions were tested individually and in simple combinations with CM-1 carbohydrates and pH.

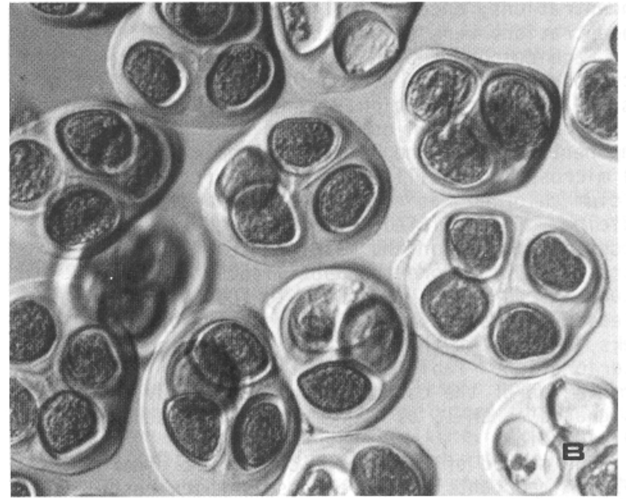

FIGURE I. a) Living cotton microspores (approximate size 100 μm) as tetrads. Cells are a light color and spherical in shape. b) Dead cells are dark and distorted.

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